INSIGHTS OF GUT MICROBIOTA: PROBIOTICS AND BIOACTIVE COMPOUNDS

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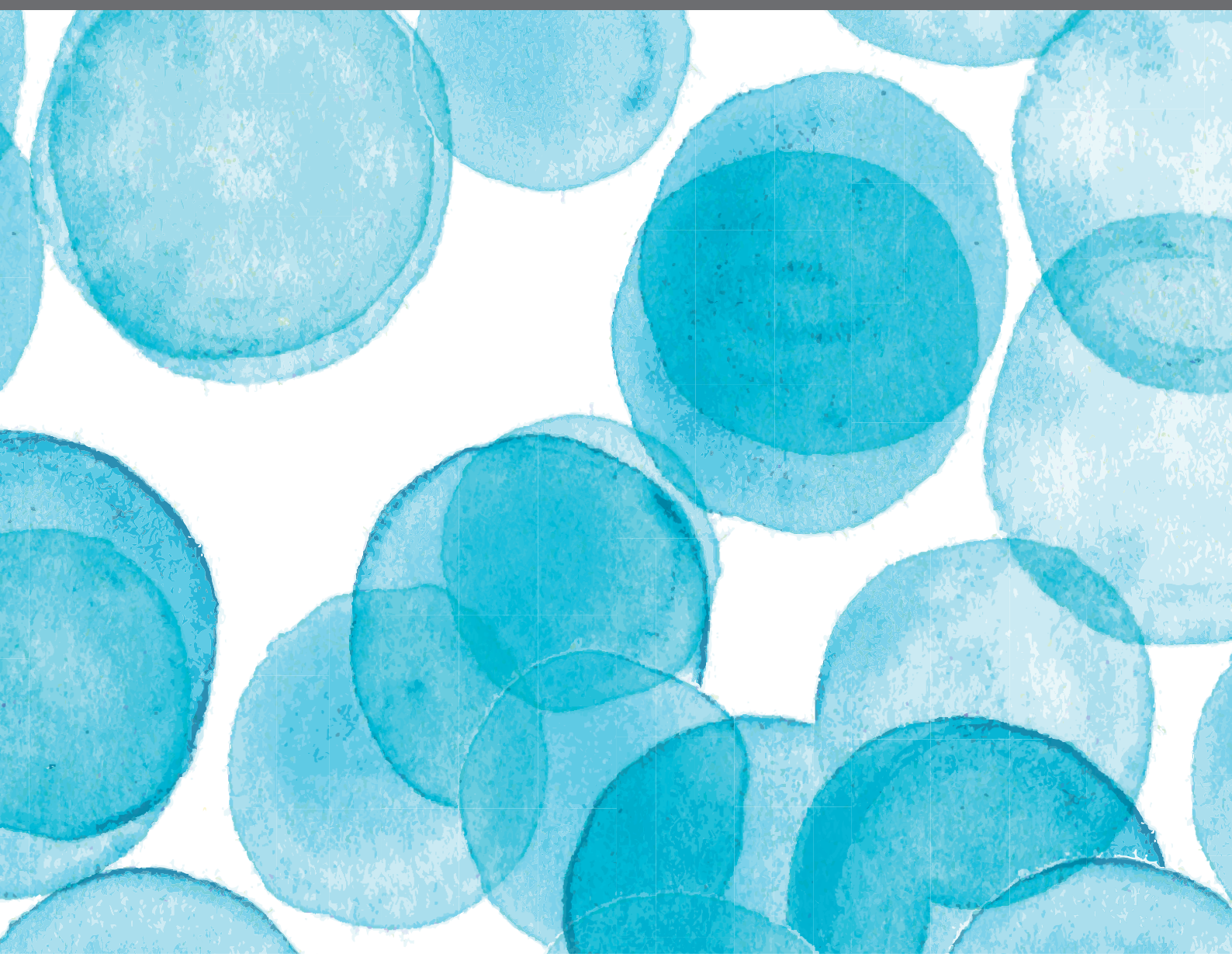
PUBLISHED IN: Frontiers in Microbiology and Frontiers in Nutrition



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ISSN 1664-8714
ISBN 978-2-88971-922-8
DOI 10.3389/978-2-88971-922-8

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INSIGHTS OF GUT MICROBIOTA: PROBIOTICS AND BIOACTIVE COMPOUNDS

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Citation: Sivieri, K., Sáyago-Ayerdi, S. G., Binetti, A. G., eds. (2021). Insights of Gut Microbiota: Probiotics and Bioactive Compounds. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-922-8

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frontiers in Microbiology

EDITORIAL
published: 01 November 2021
doi: 10.3389/fmicb.2021.780596

Editorial: Insights of Gut Microbiota: Probiotics and Bioactive Compounds

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Keywords: microbiome, functional foods, bioactive compound, prebiotic, probiotic

Editorial on the Research Topic

Insights of Gut Microbiota: Probiotics and Bioactive Compounds

The human body harbors a multitude of microorganisms, including bacteria, fungi, archaea, and viruses, which exist in a symbiotic relationship with their host. The entirety of these commensals is referred to as the microbiota, and their collective genomic information as the human microbiome. The human microbiome has emerged as a crucial component in health and disease. It is becoming increasingly recognized that the microbiome can change our health status, or switch on a wide range of diseases including cancer, cardio-metabolic diseases, allergies, and obesity. In this way, probiotics and bioactive compounds may modulate the gut microbiota. The objective of this Research Topic is to show how probiotics and bioactive compounds can modulate the intestinal microbiota. Thus, research on gut microbiota and probiotics is moving from an isolated area to one with a range of possibilities. This Research Topic focuses on studies (including original research, perspectives, minireviews, commentaries, and opinion papers) that investigate and discuss: (1) The influence of probiotics on gut microbiota; (2) The influence of bioactive compounds on gut microbiota; (3) Methods, possibilities, and approaches to change and control gut microbiota, and (4) Experimental systems and approaches in gut microbiome research.

This collection includes 20 research articles spanning diverse publication formats, including 14 Original Research Articles, two Reviews, three Mini-reviews, and one Methods. Although different, the major articles have a similar objective of finding ways to modulate the intestinal microbiota.

The importance of probiotics in human health on gut microbiota modulation is indisputable. However, is there a consensus on what the quality criteria used for probiotics to be conveyed in food or in the form of supplements are? An interesting review from Binda et al., describes the minimum criteria that apply to a probiotic strain that will be used in foods and dietary supplements; similar criteria may be applicable to other uses of probiotics. These principles are based on the consensus statement from the International Scientific Association for Probiotics and Prebiotics (ISAPP) on the scope and appropriate use of the term probiotic. This document acts as a guide for both scientists and food producers serving as a tool to summarize the steps to consider when a potential probiotic strain is intended for use, ensuring the proper use of this term in scientific publications, on food product labels, and in regulatory documents. Furthermore, aiming at the health of gut microbiota, this special topic brings several important insights. Bengoa et al., showed that exopolysaccharides (EPS) produced by two *L. paracasei* strains isolated from kefir grains have the potential to improve the short chain fat acids' (SCFAs) production using an *in vitro* EPS fermentation by human fecal microbiota. In this way, Sabater et al., highlighted that EPS produced by *Bifidobacterium animalis* subsp. *lactis* can beneficially modify the gut microbiota with a potential immune modulation effect. On the other hand, Almada-Érix et al. showed that the consumption of yogurt containing *B. coagulans* GBI-30 6086 decreases triglycerides and glucose levels and positively impacts the gut bacterial ecology in healthy rats. Therefore, Li et al., showed on heat stroke in rats that a probiotic *Bacillus licheniformis* has a preventative effect on intestinal injury

OPEN ACCESS

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *21 September 2021*
Accepted: *06 October 2021*
Published: *01 November 2021*

Citation:
Sivieri K, Sáyago-Ayerdi SG and Binetti AG (2021) Editorial: Insights of Gut Microbiota: Probiotics and Bioactive Compounds. Front. Microbiol. 12:780596. doi: 10.3389/fmicb.2021.780596

by sustaining intestinal barrier function and modulating gut microbiota. Interestingly, Diamond et al., demonstrated the beneficial potential of caffeine in microbiome and microbiome members during antibiotic exposure in animal model.

Another important topic we brought up was the importance of colonization of the intestinal microbiota in the first years of life. The delivery mode and the first 3 years of an infant's life are critical for the establishment of the intestinal microbiome. In this way, Lyu et al., evaluated how the delivery mode can affect the intestinal gut and development of intestinal epithelial cells. The authors conclude that vaginal delivery and cesarean section (C-section) can influence gut microbiota composition. In addition, they found that *B. bifidum* FL-228.1 exhibited favorable effects on the development of intestinal epithelial cell. In this sense, Cheng et al., highlighted that human milk oligosaccharides can promote the growth of *B. longum* subsp. *infantis*. Conversely, Yousuf et al., showed that routine in-hospital administration of probiotics to preterm infants resulted in the potential for colonization of the gut with probiotic post-discharge and effects on the gut microbiome, inducing the colonization by *Bifidobacterium*, thus ensuring that their intestinal microbiome resembles that of 10-day old full-term infants. Also, several large randomized controlled trials were revised by Murphy et al., showing that the relative risk for Necrotizing Enterocolitis can be reduced by the modulation of the gut microbiota with some (not all) probiotic formulations, highlighting the need to guarantee the purity and safety of commercially available probiotics, especially when they are intended to be implemented in a neonatal intensive care unit. On the other hand, the mini review realized by Ale and Binetti showed the clinical benefits of probiotic, prebiotic, and symbiotic consumption in the elderly, providing a better quality of life.

The action of probiotics and bioactive compounds on the microbiota of animals has also been investigated. Zhang et al., showed that ε-polylysine may influence the utilization of feed nutrients by Ningxiang pigs, including proteins, lipids, metabolizable energy, and fiber, by regulating the gut microbiota. In the same way, the paper published by Wang et al., deals with the effects of *Clostridium butyricum*, sodium butyrate, and butyric acid glycerides on the reproductive performance, egg quality, intestinal health, and offspring performance of yellow-feathered breeder hens; this document has already reached a milestone number of readers. The paper from Almada-Érix et al., has also been highly viewed by readers; it deals with orange juice and yogurt carrying probiotic *Bacillus coagulans* GBI-30 6086: Impact of intake on wistar male rats' health parameters and gut bacterial diversity. Something similar was discussed in the review proposed by González-Morelo et al. about molecular insights into O-linked glycan utilization by gut microbes, where the authors focus on the distinct molecular mechanism of consumption of these compounds from mucin and casein glycomacropeptide (GMP), highlighting the potential of these structures as emerging prebiotics.

Finally, this Research Topic shows significant advances made in the modulation of the gut microbiota in humans and animals, showing that the gut microbiota plays a fundamental role in health. On the other hand, the discoveries of microorganisms and key metabolites that make up the gut microbiota seem to us a future perspective for the development of effective clinical therapies. In this way, the results presented here may pave the way for creating effective clinical strategies using probiotics and bioactive compounds.

AUTHOR CONTRIBUTIONS

KS: writing, review, and edit. SS-A: writing. AB: writing and conceptualization. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors would like to thank Ibero-American Program of Science and Technology for Development, Red Iberoamericana de Aprovechamiento Integral de Alimentos Autóctonos Subutilizados (ALSUB-CYTED, 118RT0543).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Microbiology

REVIEW
published: 24 July 2020
doi: 10.3389/fmicb.2020.01662

Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements

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Still relevant after 19 years, the FAO/WHO definition of probiotics can be translated into four simple and pragmatic criteria allowing one to conclude if specific strains of microorganisms qualify as a probiotic for use in foods and dietary supplements. Probiotic strains must be (i) sufficiently characterized; (ii) safe for the intended use; (iii) supported by at least one positive human clinical trial conducted according to generally accepted scientific standards or as per recommendations and provisions of local/national authorities when applicable; and (iv) alive in the product at an efficacious dose throughout shelf life. We provide clarity and detail how each of these four criteria can be assessed. The wide adoption of these criteria is necessary to ensure the proper use of the word probiotic in scientific publications, on product labels, and in communications with regulators and the general public.

Keywords: probiotic definition, criterion, live microbes, *Lactobacillus*, *Bifidobacterium*, identification, safety, health efficacy

OPEN ACCESS

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This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *18 May 2020*
Accepted: *25 June 2020*
Published: *24 July 2020*

Citation:
Binda S, Hill C, Johansen E, Obis D, Pot B, Sanders ME, Tremblay A and Ouwehand AC (2020) Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements. Front. Microbiol. 11:1662. doi: 10.3389/fmicb.2020.01662

INTRODUCTION

Consumers are increasingly interested in maintaining health through food and dietary supplements. Use of evidence-based approaches to improve diets and lifestyles is a trend that continues to grow. This has generated an ever more varied market of foods and supplements, especially those containing probiotics. An expert consultation convened under the umbrella of the World Health Organization (WHO) and the Food and Agriculture Organization proposed a useful definition of probiotics in 2001, which was later refined in 2014 for grammatical reasons to "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). Hill et al. (2014) stipulated that probiotics must have "defined contents, appropriate viable count at end of shelf life and suitable evidence for health benefits," and further stated that all probiotics must be "safe for their intended use." These points were reiterated in 2018 by the International Scientific Association of Probiotics and Prebiotics (ISAPP) in a position statement (ISAPP, 2018). Even so, while the term "probiotic" is used widely in both food and supplement categories, it is often misused. Here we provide clarity to the minimum criteria needed for the proper use of the term probiotic. This is especially useful at a time when new "biotic" names are being introduced into the global vernacular (e.g., terms such as pharmabiotic, psychobiotic, postbiotic, synbiotic, and others).

Every part of the probiotic definition is important and can be "transposed" into easy-to-use criteria. Defining these criteria has been a key objective of different stakeholders in the probiotic field. These criteria can be presented as a decision tree, shown in **Figure 1**, serving as a tool for determining whether or not a candidate strain, or combination of strains, qualifies for probiotic status regardless of the final application. Further, they can be presented in a checklist fashion, such as in this ISAPP infographic (ISAPP, 2019) or as a list of "commandments" as has been suggested by Toscano et al. (2017).

The definition of probiotic contained herein is not restricted to traditional probiotics. Certainly, innovation will lead to candidate probiotics being isolated from novel sources, with currently unanticipated functions, and exciting, new health benefits. These so-called next generation probiotics, which may be conceptualized in some cases as live biotherapeutics, are not precluded under this definition. However, depending on the intended use, appropriate safety, legal and ethical matters must be addressed in the development of such probiotics, such as complying with the Nagoya protocol (Johansen, 2017) where applicable and in the case of isolating microbes from humans ensuring appropriate informed consent.

Notwithstanding earlier publications, there is a need to clearly and meticulously stipulate these criteria and provide details about achieving them without going into the specifics of potential mechanistic requirements. In short, probiotic strains must be (i) sufficiently characterized; (ii) safe for the intended use; (iii) supported by at least one positive human clinical trial conducted according to generally accepted scientific standards; and (iv) alive in sufficient numbers in the product at an efficacious dose throughout shelf life.

PROBIOTIC IS SUFFICIENTLY CHARACTERIZED

The key component of correct probiotic characterization is proper strain identification and naming. Probiotic strains should be named according to the currently valid bacterial nomenclature, based on the International Code of Nomenclature (Parker et al., 2019). An updated list of prokaryotic names with standing in nomenclature is available at http://www.bacterio.net/ (Parte, 2018).

Identification of probiotic microbes entails determining that a strain belongs to an established, named genus and species, and subspecies for species in which subspecies have been described. Since some probiotic activities might be strain specific, a proper typing of the strain is furthermore required. Proper strain designation is therefore composed of two main parts: the official genus, species (and subspecies) names, according to the nomenclatural rules, followed by a strain designation which could be the catalog number of a recognized culture collection or a commercial strain designation. For this purpose, we recommend that the strain should be deposited in a recognized culture collection, for safe-keeping and so that the strain is available for research purposes, but not necessarily for commercial use. The use of multiple strain designations for a single strain should be avoided as this is a cause of confusion. Manufacturers should also ensure maintenance of genetic purity of their strains so that products contain the same strain with the same properties over time.

Identification technologies may vary according to the organism and will develop over time, but several molecular methods are currently widely applicable as phenotypic techniques alone are insufficient for proper identification. Sequencing of the 16S ribosomal DNA is a well-known and reliable way to identify species, assuming reliable reference sequences are used. The sequence obtained can first be matched to large reference databases that cover almost the entire known bacterial diversity, but final validation is preferably done using curated databases such as PATRIC (Wattam et al., 2017). In the case of doubt or for a more detailed identification, involving other molecular and phenotypic traits, Bergey's Manual of Determinative Bacteriology can be consulted.[1] Further, Mattarelli et al. (2014) described the "Recommended minimal standards for description of new taxa of the genera *Bifidobacterium*, *Lactobacillus* and related genera." This is a useful resource for techniques that can be applied to proper identification as well.

The "gold standard" for strain identification is whole genome sequencing (WGS) including any extrachromosomal elements. A fully sequenced genome allows the identification of microbes to the species and strain level. Reference databases are available, e.g., at NCBI.[2] Having a complete genome sequence has many advantages as it allows robust and precise strain specific identification and facilitates a search for the presence or absence of risk factors (see section "Probiotic is Safe for Intended Use" on safety). Furthermore, it will help to identify possible plasmids, common in some lactic acid bacteria and possibly important for probiotic activity. While WGS is the preferred method, other typing methods may allow comparison of individual strains. Multi-locus sequence typing or pulsed-field gel electrophoresis allow comparison of strains, but not *de novo* identification of species or genus. Identification should preferably be done by specialized or appropriately accredited laboratories, which can access the required reference databases and use current validated and calibrated methods and equipment. It should also be stressed that for probiotic mixtures, it is important that each individual strain in the mixture is properly identified, especially when each strain can be eligible for status as a probiotic.

The characterization of probiotic strains should support their probiotic activity. While clinical outcomes are required for a claim of probiotic functionality (see section "Probiotic is Supported by at Least one Human Clinical Trial According to Generally Accepted Scientific Standards" regarding clinical evidence), testing for characteristics considered important for probiotic efficacy could be indicative of possible mechanisms that underlie the observed clinical findings. Such characterizations could include survival at relevant body sites, the production of lactic acid or other short chain fatty acids, adhesion to mucus or intestinal epithelial cells, interaction with human immune cells, resistance to digestive enzymes, bile or acid,

[1] https://www.bergeys.org/

[2] http://www.ncbi.nlm.nih.gov/genbank/

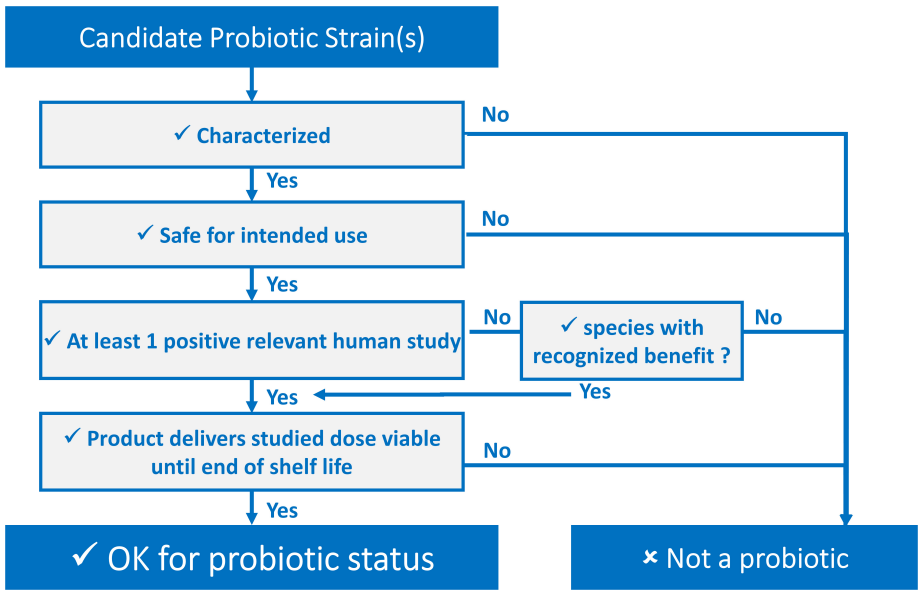

FIGURE 1 | Decision tree to determine if a candidate probiotic fulfills the definition criteria.

antibacterial activity via competitive exclusion or production of bacteriocins or hydrogen peroxide. To this end, the Belgian Superior Health Council published a useful report on approaches to characterize probiotics (Huys et al., 2013), focusing on identification, strain typing and safety assessment. However, it must be emphasized that such phenotypic characterization is not a requirement for probiotic status. These assays may provide indications of function or be useful in initial screening strategies, but they are not validated biomarkers of probiotic functionality. Strains possessing any or all of these characteristics cannot use the term probiotic solely based on the presence of these genotypes or phenotypes.

Recent meta-analyses have confirmed that available evidence for some health effects is most rigorously linked to specific strains of probiotics (McFarland and Evans, 2018; van den Akker et al., 2018). In some instances, health benefits may not be limited to specific strains, but can be shared among wider taxonomic groups (Sanders et al., 2017). This function may be linked to a single property of the probiotic microbe. An example is the presence of the enzyme lactase in the case of mediation of lactose intolerance symptoms. The presence of a trait such as this may be species rather than strain-specific. Even in the case of a health benefit mediated by a shared trait, the probiotic must be identified to the strain level.

PROBIOTIC IS SAFE FOR INTENDED USE

Providing consumers with foods and dietary supplements that meet applicable safety standards is a basic responsibility of probiotic manufacturers. Establishing that a specific probiotic is safe for use in foods and dietary supplements requires, as a starting point, proper identification to the strain level, and further documenting safe use through historical evidence or experimentation. Historical data of safe use can be an important factor in an overall assessment of safety for an intended use. In its absence, safety must be determined based on scientific principles, including the conduct of adequate phase 1 studies (Brodmann et al., 2017). Strain safety is assessed on a case-by-case basis and no specific requirements for sufficient evidence can be made, but a probiotic strain needs to comply with the safety requirements stipulated by the national/regional regulator as discussed below.

Probiotic Species and Strains Must Be Safe for Human Consumption

For daily use of probiotics by the general healthy population, potential safety concerns arising from the administration of live micro-organisms must be addressed. Many species of lactic acid bacteria, bifidobacteria and yeasts, representing most of the commercially available probiotic strains, are judged to be safe for use in foods and supplements. This is because they belong to genera and species with a documented history of safe use, either as probiotics or as starter cultures (Bourdichon et al., 2018). Going beyond history of safe use, the European Food Safety Authority (EFSA) has maintained lists of species presumed to be safe for human consumption in foods under the "Qualified Presumption of Safety" (QPS) concept since 2007 (EFSA, 2007). The QPS approach is an evidence-based, thorough and regularly updated approach to communicate on the safety of specific species of micro-organisms. The list is a reasonable basis for establishing safety of food strains when belonging to a QPS species, provided that the strain-specific testing described below is also conducted. The list results from historical data, from regular monitoring of the body of knowledge and through extensive scientific literature reviews, applied to a wide array of

micro-organisms traditionally found in the food chain (EFSA, 2020b). It should be noted that the scope of QPS is food consumption by the general, healthy population and does not specifically take into consideration potential risks for vulnerable populations (EFSA, 2005) or non-food uses of probiotics. In addition, the QPS list is not exhaustive, as it is based on submissions to EFSA for premarket approval in the EU market, and many microorganisms used in traditional fermented foods are not included in the list (Bourdichon et al., 2018). In Europe, if a strain does not belong to a QPS species, the Novel Food regulation (EU, 2015) may apply before it can be brought to market. Other jurisdictions have other procedures to assess safety of probiotics, such as the generally recognized as safe (GRAS) regulation in the United States.

Species- and Strain-Specific Safety Criteria for Probiotics

Any safety evaluation is predicated on proper species identification, according to the principles outlined in section "Introduction." In addition, identification of genus or species-specific risk factors, and testing at strain level is required. Most important among these is the absence of acquired antimicrobial resistance genes or known virulence factors. Within the EU, EFSA has issued several guidelines describing phenotypic cut-off susceptibility and resistance values for relevant antibiotics and methods for determining these (EFSA, 2018). The guidelines should also be used for the assessment of bacterial and antimycotic susceptibility for yeasts (EFSA, 2018). Standardized analytical methods are available for the phenotypic screening of candidate bacterial strains (ISO-IDF, 2010). If resistance above cut-off values is observed, further characterization is required. WGS of the strain will confirm the presence or absence of known genes involved in the observed resistance. In cases where putative resistance genes are detected, it is recommended to determine if transposable elements are in their genomic vicinity. If this is the case it cannot be excluded that the resistance gene is transferable, and commercialization of the strain is not recommended. Otherwise, the genome sequence can assist in identifying putative antimicrobial resistance genes by searching at least two databases. For microorganisms not well-represented in databases, a Hidden-Markov model database is recommended. Depending on their taxonomy and their intended use, the strain's genome may need to be assessed for the presence of genes coding for known virulence factors such as toxins, invasion, and adhesion factors (EFSA, 2020a). In those cases where antibiotic resistance cut-off values are not known, it would be the responsibility of the producer to ensure that the proposed probiotic strain(s) do not contain transferrable antibiotic resistance genes, and that the resistance profile is consistent with other members of the same species. In some cases it may be necessary to generate new data on the susceptibility profiles of the considered taxon, including making sure that susceptibility testing methods are relevant and adapted to the physiology of the considered micro-organisms.

Other phenotypic properties may be assessed at strain level for safety, such as the ability to form biogenic amines and D-lactate. Both can be conveniently tested through analysis of the genome or through standardized phenotypic tests. In addition, hemolytic activity and bile salt hydrolase activity are sometimes assessed at strain level, but their relevance to safety remains to be determined (Huys et al., 2013).

In vivo Safety Tests

In the case of most current probiotics belonging to QPS species and with a documented history of safe use in foods, the value of *in vivo* safety tests is unclear, especially given the European Union's position stating that for ethical and efficiency reasons, unnecessary research should not be performed on animals (EU, 2010). Little or no effect from QPS species is to be expected in healthy animal models, such as mice or rats (Shokryazdan et al., 2016).

Human intervention studies on the other hand allow for proper documentation of safety and tolerance of probiotics through rigorous monitoring and reporting of adverse events. Biological and clinical parameters, including vital signs, can be monitored to collect valuable safety data. Unexpected deviations from baseline or standard values might indicate a possible safety concern. Documenting these safety endpoints must be performed during any type of clinical intervention, analyzed and reported according to accepted scientific standards for human studies. Sponsors, investigators, authors, and journal editors should facilitate the systematic reporting of safety and tolerance data in human clinical interventions for probiotics. It should be noted that any study, particularly studies of longer duration and involving large numbers of subjects, will surely observe adverse events. The key point is to determine if the adverse events are different between the intervention groups (i.e., probiotic and placebo) and/or are considered to be intervention-related. To date, only rare, mild, and transient probiotic-related adverse events have been reported in studies with healthy subjects (Goldenberg et al., 2017). Specific sensitive populations exist (e.g., the young, old, pregnant, and immune compromised population) and medical supervision of probiotic intervention and use is advised in such populations (Sanders et al., 2016).

With a significant number of strains from *Lactobacillus*, *Bifidobacterium*, and yeast species having a long history of safe use and having been the subject of thorough assessments and monitoring, it can be concluded that there are no major safety concerns for their use in foods and dietary supplements for the general population. Safety evaluations focus on the intended use, which here is food and dietary supplements; other uses may have different safety requirements e.g., depending on their delivery format or dose.

PROBIOTIC IS SUPPORTED BY AT LEAST ONE HUMAN CLINICAL TRIAL ACCORDING TO GENERALLY ACCEPTED SCIENTIFIC STANDARDS

The ability to confer a health benefit to the host is a fundamental part of the definition of a probiotic since 2001 (FAO/WHO, 2002)

and was reaffirmed in 2014 (Hill et al., 2014). By health benefit we here mean a positive effect on some measure of a person's health from, in this case, the use of probiotics. This phrasing is non-proscriptive by design, to allow innovation in exploring any number of possible health endpoints. At least one human trial demonstrating a health benefit is required to qualify the candidate microbial strain(s) for probiotic status, preferably followed by confirmatory trial(s). Herein, we qualify this requirement by stating that the trial must be conducted according to generally accepted scientific standards. In rare circumstances, as recognized by certain authorities, the term "probiotic" may be appropriately used by strains of a species (or other taxonomic group), where several members of that species have been shown to confer a benefit driven by a shared mechanism (Hill et al., 2014; Sanders et al., 2017). For example, lactase activity expressed by strains of *Streptococcus thermophilus* or *L. delbrueckii* subsp. *bulgaricus*, which leads to reduced symptoms associated with lactose maldigestion, is a common property of these species. Strains of *S. thermophilus* and *L. bulgaricus* can be considered "probiotic" based on this benefit. Further, to correctly use the term "probiotic" to describe such strains, the organism must be identified at strain level and shown to express the relevant trait. A valid demonstration of a health benefit depends both on the quality and soundness of the trial itself (i.e., how well it was designed and conducted) and on the capacity of the scientific community to critically appraise published trial results (i.e., how well it was reported). Several tools exist to facilitate the design, reporting, risk of bias (RoB) assessment and critical appraisal of clinical trials used to support probiotic status (**Table 1**).

TABLE 1 | Tools to facilitate design, reporting, managing risk of bias, and critical appraisal of human intervention studies with probiotics.

Tool name	References and links
Clinical trial protocol design guidelines	
International council for harmonization of technical requirements for pharmaceuticals for human use E6 (R2)	https://database.ich.org/sites/default/files/E6_R2_Addendum.pdf
Statistical principles for clinical trials E9	https://database.ich.org/sites/default/files/E9_Guideline.pdf
Structure and content of clinical study reports E3	https://database.ich.org/sites/default/files/E3_Guideline.pdf
SPIRIT 2013	https://www.spirit-statement.org/ https://www.spirit-statement.org/publications-downloads/
Critical appraisal tools (quality assessment)	
JBI checklists (Joanna Briggs Institute, 2017)	(Tufanaru et al., 2017) https://joannabriggs.org/ebp/critical_appraisal_tools, https://joannabriggs.org/sites/default/files/2019-05/JBI_RCTs_Appraisal_tool2017_0.pdf
CASP checklists (Critical appraisal skills program, 2018)	https://casp-uk.net/wp-content/uploads/2018/01/CASP-Randomised-Controlled-Trial-Checklist-2018.pdf
Critical appraisal checklists (SURE, 2018)	https://www.cardiff.ac.uk/__data/assets/pdf_file/0005/1142969/SURE-CA-form-for-RCTs-and-other-experimental-studies_2018.pdf
BMJ Best practice toolkit (BMJ publishing group limited, 2019)	https://bestpractice.bmj.com/info/us/toolkit/learn-ebm/appraising-2-armed-randomized-controlled-trials/
SIGN checklists and notes (Scottish Intercollegiate Guidelines Network, 2001-2019)	(Harbour and Miller, 2001) https://www.sign.ac.uk/assets/checklist_for_controlled_trials.doc
Critical appraisal tools (Centre for Evidence-Based Medicine, 2020)	https://www.cebm.net/wp-content/uploads/2018/11/RCT.pdf
EQUATOR (Enhancing the quality and transparency of health research) Network	https://www.equator-network.org
Risk of Bias assessment tool	
RoB 2 tool	(Sterne et al., 2019) https://methods.cochrane.org/bias/resources/rob-2-revised-cochrane-risk-bias-tool-randomized-trials, https://sites.google.com/site/riskofbiastool/welcome/rob-2-0-tool
Reporting guidelines	
CONSORT 2010	(Schulz et al., 2010) http://www.consort-statement.org/consort-2010

Considerations for Protocol Design

Recognized guidelines for clinical trial design (and conduct) were originally developed to ensure participants' welfare and ethical trial conduct and have been available and often mandatory for several decades, e.g., the Good Clinical Practice guidelines of the International Council for Harmonisation (ICH-GCP) and country-specific, legally binding versions (Vijayananthan and Nawawi, 2008). Compliance to ICH-GCP guidelines, in addition to the unequivocal ethical value it provides, also contributes to ensuring the generation of higher quality and more reliable data. An internationally recognized tool, endorsed by journals, funders, regulators and academic institutions worldwide, was developed specifically for the design of trial protocols that comply with the recommendations of the ICH-GCP, the WHO, and the International Committee of Medical Journal Editors (ICMJE). The "Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT)" 2013 checklist contains a list of 33 elements that should be included in all clinical trial protocols (Chan et al., 2013). In accordance with the SPIRIT 2013 checklist (Item 2), the publication of the study protocol in a public database (e.g., ClinicalTrials.gov) prior to the start of the study is highly recommended[3] and is viewed as a way of fostering the design of higher-quality studies while contributing to more transparent reporting of results. Furthermore, the publication of clinical trial protocols in peer-reviewed journals, which usually require that the protocol should be registered in a public registry, also constitutes a good practice that should be further encouraged in the probiotics field.

From a scientific standpoint, several trial design challenges frequently appear to prevent drawing formal conclusions about a health benefit in probiotics trials (Brussow, 2019). These may include details of the design of the study [randomized

[3]http://www.icmje.org/news-and-editorials/clin_trial_sep2004.pdf

controlled trials (RCTs) vs. non-randomized trials; cross-over vs. parallel arms design], the participant's allocation concealment, blinding (double-blind vs. single-blind or open label), the choice of controls (placebo vs. comparator treatment), the dosing and administration regimen (concentration used, administration schedule, start, and duration of the supplementation period), power calculations for the primary outcome, or the choice of population (health status, age, and gender). Due the inherent specificity of candidate microbial strains, no guideline specific for the whole probiotics field can be developed regarding the preference for a certain study design type, or of a specific dosing regimen over another. However, a careful consideration of these parameters in parallel with the microbial strain and target population characteristics is warranted at the study design stage (Shane et al., 2010). To this end, it may be helpful to gain prior knowledge of the accepted standards of trial reporting as well as of the tools available for the critical appraisal of published trials (**Table 1**).

Considerations for Trial Reporting

Several international journals require authors to report the results of their trials according to an established and recognized set of guidelines, namely The Consolidated Standards of Reporting Trials (CONSORT), which has become the mainstay for reporting and publishing trial results (Schulz et al., 2010). While the 25-point CONSORT checklist was not created as a guideline for trial design and conduct, prior knowledge of the elements that must be reported can facilitate the design. The CONSORT 2010 checklist was considered during the development of the SPIRIT 2013 guideline for protocol design to facilitate the passage from SPIRIT-compliant protocol to a CONSORT-compliant report (Chan et al., 2013).

Compliance with the CONSORT 2010 guidelines for reporting trials will facilitate subsequent critical appraisal of the results and contribute to generating stronger conclusions from meta-analyses and systematic reviews. Briefly, CONSORT covers all aspects of trial design and conduct, data collection and analyses, as well as reporting. For example, CONSORT requires a participant flowchart explicitly stating numbers of participants for each step from recruitment to study completion, the exclusions, losses to follow up, and sizes of the intent-to-treat or per protocol populations. This information is crucial for future quality and RoB assessments needed afterward (e.g., systematic reviews for evidence-based medicine guidelines or regulatory purposes). CONSORT also stipulates that the authors should highlight the limitations of their study, such as the sources of bias and uncertainties that may influence the interpretation of the results. A description of results generalizability is expected, as well as a clear perspective of health benefits versus risks (implying a detailed reporting of the adverse events).

Critical Appraisal and RoB Assessment of Published Trials

Critical appraisal and RoB assessment of clinical studies are important components of evidence-based medicine. They allow the determination, in an objective manner, of the weight of a trial's findings (Buccheri and Sharifi, 2017). Numerous tools have been developed for these purposes, which are mostly designed for authors of systematic reviews and meta-analyses or best practice guidelines but can be useful when assessing the quality of trials that have been published without the use of a reporting guideline such as CONSORT 2010. This concerns trials published before 2010, but unfortunately also a number of more recent trials. Compliance to the CONSORT 2010 guidelines remains low in the medical literature in general (Jin et al., 2018).

The difference between critical appraisal (i.e., quality assessment) tools and RoB assessment tools may be considered as ambiguous, but the two approaches are clearly distinct. For example, the RoB assessment tools used by authors of Cochrane reviews are designed to address whether the results of the trial are free of bias and credible (Higgins et al., 2019; Sterne et al., 2019). On the other hand, quality assessment tools often include parameters relating both to reporting quality (e.g., obtaining ethical approval or describing power calculations) as well as to the quality, transparency, and consistency of the research (e.g., randomization and allocation concealment, proper control selection, and missing outcome data). The latter parameters are directly related to potential sources of bias assessed by RoB tools. Generally, the weight attributed to the results of a trial is proportional to how efficiently sources of bias have been avoided (Higgins et al., 2019). Considering that lack of randomization, blinding or controls are identified as significant sources of bias in clinical trials, double-blind RCTs have become the "gold standard" design to demonstrate health benefits in a reliable manner, as demonstrated by the higher score attributed to RCTs over other designs when grading the quality of evidence from clinical trials (Guyatt et al., 2008). Other study designs, such as open-label and uncontrolled studies, are possible and have been used in the past. While the results from such studies may not be robust enough to be used alone to qualify a probiotic designation, they can provide useful supportive documentation.

PROBIOTIC IS ALIVE IN THE PRODUCT AT AN EFFICACIOUS DOSE THROUGHOUT SHELF LIFE

While the three previously described criteria refer specifically to a microbial strain to be considered as a probiotic, this fourth criterion applies to the product that delivers the probiotics. The definition of probiotics does not include a reference to a specific dose, but rather states that probiotics should be administered in amounts that are adequate to result in a health benefit for the host. Thus, it is conceivable, given that probiotics are living microbes capable of self-replication within the host, that over time a few probiotic cells could be sufficient to elicit a beneficial effect if they grow sufficiently within the host. This is certainly true for pathogenic microbes causing disease, which can cause deleterious effects on host health at extremely low doses because of their virulence and capacity to replicate within the host.

Dose ranging studies were intended to determine the tolerability, efficacy, and safety profile of an active substance that can be delivered in fixed concentrations and that normally

cannot multiply post-administration (Ting, 2006). Consequently, dose ranging studies are a common feature in clinical trials but are less common in food and dietary supplement trials. This is largely a result of the presumption of safety for food ingredients. In clinical settings, dose ranging studies are usually performed after the maximum tolerable dose (MTD) has been elucidated for the bioactive under investigation. We are not aware of any oral MTD study that has been performed in humans for any probiotic strain or strain combination (section "Probiotic is Sufficiently Characterized"). Given that probiotics have an excellent safety profile and the fact that they have rarely been subjected to either MTD or dose ranging studies, it is common for most studies to simply choose a daily dose between 10^8 and 10^{11} colony forming units (CFU), which reflect effective doses in past studies. While there may be an interest in determining an optimal dose that leads to a specific health benefit, this is not an essential criterion.

Quantification of the viability of probiotic strains should be done using standardized enumeration methods such as plating; CFU counting on selective growth media, e.g., for *Lactobacillus acidophilus* (ISO 20128; **Table 2**) and *Bifidobacterium* (ISO 29981 or IDF 220:2010; **Table 2**) or by flow cytometry (ISO 19344:2015; **Table 2**). Methods have been reviewed by various authors (Davis, 2014; Zielińska et al., 2018). The viability of probiotic strains at the efficacious dose should be documented in the test products during clinical investigations and guaranteed until the end of shelf life in commercial products according to quality procedures.

For the quantification of probiotic combinations, culture-independent metagenomics methods based on high-throughput next-generation sequencing have been developed (Patro et al., 2016) although these methods do not ensure that what is counted is viable. These methods can reveal interesting information on potential contaminants (Quigley et al., 2013) but may lack methodological validation (Sohier et al., 2014). They can, however, be performed by accredited laboratories which guarantees a certain level of consistency and reproducibility. Molecular methods to determine viability in complex mixtures are under development such as e.g., propidium monoazide (PMA)-PCR (Scariot et al., 2018). These are, however, experimental and not standardized.

In general, fecal recovery is often used as a surrogate marker to reflect sufficient dose for gastrointestinal health targets. Dose ranging could be possible for those probiotics which have a readily determined endpoint of efficacy (e.g., serum cholesterol levels), although once again the issue of replication *in situ* could cause problems with interpretation. One study in which dose ranging was performed was an IBS trial involving *Bifidobacterium longum* subsp. *infantis* 35624 in which three doses were tested, 10^6, 10^8, and 10^{10} CFU. This had an interesting outcome in that the 10^8 CFU dose was effective, whereas the other two doses were not. This anomalous result was likely because the capsules containing the highest dose did not dissolve, and therefore only the results of the other two doses could be assessed (Whorwell et al., 2006).

An alternative to classic dose ranging studies is to examine the large body of documented probiotic trials in humans and calculate the doses used in each study and the clinical outcomes. Such an analysis was recently performed, and the conclusions were nuanced (Ouwehand, 2017). For some probiotic/health state combinations there was evidence of a clear dose response, but for other combinations the data were not compelling.

If a particular study elicits the desired health benefit, then that dose would serve as the minimum dose for which a health claim should be permitted. Products using a higher dose should be also able to make the same claim, but claims should not be permitted for any dose lower than that tested in humans. Here, we will not further discuss health claims; that belongs in the realm of regulators. While regulators in general adhere to the probiotic definition, they tend to interpret the requirements differently in their respective jurisdictions. An analysis of this falls outside the scope of the current paper.

What influence, if any, the delivery format has on a probiotic effect is an interesting topic. To date, few studies have been conducted on the direct comparison of a probiotic delivered in different matrices related to the same clinical endpoint. Two reviews have addressed the matter with one concluding that there is currently no evidence that the delivery matrix has a substantial effect on probiotic efficacy (Sanders et al., 2014) and the other concluded that there may be strain dependent matrix effects (Flach et al., 2018). Both papers agree that data on the topic is scarce.

TABLE 2 | Examples of standard methods for probiotic enumeration.

Standard	Taxon name	Web pages
ISO 20128:2006	*Lactobacillus acidophilus*	https://www.iso.org/obp/ui/#iso:std:iso:20128:ed-1:v1:en
ISO 29981 = IDF 220:2010	*Bifidobacterium*	https://www.iso.org/standard/45765.html
ISO 19344:2015 = IDF 232	Milk and milk products - Starter cultures, probiotics and fermented products - Quantification of lactic acid bacteria by flow cytometry	https://www.iso.org/obp/ui/#iso:std:iso:19344:ed-1:v1:en https://www.iso.org/standard/64658.html

CONCLUSION

Probiotics are the subject of global investigative research, innovative product design, effective marketing, regulatory scrutiny, focused consumer interest and use by healthcare practitioners. It would be beneficial to all involved in these undertakings to clearly understand the criteria needed for the word "probiotic" to be used responsibly. This paper describes the minimum criteria that apply to a probiotic strain that will be used in foods and dietary supplements and similar criteria may be applicable to other uses of probiotics. Specifically, the strain must be identified using recognized scientific methods, named according to valid current nomenclature, and named with a retrievable strain designation. Methods will vary depending on species of the probiotic and are likely to change as technologies evolve. Also, we recommend that it should be deposited in an international culture collection. Further, the strain must have demonstrated safety for its intended use and a demonstrated health benefit based on at least one study

that meets generally accepted scientific standards or as per recommendations and provisions of local/national authorities when applicable. Sufficient levels of the probiotic strain(s) must be contained in the final product throughout the shelf life in order to be able to deliver the claimed (and evidence-based) health benefit. Products should be manufactured according to applicable good manufacturing requirements to assure safety, purity, and stability (Jackson et al., 2019) and should be labeled in a manner that communicates essential information on product contents (specific strains, level of live probiotic delivered at end of shelf life, and statements about health benefits as allowed) to the end-user. Adherence to these principles will assure that the marketplace does not contain products that misuse the term "probiotic." Some local regulatory contexts can define probiotics in a different manner, but it is the responsibility of the product manufacturer to produce and market probiotics that follow local rules and regulations and are in line with the above defined principles.

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization, wrote sections for the manuscript, reviewed and edited the manuscript, and read and agreed to the final version of the manuscript.

REFERENCES

Bourdichon, F., Alper, I., Bibiloni, R., Dubois, A., Laulund, S., Miks, M., et al. (2018). Inventory of microbial food cultures with safety demonstration in fermented food products. *Bull. Int. Dairy Federat.* 495, 5–71.

Brodmann, T., Endo, A., Gueimonde, M., Vinderola, G., Kneifel, W., de Vos, W. M., et al. (2017). Safety of novel microbes for human consumption: practical examples of assessment in the European Union. *Front. Microbiol.* 8:1725. doi: 10.3389/fmicb.2017.01725

Brussow, H. (2019). Probiotics and prebiotics in clinical tests: an update. *F1000Res* 8:F1000 Faculty Rev-1157. doi: 10.12688/f1000research.19043.1

Buccheri, R. K., and Sharifi, C. (2017). Critical Appraisal Tools and Reporting Guidelines for Evidence-Based Practice. *Worldviews Evid. Based Nurs.* 14, 463–472. doi: 10.1111/wvn.12258

Chan, A. W., Tetzlaff, J. M., Altman, D. G., Laupacis, A., Gotzsche, P. C., Krleza-Jeric, K., et al. (2013). SPIRIT 2013 statement: defining standard protocol items for clinical trials. *Ann. Intern. Med.* 158, 200–207. doi: 10.7326/0003-4819-158-3-201302050-201302583

Davis, C. (2014). Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. *J. Microbiol. Methods* 103, 9–17. doi: 10.1016/j.mimet.2014.04.012

EFSA (2005). Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives. *EFSA J.* 3:226. doi: 10.2903/j.efsa.2005.226

EFSA (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA J.* 5:587. doi: 10.2903/j.efsa.2007.587

EFSA (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA J.* 16:5206. doi: 10.2903/j.efsa.2018.5206

EFSA (2020a). *EFSA Statement on the Requirements for Whole Genome Sequence Analysis of Microorganisms Intentionally Used in the Food Chain*. Parma: EFSA.

EFSA (2020b). Scientific Opinion on the update of the list of QPS recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). *EFSA J.* 18:5965. doi: 10.2903/j.efsa.2020.5965

EU (2010). *On the Protection Of Animals Used for Scientific Purposes*, ed. E. Parliament, (Brussels: European Parliament).

EU (2015). *On novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of theCouncil and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001*, ed. E. Parliament, (Brussels: EU).

FAO/WHO (2002). *Guidelines for the Evaluation Of Probiotics in Food*. Paris: FAO, 1–11.

Flach, J., van der Waal, M. B., van den Nieuwboer, M., Claassen, E., and Larsen, O. F. A. (2018). The underexposed role of food matrices in probiotic products: reviewing the relationship between carrier matrices and product parameters. *Crit. Rev. Food Sci. Nutr.* 58, 2570–2584. doi: 10.1080/10408398.2017.1334624

Goldenberg, J. Z., Yap, C., Lytvyn, L., Lo, C. K., Beardsley, J., Mertz, D., et al. (2017). Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. *Cochrane Database. Syst. Rev.* 12:CD006095. doi: 10.1002/14651858.CD006095.pub4

Guyatt, G. H., Oxman, A. D., Vist, G. E., Kunz, R., Falck-Ytter, Y., Alonso-Coello, P., et al. (2008). GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *BMJ* 336, 924–926. doi: 10.1136/bmj.39489.470347.AD

Harbour, R., and Miller, J. (2001). A new system for grading recommendations in evidence based guidelines. *BMJ* 323, 334–336. doi: 10.1136/bmj.323.7308.334

Higgins, J. P. T., Thomas, J., Chandler, J., Cumptson, M., Li, T., Page, M. J., et al. (2019). *Cochrane Handbook for Systematic Reviews of Interventions*. London: Cochrane.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Huys, G., Botteldoorn, N., Delvigne, F., De Vuyst, L., Heyndrickx, M., Pot, B., et al. (2013). Microbial characterization of probiotics–advisory report of the Working Group "8651 Probiotics" of the Belgian Superior Health Council (SHC). *Mol. Nutr. Food Res.* 57, 1479–1504. doi: 10.1002/mnfr.201300065

ISAPP (2018). *Minimum Criteria for Probiotics [Online]*. Sacramento, CA: International Scientific Association for Probiotics and Prebiotics.

ISAPP (2019). *Probiotic Checklist: Making a Smart Selection [Online]*. Sacramento, CA: International Scientific Association of Probiotics and Prebiotics.

ISO-IDF, (2010). *Milk and Milk Products — Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-Enterococcal Lactic Acid Bacteria (LAB)*. London: ISO.

Jackson, S. A., Schoeni, J. L., Vegge, C., Pane, M., Stahl, B., Bradley, M., et al. (2019). Improving End-User Trust in the Quality of Commercial Probiotic Products. *Front. Microbiol.* 10:739. doi: 10.3389/fmicb.2019.00739

Jin, Y., Sanger, N., Shams, I., Luo, C., Shahid, H., Li, G., et al. (2018). Does the medical literature remain inadequately described despite having reporting guidelines for 21 years? - A systematic review of reviews: an update. *J. Multidiscip. Healthc.* 11, 495–510. doi: 10.2147/JMDH.S155103

Johansen, E. (2017). Future access and improvement of industrial lactic acid bacteria cultures. *Microb. Cell Fact.* 16:230. doi: 10.1186/s12934-017-0851-851

Mattarelli, P., Holzapfel, W., Franz, C. M., Endo, A., Felis, G. E., Hammes, W., et al. (2014). Recommended minimal standards for description of new taxa of the genera Bifidobacterium, Lactobacillus and related genera. *Int. J. Syst. Evol. Microbiol.* 64(Pt 4), 1434–1451. doi: 10.1099/ijs.0.060046-60040

McFarland, L. V., and Evans, C. T. (2018). Goldstein strain-specificity and disease-specificity of probiotic efficacy: A systematic review and meta-analysis. *EJC. Front. Med. (Lausanne)* 5:124. doi: 10.3389/fmed.2018.00124

Ouwehand, A. C. (2017). A review of dose-responses of probiotics in human studies. *Benef. Microbes* 8, 143–151. doi: 10.3920/BM2016.0140

Parker, C. T., Tindall, B. J., and Garrity, G. M. (2019). International Code of Nomenclature of Prokaryotes. *Int. J. Syst. Evol. Microbiol.* 69, S1–S111.

Parte, A. C. (2018). LPSN - List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *Int. J. Syst. Evol. Microbiol.* 68, 1825–1829. doi: 10.1099/ijsem.0.002786

Patro, J. N., Ramachandran, P., Barnaba, T., Mammel, M. K., Lewis, J. L., and Elkins, C. A. (2016). Culture-Independent Metagenomic Surveillance of Commercially Available Probiotics with High-Throughput Next-Generation Sequencing. *mSphere* 1:e00057-16. doi: 10.1128/mSphere.00057-16

Quigley, L., McCarthy, R., O'Sullivan, O., Beresford, T. P., Fitzgerald, G. F., Ross, R. P., et al. (2013). The microbial content of raw and pasteurized cow milk as determined by molecular approaches. *J. Dairy Sci.* 96, 4928–4937. doi: 10.3168/jds.2013-6688

Sanders, M. E., Benson, A., Lebeer, S., Merenstein, D. J., and Klaenhammer, T. R. (2017). Shared mechanisms among probiotic taxa: implications for general probiotic claims. *Curr. Opin. Biotechnol.* 49, 207–216. doi: 10.1016/j.copbio.2017.09.007

Sanders, M. E., Klaenhammer, T. R., Ouwehand, A. C., Pot, B., Johansen, E., Heimbach, J. T., et al. (2014). Effects of genetic, processing, or product formulation changes on efficacy and safety of probiotics. *Ann. N. Y. Acad. Sci.* 1309, 1–18. doi: 10.1111/nyas.12363

Sanders, M. E., Merenstein, D. J., Ouwehand, A. C., Reid, G., Salminen, S., Cabana, M. D., et al. (2016). Probiotic use in at-risk populations. *J. Am. Pharm. Assoc.* 56, 680–686. doi: 10.1016/j.japh.2016.07.001

Scariot, M. C., Venturelli, G. L., Prudencio, E. S., and Arisi, A. C. M. (2018). Quantification of Lactobacillus paracasei viable cells in probiotic yoghurt by propidium monoazide combined with quantitative PCR. *Int. J. Food Microbiol.* 264, 1–7. doi: 10.1016/j.ijfoodmicro.2017.10.021

Schulz, K. F., Altman, D. G., Moher, D., and Group, C. (2010). CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. *Ann. Intern. Med.* 152, 726–732. doi: 10.7326/0003-4819-152-11-201006010-201006232

Shane, A. L., Cabana, M. D., Vidry, S., Merenstein, D., Hummelen, R., Ellis, C. L., et al. (2010). Guide to designing, conducting, publishing and communicating results of clinical studies involving probiotic applications in human participants. *Gut Microbes* 1, 243–253. doi: 10.4161/gmic.1.4.12707

Shokryazdan, P., Faseleh Jahromi, M., Liang, J. B., Kalavathy, R., Sieo, C. C., and Ho, Y. W. (2016). Safety assessment of two new lactobacillus strains as probiotic for human using a rat model. *PLoS One* 11:e0159851. doi: 10.1371/journal.pone.0159851

Sohier, D., Pavan, S., Riou, A., Combrisson, J., and Postollec, F. (2014). Evolution of microbiological analytical methods for dairy industry needs. *Front. Microbiol.* 5:16. doi: 10.3389/fmicb.2014.00016

Sterne, J. A. C., Savovic, J., Page, M. J., Elbers, R. G., Blencowe, N. S., Boutron, I., et al. (2019). RoB 2: a revised tool for assessing risk of bias in randomised trials. *BMJ* 366:l4898. doi: 10.1136/bmj.l4898

Ting, N. (2006). *Dose Finding in Drug Development*. New York, NY: Springer.

Toscano, M., De Grandi, R., Pastorelli, L., Vecchi, M., and Drago, L. (2017). A consumer's guide for probiotics: 10 golden rules for a correct use. *Dig. Liver Dis.* 49, 1177–1184. doi: 10.1016/j.dld.2017.07.011

Tufanaru, C., Munn, Z., Aromataris, E., Campbell, J., and Hopp, L. (2017). "Systematic reviews of effectiveness," in *Joanna Briggs Institute Reviewer's Manual*, eds E. Aromataris, and Z. Munn, (Adelaide: Joanna Briggs Institute).

van den Akker, C. H. P., van Goudoever, J. B., Szajewska, H., Embleton, N. D., Hojsak, I., Reid, D., et al. (2018). Espghan working group for probiotics, prebiotics & committee on nutrition. probiotics for preterm infants: A strain-specific systematic review and network meta-analysis. *J. Pediatr. Gastroenterol. Nutr.* 67, 103–122, doi: 10.1097/MPG.0000000000001897

Vijayananthan, A., and Nawawi, O. (2008). The importance of Good Clinical Practice guidelines and its role in clinical trials. *Biomed. Imaging Interv. J.* 4:e5. doi: 10.2349/biij.4.1.e5

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., et al. (2017). Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res.* 45, D535–D542. doi: 10.1093/nar/gkw1017

Whorwell, P. J., Altringer, L., Morel, J., Bond, Y., Charbonneau, D., O'Mahony, L., et al. (2006). Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. *Am. J. Gastroenterol.* 101, 1581–1590. doi: 10.1111/j.1572-0241.2006.00734.x

Zielińska, D., Ołdak, A., Rzepkowska, A., and Zieliński, K. (2018). "Enumeration and identification of probiotic bacteria in food matrices," in *Advances in Biotechnology for Food Industry*, eds A. M. Holban, and A. M. Grumezescu, (London: Academic Press), 167–196. doi: 10.1016/b978-0-12-811443-8.00006-2

Conflict of Interest: At the time of writing, SB and DO were employed by Danone Nutricia Research. EJ was employed by Chr. Hansen A/S. BP was employed by Yakult Europe BV. AT was employed by Rosell Institute for Microbiome and Probiotics. AO was employed by DuPont Nutrition and Biosciences. CH conducts academic research funded by commercial companies. MS consults with companies that manufacture probiotics or probiotic-containing products and serves as the executive science officer for ISAPP.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 September 2020
doi: 10.3389/fmicb.2020.574137

Persistence of Suspected Probiotic Organisms in Preterm Infant Gut Microbiota Weeks After Probiotic Supplementation in the NICU

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *19 June 2020*
Accepted: *26 August 2020*
Published: *25 September 2020*

Citation:
Yousuf EI, Carvalho M, Dizzell SE, Kim S, Gunn E, Twiss J, Giglia L, Stuart C, Hutton EK, Morrison KM and Stearns JC (2020) Persistence of Suspected Probiotic Organisms in Preterm Infant Gut Microbiota Weeks After Probiotic Supplementation in the NICU. Front. Microbiol. 11:574137. doi: 10.3389/fmicb.2020.574137

Probiotics are becoming a prevalent supplement to prevent necrotizing enterocolitis in infants born preterm. However, little is known about the ability of these live bacterial supplements to colonize the gut or how they affect endogenous bacterial strains and the overall gut community. We capitalized on a natural experiment resulting from a policy change that introduced the use of probiotics to preterm infants in a single Neonatal Intensive Care Unit. We used amplicon sequence variants (ASVs) derived from the v3 region of the 16S rRNA gene to compare the prevalence and abundance of *Bifidobacterium* and *Lactobacillus* in the gut of preterm infants who were and were not exposed to a probiotic supplement in-hospital. Infants were followed to 5 months corrected age. In the probiotic-exposed infants, ASVs belonging to species of *Bifidobacterium* appeared at high relative abundance during probiotic supplementation and persisted for up to 5 months. In regression models that controlled for the confounding effects of age and antibiotic exposure, probiotic-exposed infants had a higher abundance of the suspected probiotic bifidobacteria than unexposed infants. Conversely, the relative abundance of *Lactobacillus* was similar between preterm groups over time. *Lactobacillus* abundance was inversely related to antibiotic exposure. Furthermore, the overall gut microbial community of the probiotic-exposed preterm infants at term corrected age clustered more closely to samples collected from 10-day old full-term infants than to samples from unexposed preterm infants at term age. In conclusion, routine in-hospital administration of probiotics to preterm infants resulted in the potential for colonization of the gut with probiotic organisms post-discharge and effects on the gut microbiome as a whole. Further research is needed to fully discriminate probiotic bacterial strains from endogenous strains and to explore their functional role in the gut microbiome and in infant health.

Keywords: probiotics, early preterm infants, gut microbiota, *Bifidobacterium*, *Lactobacillus*

Abbreviations: NICU, neonatal intensive care unit; NEC, necrotizing enterocolitis; ASV, amplicon sequence variant.

INTRODUCTION

In healthy full-term infants, bacteria begin to colonize the gut at birth (Perez-Muñoz et al., 2017) and complex microbial communities are formed dynamically as the infant develops (Bäckhed et al., 2015). Preterm birth alters bacterial colonization due to factors including immaturity of the gut environment, exposure to antibiotics, and supplemental feeding with formula (Groer et al., 2014). Infants born preterm are at risk of developing sepsis and necrotizing enterocolitis (NEC), leading causes of mortality and morbidity in this population (Kona and Matlock, 2018). Probiotics, containing strains of *Lactobacillus* sp. and/or *Bifidobacterium* sp. are effective at reducing the incidence of NEC (Deshpande et al., 2010; AlFaleh and Anabrees, 2014; Olsen et al., 2016; Sawh et al., 2016), and may also reduce sepsis in very low birthweight infants (Kona and Matlock, 2018). The mechanisms for how probiotic organisms protect against NEC are largely unknown but may include their ability to increase mucus production, prevent the adherence of enteric pathogens to the gut epithelium (Ewaschuk et al., 2008) and increase barrier function of gut epithelial cells (Mack et al., 1999). Due to their effectiveness against NEC, probiotics are now administered to preterm infants in many neonatal intensive care units (NICUs) around the world. In fact, recent clinical practice guidelines from the American Gastroenterology Association (AGA) suggest that certain probiotic *Lactobacillus* and *Bifidobacterium* strains (and combinations of these strains) be used for the prevention of NEC in preterm and low birth weight infants (Su et al., 2020).

What is not known is whether probiotic strains establish long-term or permanent colonization in the preterm infant gut and if so, what impact such colonization has on infant health and development. Increased DNA from species of *Bifidobacterium* has been seen in preterm infant stool during probiotic supplementation but this often becomes reduced after supplementation has ceased (Li et al., 2004; Mohan et al., 2006; Plummer et al., 2018; Strus et al., 2018). In one small study, a persistent suspected probiotic signal from the genus *Bifidobacterium* (but not *Lactobacillus)* was identified in the post-discharge fecal sample of four infants after discontinuation of a probiotic containing *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (Abdulkadir et al., 2016). No comparator control group was included at the post-discharge timepoint. Therefore, it is unclear if this represented probiotic or endogenous bifidobacterial colonization.

In the current paper, we describe the presence of suspected probiotic bacterial signatures (based on 16S rRNA gene sequences) in the gut of early preterm infants several weeks after discontinuation of the probiotic supplement and investigated the effect of FloraBABY probiotic supplementation on the development of the gut microbiome as a whole. Overall, our results suggest that the administration of probiotic strains to early preterm infants induces earlier colonization by *Bifidobacterium* than would occur in the absence of probiotic supplementation and that this generates a gut microbiome more similar to 10 day old full-term infants.

MATERIALS AND METHODS

Study Participants and Design

Preterm infants were enrolled in the study within 72 h after birth at either McMaster Children's Hospital or St. Joseph's Healthcare Hamilton. Exclusion criteria included triplets or higher order multiples and diagnoses of surgical bowel diseases and/or structural bowel abnormalities. Recruitment of 69 preterm infants took place between April 2017 and February 2018. Infants who developed surgical NEC during the study were excluded from further study. Ethics approval for the study was obtained by the Hamilton Integrated Research Ethics Board and parents provided written, informed consent at the time of enrollment.

At enrollment, parents completed a baseline questionnaire on prenatal exposures. Data about the pregnancy and birth and the infant's in-hospital progress, including nutrition, medication exposure and growth, were collected from antenatal records, birth records, and the maternal and infant charts. Information about infant diet and medication use (including probiotic supplements) following hospital discharge were collected from parents at the first study visit, which took place as close to the term corrected age of 40 weeks as possible, and at 6 weeks, 12 weeks, and 5 months corrected age. These visits took place at McMaster Children's Hospital (Hamilton, ON, Canada) or at the participant's home.

In November 2017, there was a practice change in the Neonatal Intensive Care Unit (NICU) of McMaster Children's Hospital (Hamilton, ON, Canada) resulting in the routine use, of a probiotic treatment for infants born at either less than 34 weeks gestational age or with birthweight less than 2 kg. Infants were excluded from probiotic supplementation if they had any of: congenital gastro-intestinal (GI) anomalies that had not undergone surgical repair, were NPO, had confirmed sepsis, were diagnosed or suspected to have congenital or acquired immunodeficiency syndrome (i.e., HIV, SCID) or had suspected Cow's Milk Protein Allergy, or other enteropathy. The commercially available FloraBABY probiotic (Renew Life Canada, Brampton ON, Canada) was used. According to the manufacturer, this contains 0.5 g (2 billion CFU bacteria) per single dose sachet, including: *Bifidobacterium breve* (HA-129), *Lactobacillus rhamnosus* (HA-111), *Bifidobacterium bifidum* (HA-132), *Bifidobacterium longum* subsp. *infantis* (HA-116), and *Bifidobacterium longum* subsp. *longum* (HA-135). The FloraBABY supplement was prepared by nursing staff at the infant's bedside from single dose sachets by mixing with 1 mL of either expressed breastmilk or sterile water. Following introduction, the probiotic was provided daily to the infant until discharge or transfer to another hospital. The study participants for this analysis were a subgroup of infants enrolled in the Baby & Pre-Mi pilot study. Inclusion in this sub-study was based on admission to the NICU at McMaster Children's Hospital (MUMC) either prior to or after the practice change, gestational age under 32 weeks (early preterm), and collection of stool samples in-hospital and at the term study visit.

Stool Sample Collection

Following enrollment in the Baby & Pre-Mi study, stool samples were collected every other day until the infant was either discharged from hospital or had reached term corrected age. Diapers with stool were transferred into pre-labeled plastic bags by nursing staff and immediately stored in a −20°C freezer located in the NICU. The sample was then transferred by research personnel to the laboratory and continued to be stored at −20°C until processing. In addition to the stool samples collected in-hospital, samples were also collected at visits that occurred at term, 6 weeks, 12 weeks, and 5 months corrected age. Parents collected the stool sample with supplied, standardized materials and were instructed to store the a sample in a household freezer and then bring the frozen sample to the study visit. If the infant was still in-hospital at the time of the study visit, the sample was collected by the infant's nurse.

Term Comparator Cohort

Stool samples collected from a cohort of 51 full-term infants from the Baby & Mi pilot study were utilized for comparison with our early preterm cohort. This study is also a longitudinal, prospective study wherein women with uncomplicated pregnancies were recruited during pregnancy from midwifery practices, and infants born at term (>37 weeks gestation) were subsequently enrolled. Ethics approval for the Baby & Mi study was obtained from all participating sites and parents provided written, informed consent at the time of enrollment. The development of the gut microbiome up to 12 weeks of life for this cohort has been reported elsewhere (Stearns et al., 2017). For inclusion in the comparator group for the current study, infants had to be healthy, vaginally born, been breastfed to at least 5 months and not have received intrapartum antibiotics. 16S rRNA gene data from 199 stool samples collected at 10 days, 6 weeks, 12 weeks, and 5 months of life were used in this analysis.

DNA Extraction, Sequencing of Bacterial Tags and Analysis

DNA was extracted from 0.1 g of stool with mechanical lysis with 2.8 mm ceramic beads and 0.1 mm glass beads for 3 min at 3000 rpm in 800 μl of 200 mM sodium phosphate monobasic (pH 8) and 100 μl guanidinium thiocyanate EDTA N-lauroylsarcosine buffer (50.8 mM guanidine thiocyanate, 100 mM ethylenediaminetetraacetic acid and 34 mM N-lauroylsarcosine) as previously described (Stearns et al., 2015, 2017). This extract was then purified with the MagMAX-96 DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA) on the MagMAX Express-96 Deep Well Magnetic Particle Processor (Applied Biosystems, Foster City, CA). The DNA was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Mississauga, ON Canada). Amplification of the bacterial 16S rRNA gene v3 region (150 base pair) tags was performed as previously described (Bartram et al., 2011) with the following changes: 5 pmol of primer, 200 μM of each dNTP, 1.5 mM MgCl2, 2 μl of 10 mg/ml bovine serum albumin, and 1.25 U Taq polymerase (Life Technologies, Carlsbad, CA, United States) were used in a 50 μl reaction volume. The PCR program used was as follows: 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, then a final extension step at 72°C for 10 min. Illumina libraries were sequenced in the McMaster Genomics Facility with 250 base pair sequencing in the forward and reverse directions on the Illumina MiSeq instrument. The completed run was de-multiplexed with Illumina's Casava software. Adapter, primer and barcode sequences were trimmed from sequencing reads with cutadapt (Martin, 2011) then ASVs were inferred from the sequenced data using the DADA2 pipeline (Callahan et al., 2016). The vegan package (v2.5-6) in R was used to calculate alpha diversity metrics, including observed richness and Shannon diversity index (Oksanen et al., 2018). Observed richness was estimated from ASV counts using the rarefy function, with a sample depth of 5, 000 sequences, while Shannon diversity index was calculated with the diversity function. One preterm infant sample did not meet the sample depth and was not included in observed richness calculations. Beta diversity was based on Bray-Curtis dissimilarity matrices calculated from relative abundance values of all ASVs.

Bacterial Species Phylogeny

In order to resolve the species distribution of the most abundant ASVs assigned to the *Bifidobacterium* genus, a reference tree was made from reference sequences of *Bifidobacterium* species from the ribosomal database project (RDP) (Cole et al., 2014). All full-length 16S rRNA gene reference sequences for bifidobacterial species (46 in total) from the RDP were aligned using MUSCLE (Edgar, 2004) then used to create an approximate maximum likelihood phylogeny with FastTree (Price et al., 2010) using the Generalized Time Reversible model.

Statistics

To assess differences between bacterial communities in each sample (beta diversity), principal coordinate analysis plots were generated in the package phyloseq (v30.0). Differences in permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis dissimilarities with 99, 999 permutation were assessed using the adonis function in the vegan package. Differences in alpha diversity metrics between preterm groups were assessed using linear mixed modeling (lme4 (v1.1-21) and lmerTest (v.3.1-1) packages), with postmenstrual age, cohort, and percent days on antibiotics as fixed effects, and participant as a random effect. Mixed effects models with a negative binomial distribution and log link function were constructed using the package glmmTMB (v0.2.3) to model bacterial abundance data of preterm infants. ASV counts were the response variable, participant was the random effect, and the exposure variables included cohort, postmenstrual age, and percent days on antibiotics. This mixed effect model, including postmenstrual age as the fixed effect and participant as the random effects, was also used to model *Bifidobacterium* abundance in full-term infants. The total number of reads per sample was log-transformed and used as the offset to account for differences in sequencing depth. Comparative analysis was done to look at differences in beta diversity with PERMANOVA using Bray-Curtis distances. Samples were first stratified by collection time point, then

pairwise comparisons were completed between preterm groups and the full-term comparator group. Differences with a *p*-value below 0.05 were considered significant.

RESULTS

Baseline and Study Visit Characteristics of Probiotic-Exposed and Unexposed Preterm Infants

Twenty-two (22) early preterm infants met the criteria for this study. The gestational age at birth ranged from 22 weeks + 6 days to 30 weeks + 3 days (**Table 1**). Of these infants, 14 never received the FloraBABY probiotic and 8 infants were enterally administered the probiotic through supplementation of expressed breast milk or sterile water. Infant characteristics were similar between probiotic-exposed and unexposed groups (**Table 1**). Age of the infant at the time of the first administration of probiotics ranged from 30.29 to 36.14 weeks postmenstrual age. Following this first introduction, infants received the probiotic daily until hospital discharge for a duration of between 3.29 and 13.57 weeks; and postmenstrual age at the time of cessation of probiotic use ranged from 35.86 to 49.57 weeks (**Figure 1**). A total of 573 stool samples collected during infant hospitalization were included in this analysis. Profiling of 16S rRNA gene was completed in an average (SD) of 26.13 (7.68) samples for each exposed infant and 26.00 (14.58) samples for each unexposed infant. An additional 75 samples were collected at each of the four study visits: term corrected age of 40 weeks (Visit 1), 6 weeks (Visit 2), 12 weeks (Visit 3), and 5 months (Visit 4) corrected age (**Figure 1**). In the probiotic-exposed cohort, 20 of 25 study visit samples were collected following discontinuation of the probiotic. All infants received breastmilk during hospitalization until at least 37 weeks postmenstrual age (**Supplementary Figure S1A**) although two infants were weaned from breastmilk during the probiotic supplementation period. Two infants from the probiotic-exposed group did not receive oral or IV antibiotics during their hospitalization and all infants in the unexposed group received antibiotics (**Supplementary Figure S1B**). All infants that received antibiotics were administered courses of aminoglycoside and *b*-lactam antibiotics during the first 72 h of life. Additionally, 4 infants in the probiotic-exposed group and 12 infants in the unexposed group received additional and variable courses of antibiotics during hospitalization or following discharge (**Supplementary Figure S1B**).

TABLE 1 | Participant characteristics of preterm infants.

	Probiotic-exposed (*n* = 8)	Unexposed to probiotic (*n* = 14)	*p*-value
Gestational age at birth, weeks	28.1 ± 1.65	27.5 ± 2.03	0.47
Cesarean delivery, N (%)	6 (75.0%)	10 (71.4%)	0.99
Birth weight, g	975 ± 284	1025 ± 308	0.71
Birth weight z-score	−0.46 ± 0.60	0.12 ± 0.82	0.09
Male, N (%)	3 (37.5%)	4 (28.6%)	0.99
Twins, N (%)	4 (50.0%)	2 (14.3%)	0.14
In-hospital samples collected during FloraBABY supplementation, N (%)	116/209 (55.5%)	0/364 (0%)	**<0.001**
Antibiotic exposure, days (N)	8.63 ± 9.44 (8)	15.3 ± 12.3 (13)	0.21
Weaned from breastmilk, N (%)	6 (75.0%)	7 (50.0%)	0.38
Postmenstrual age (PMA) at weaning from breastmilk, weeks	41.3 ± 3.35	44.8 ± 5.97	0.23

The data are presented as N (%) for categorical parameters and as mean ± SD for continuous variables. P-value < 0.05 using Student's t-test or Mann-Whitney (continuous) or Fisher's exact test (categorical) were considered to be statistically significant.

Comparison of the Relative Abundance of Bifidobacterium ASVs Between Probiotic-Exposed and Unexposed Infants During Hospitalization

We identified a total of 127 ASVs classified to the genus *Bifidobacterium* within our preterm cohort. Of the 457 in-hospital samples collected from all 22 infants in the absence of probiotic exposure (i.e., prior to exposure or in those never exposed), 224 samples from 21 infants had detectable levels of at least one *Bifidobacterium* ASV and this genus made up a mean of 2% of the microbial community. This indicates that *Bifidobacterium* sp. are naturally prevalent, but not abundant in the preterm infant gut microbiome between 1 and 18 weeks postnatally. Four ASVs (ASV 202, ASV 203, ASV 204, and ASV 205) assigned to *Bifidobacterium* sp. had a greater relative abundance than the other bifidobacterial ASVs in our dataset (**Supplementary Figure S2**). The relative abundance of these four ASVs was 0.005–5% before probiotic exposure and increased to 6–21% after exposure. In contrast, in unexposed infants, the relative abundance of these ASVs was 0.02%. The total relative abundance of all other ASVs belonging to the genus *Bifidobacterium* was below 1.4% in both the probiotic-exposed and unexposed groups, up to the term corrected age visit (**Supplementary Figure S2**).

Comparison of the Relative Abundance of Lactobacillus ASVs Between Probiotic-Exposed and Unexposed Infants During Hospitalization

We identified a total of 38 ASVs that were classified to the genus *Lactobacillus* in our preterm cohort. In the absence of probiotic exposure, the average relative abundance of *Lactobacillus* ASVs was 0.9% and the prevalence of these ASVs was 55%. This indicates that *Lactobacillus* sp. were prevalent in the preterm infant gut, yet they were not dominant members of microbial communities profiled in the stool. Of the 38 ASVs belonging to *Lactobacillus*, ASV 2940 had a higher average relative abundance than all other *Lactobacillus* ASVs in probiotic-exposed infants

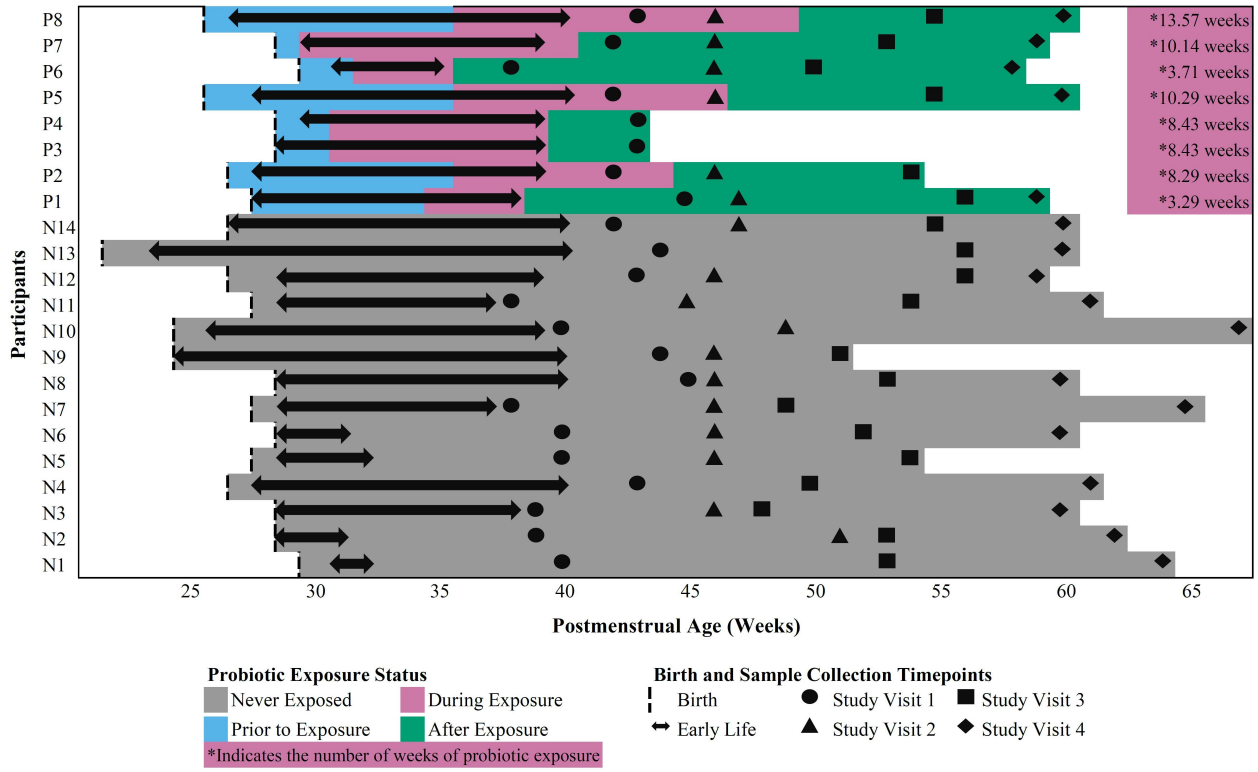

FIGURE 1 | Outline of in-hospital and study visit sample collection across postmenstrual age in probiotic-exposed and unexposed preterm infants.

(**Supplementary Figure S3**) compared to non-exposed preterm infants. During probiotic administration, the prevalence of *Lactobacillus* ASV 2940 increased to 98% with an average relative abundance of 2% of the microbial community (**Supplementary Figure S3**). From birth to term corrected age (Visit 1), the total relative abundance of all remaining ASVs belonging to the genus *Lactobacillus* remained low.

Bifidobacterium and Lactobacillus ASV Sequence Identity With Reference 16S rRNA Genes

In order to explore the possibility that the dominant *Bifidobacterium* and *Lactobacillus* ASVs that appeared in probiotic-exposed infants may be the probiotic strains themselves, we determined the sequence identity between ASV sequences and reference sequences of 16S rRNA genes for *Bifidobacterium* and *Lactobacillus* strains from the Ribosomal Database Project (Oksanen et al., 2018). As we did not have the 16S rRNA gene sequence for the commercial FloraBABY product we relied on reference sequences as these can provide an indication of the species classification. We completed a multiple-sequence alignment between the full-length reference 16S rRNA gene sequences and included the short ASV sequences, derived from amplification of the v3 region of the 16S rRNA gene, for all ASVs with an average relative abundance above 1% in the preterm or full-term cohorts that were classified as either *Bifidobacterium* and *Lactobacillus*. We were able to resolve species separation within the *Bifidobacterium* genus with a simple phylogeny, although strain resolution was not possible (**Supplementary Figure S4**). The four bifidobacterial ASVs that appeared in the probiotic-exposed preterm infants bore the closest sequence similarity to reference sequences of *Bifidobacterium longum* (ASV 202 and 203), *B. bifidum* (ASV 204) and *B. breve* (ASV 205) which matched the species designation of the strains present in the probiotic supplement according to the label. We were unable to resolve species of *Lactobacillus*.

Impact of Probiotic Supplementation on the Abundance of Bifidobacterium and Lactobacillus After Discontinuation of the Probiotic Supplement

In order to determine the longer-term influence of probiotic supplementation on the abundance of the four bifidobacterial ASVs highlighted above, we examined samples obtained from probiotic-exposed and unexposed preterm infants from term to 5 months corrected age. Negative binomial regression was used to model bacterial count data. Regression models included individual as a random effect and probiotic exposure status, postmenstrual age, and percent of days on antibiotics as fixed effects. To look at the long-term effects of probiotics following discontinuation of use, only samples collected after a minimum 2-week washout period were included in our regression models.

TABLE 2 | Effect of probiotic exposure on the abundance of *Bifidobacterium* and *Lactobacillus* ASVs and genera in preterm infants.

	Cohort†	Postmenstrual age	Probiotic-exposed: Postmenstrual age	Percent days on antibiotics
Taxa	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
Bifidobacterium	7.74 (−1.31, 16.8)	0.132 (8.45e-04, 0.263)	−0.122 (−0.0285, 0.0416)	0.00133 (−0.00938, 0.0964)
Bifidobacteriumlongum ASV 202	9.84 (0.560, 19.1)*	0.153 (0.0534, 0.252)**	−0.129 (−0.305, 0.0462)	−0.0751 (−0.226, 0.0754)
Bifidobacteriumlongum ASV 203	29.0 (10.5, 47.5)**	0.476 (0.295, 0.658)***	−0.487 (−0.845, −0.129)**	−0.00342 (−0.228, 0.159)
Bifidobacteriumbifidum ASV 204	15.6 (7.04, 24.2)***	0.233 (0.134, 0.332)***	−0.228 (−0.390, −0.0647)**	−0.0650 (−0.195, 0.0648)
Bifidobacteriumbreve ASV 205	21.12 (7.01, 35.4)**	0.243 (0.105, 0.382)***	−0.323 (−0.593, −0.0538)*	−0.160 (−0.367, 0.0480)
Sum of other *Bifidobacterium* ASVs	−0.0484 (−12.8, 12.7)	0.0923 (-0.0268, 0.211)	0.00325 (−0.242, 0.249)	0.0264 (−0.0654, 0.118)
Lactobacillus	6.82 (−6.29, 19.9)	0.0785 (−0.0261, 0.183)	−0.117 (−0.371, 0.136)	−0.170 (−0.320, −0.0206)*
Lactobacillus ASV 2940	4.50 (−10.2, 19.2)	0.0688 (−0.0494, 0.187)	−0.0723 (−0.355, 0.210)	−0.174 (−0.322, −0.0258)*
Other *Lactobacillus*	−17.0 (−38.9, 4.96)	0.00255 (−0.0689, 0.120)	0.0358 (−0.0738, 0.790)	−0.0243 (−0.457, −0.0282)*

*Negative binomial regression was used to model Bifidobacterium and Lactobacillus count data with an offset for sequencing depth. †Comparison of probiotic-exposed preterm infants to unexposed preterm infants. *p < 0.05. **p < 0.01. ***p < 0.001.*

Samples from unexposed infants were found to have a significantly higher percentage of days on antibiotics by the first study visit ($p = 0.01$), and the unexposed infants were on average 3 weeks older at the fourth study visit ($p = 0.003$), compared to the probiotic-exposed infants (**Supplementary Table S1**). After correcting for antibiotic exposure and repeated sampling, probiotic exposure and postmenstrual age were positively related to the counts of *Bifidobacterium longum* ASV 202, *Bifidobacterium longum* ASV 203, *Bifidobacterium bifidum* ASV 204 and *Bifidobacterium breve* ASV 205 with an interaction effect between age and exposure status (**Table 2**). Postmenstrual age had a stronger relationship with bifidobacterial abundance in unexposed preterm infants compared to probiotic-exposed infants. Furthermore, the positive relationship of bacterial abundance and postmenstrual age in the unexposed infants suggests that the abundance of bifidobacteria increased naturally over time. In probiotic-exposed preterm infants the *Bifidobacterium* genus and *Bifidobacterium longum* ASV 203 and *Bifidobacterium bifidum* ASV 204 did not increase in abundance over time, while *Bifidobacterium breve* ASV 205 decreased over time. For *Bifidobacterium longum* ASV 202, probiotic exposure and age each had a positive effect (**Supplementary Figure S5**). Changes in *Bifidobacterium* abundance over time in healthy, vaginally-born full term infants that were breastfed to at least 5 months, and not exposed to the probiotic supplement ($n = 51$) (Stearns et al., 2017) were similarly modeled. The abundance of *Bifidobacterium* in the full-term cohort was not found to be significantly related to postmenstrual age ($p = 0.06$).

Neither postmenstrual age nor probiotic status were related to the abundance of the *Lactobacillus* ASV 2940, the sum of all other *Lactobacillus* ASVs, or the *Lactobacillus* genus. Antibiotic exposure, however, had a significant negative effect on the abundance of these bacterial groups (**Table 2**).

The Effect of Probiotic Persistence on the Gut Microbial Community

The persistence of a suspected probiotic signal in the microbiota of preterm infants begs the question of whether the overall gut microbial diversity and community structure are altered with probiotic administration and how long such an alteration lasts. In order to explore the effects of postmenstrual age, probiotic exposure, and antibiotic exposure on alpha diversity of gut microbial communities in preterm infants following cessation of probiotic use, linear mixed models were used with the individual as a random effect and probiotic exposure, postmenstrual age and percent of days on antibiotics as fixed effects. We found a positive and significant effect of postmenstrual age on species richness, but not Shannon diversity, and no significant relationship was observed between probiotic exposure and alpha diversity metrics (**Table 3**).

TABLE 3 | Effect of postmenstrual age, antibiotics, and probiotic exposure on alpha diversity in preterm infants.

	Species richness β (95% CI)	Shannon diversity β (95% CI)
Cohort†	30.1 (−14.0, 74.3)	0.00164 (−1.78, 1.83)
Postmenstrual age	0.706 (0.289, 1.11)*	0.00954 (−0.00739, 0.0263)
Probiotic-exposed: Postmenstrual age	−0.505 (−1.35, 0.337)	−0.00169 (−0.0371, 0.0324)
Percent days on antibiotics	−0.254 (−0.870, 0.365)	−0.00759 (−0.0304, 0.0157)

†*Comparison of probiotic-exposed preterm infants to unexposed preterm infants. *p < 0.05. **p < 0.01. ***p < 0.001.*

Beta diversity between probiotic exposed and unexposed preterm infants and full-term infant gut microbiome samples was explored at postmenstrual-matched ages. As with the analyses above, a minimum 2-week washout period from probiotics was used as a cutoff for sample inclusion. First, principal coordinate analysis (PCoA) on Bray-Curtis dissimilarities was used to visualize clustering of samples based on microbial community. After the washout period, the samples collected near term-age from preterm infants exposed to probiotics clustered more closely to samples from 10-day-old full-term infants than did samples at term corrected age from preterm infants never exposed to

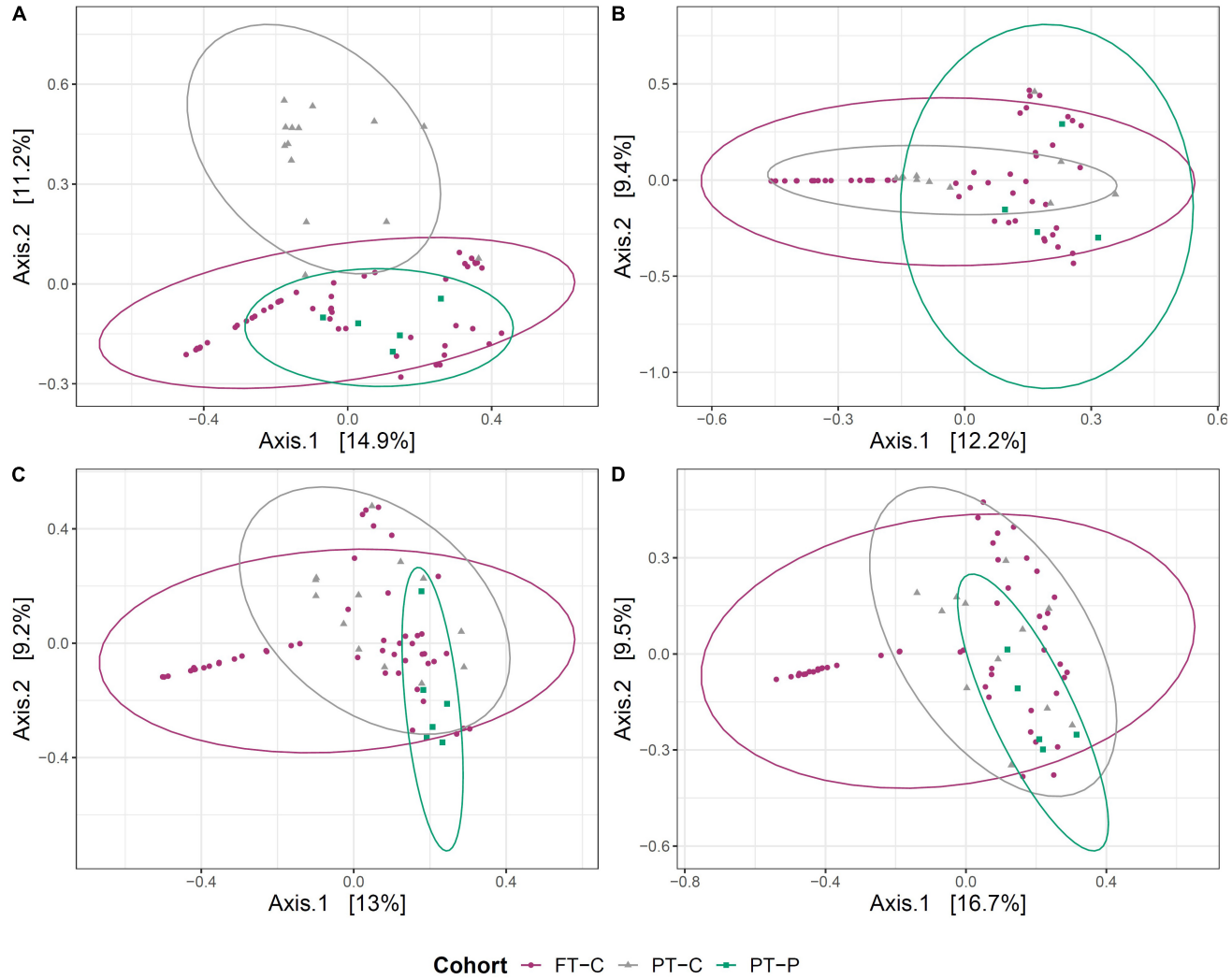

FIGURE 2 | Clustering of the gut microbial community of probiotic exposed preterm infants (after the washout period), unexposed preterm infants and full-term infants. Principal coordinate analysis (PCoA) plots, based on Bray-Curtis dissimilarities of preterm infants not exposed to the probiotic (PT-C), preterm infants exposed to the probiotic (PT-P) and full-term infants (FT-C) at around term **(A)**, 6 weeks **(B)**, 12 weeks **(C)**, and 5 months corrected age **(D)**, compared with samples from full-term infants at 10 days, 6 weeks, 12 weeks, and 5 months postnatal age, respectively.

probiotics (**Figure 2A**). This clustering did not, however, persist at 6 weeks, 12 weeks or 5 months (**Figures 2B–D**).

Permutational analysis of variance (PERMANOVA) was used to test the association of variation in microbial communities with cohort, postmenstrual age and, in the case of preterm infants, antibiotic exposure. Antibiotic exposure was not included within models with full-term infants, because only one of 51 infants born full-term had been exposed to antibiotics during the study period. Within preterm infants, at the term corrected age, 20.9% of the variation between gut microbial communities was associated with probiotic exposure ($p \leq 0.001$) and 11.4% was associated with antibiotic exposure ($p = 0.006$) (**Supplementary Table S2**). The magnitude of the variance explained by probiotic exposure in preterm infants decreased over time to 13.1% at 6 weeks corrected age, 12.9% at 12 weeks corrected age and 8.5% at 5 months corrected age (**Supplementary Table S2**). Postmenstrual age only had a significant effect at 5 months of age and was responsible for 11.3% of the variance observed in community structure between preterm groups ($p = 0.04$). When unexposed preterm infants at the term corrected age were compared with 10-day-old full-term infants, 8.7% of the variation in the gut microbiome was associated with cohort, and this proportion decreased over time to 3.9% at 6 weeks, 3.0% at 12 weeks and 2.8% at 5 months (**Supplementary Table S2**). In contrast, when the probiotic exposed preterm infants at the term corrected age were compared with 10 day old full-term infants, 3.2% of the variation in the gut microbiome was associated with cohort, which changed slightly over time to 2.4% at 6 weeks, 4.0% at 12 weeks and 3.5% at 5 months. This suggests that prior administration of the probiotic had a considerable effect on the gut microbiome of preterm

infants, but this effect decreased over the 5-month study period. That said, these findings also highlight that there were significant differences between the gut microbiome of preterm infants at the term corrected age and 10 day-old full-term infants, even when probiotics were administered in early life. At term age, the gut microbiome of probiotic exposed infants was more similar to that of 10-old full-term infants than the gut microbiome of unexposed preterm infants at term age. No significant effect of postmenstrual age or antibiotics was observed on differences in bacterial community structure between preterm and full-term infants.

DISCUSSION

Infants born very preterm often have a delay in colonization with *Bifidobacterium*, a dominant bacterial genus within the gut microbiome of breastfed full-term infants (Bäckhed et al., 2015; Yassour et al., 2016; Stearns et al., 2017). The delay in the arrival of bifidobacteria may contribute to the establishment of more pathogenic bacteria (Butel et al., 2007) and a susceptibility to sepsis (Mai et al., 2013; Stewart et al., 2017). Routine administration of multi-strain probiotic supplements with *Bifidobacterium* sp. and *Lactobacillus* sp. are effective in reducing the incidence of NEC in preterm infants (Aceti et al., 2015; Chang et al., 2017). However, long-term colonization of the preterm infant gut with bacterial strains from probiotic supplements has not been definitively shown to date. In adults, discontinuing a probiotic reduces the detection of that probiotic signal in stool (Bouhnik et al., 1992; Kullen et al., 1997; Charbonneau et al., 2013), although recently, more variability and possible probiotic colonization of the adult gut has been suggested (Maldonado-Gomez et al., 2016; Zmora et al., 2018). The preterm gut environment could be more permissive to colonization with supplemented bacteria, since the bacteria found there are less abundant and not yet organized into complex communities (Ho et al., 2018). Whether probiotic organisms establish persistent colonization is still unclear, largely due to the fact that molecular profiling of the gut microbiome (e.g., 16S rRNA gene surveys) is unable to distinguish between probiotic and endogenous strains of bacteria.

In this exploratory study, we compared the fecal microbiome of preterm infants exposed and unexposed to probiotics as part of their care following birth. We were able to take advantage of a "natural experiment" that occurred because of a change in clinical practice that stipulated routine probiotic supplementation in this NICU population. The probiotic investigated here (FloraBABY) has been shown to reduce the rate of NEC in a large prospective cohort study (Janvier et al., 2014), although the effect of this probiotic on gut microbial composition has not been previously explored. We also compared preterm cohorts with a cohort of full-term infants that followed the same longitudinal data collection protocol out to 5 months of age. Samples were collected according to postnatal age in full-term infants and corrected age in preterm infants to reflect current pediatric guidelines that recommend preterm infant growth be modeled after healthy term-born infants (American Academy of Pediatrics, 1977). We set out to determine whether probiotic strains given as a supplement were colonizing the preterm infant gut, and to determine if probiotic supplementation exerted a consistent effect on the overall gut microbiome in the post-discharge period. Our results suggest that the administration of FloraBABY to preterm infants increases the abundance of *Bifidobacterium* but not *Lactobacillus* in the infant gut for many weeks after the discontinuation of the probiotic. Further, the gut microbiome at term corrected age in probiotic-exposed preterm infants more closely resembled that of 10-day-old full-term infants than unexposed preterm infants.

In the absence of probiotic exposure, *Bifidobacterium* sp. abundance in our preterm cohort was low during the first months of life, consistent with previous studies (Stewart et al., 2015; Patel et al., 2016; Butcher et al., 2018). Probiotic exposure was associated with a higher abundance of *Bifidobacterium* and *Lactobacillus* in the stool. Increased relative abundance of four ASVs belonging to the genus *Bifidobacterium* (ASV 202 - ASV 205) and one ASV assigned to the genus *Lactobacillus* (ASV 2940) coincided with the period of probiotic administration (**Supplementary Figures S2, S3**). The suspected probiotic bifidobacterial strain ASVs bore sequence similarity to reference sequences similar to species listed in the ingredients of the probiotic supplement (e.g., *B. longum*, *B. breve*, and *B. bifidum*; **Supplementary Figure S4**) and were distinct from other naturally occurring strains of *Bifidobacterium* that were present during hospitalization in the absence of probiotic supplementation. Although we were able to discriminate between suspected endogenous bifidobacterial ASVs and suspected probiotic bifidobacterial ASVs at early timepoints in the preterm infant samples, we found that the amplicon-based profiles, from short sequences of the 16S rRNA gene, were unable to discriminate between probiotic-derived and some suspected endogenous strains of *B. longum*, *B. breve* and *B. bifidum* that appeared naturally in unexposed preterm infants beginning at 6 weeks corrected age. Species-level resolution could not be obtained for the suspected probiotic *Lactobacillus* strain ASV. Our data also indicated the appearance of suspected probiotic *Bifidobacterium* ASVs in some infants prior to the start of probiotic supplementation (**Supplementary Figures S2A,C,D**), suggesting potential cross-colonization within the NICU. This type of cross-colonization has been suspected before in large randomized controlled trials (Costeloe et al., 2016; Plummer et al., 2018).

Both probiotic exposure and postmenstrual age were directly related to increased abundance of *Bifidobacterium* ASVs in preterm infants (**Table 2**). Previous studies have shown that colonization of the preterm infant gut by *Bifidobacterium* is dependent on postmenstrual age (Butel et al., 2007; Korpela et al., 2018) and that daily probiotic administration until 34 weeks postmenstrual age can increase the *Bifidobacterium* abundance in preterm infants compared to control groups (Watkins et al., 2019). Here we confirm the effect of postmenstrual age on the abundance of bifidobacteria and demonstrate that probiotic exposure increased the initial abundance of

bifidobacteria in preterm infants. In the case of *Bifidobacterium longum* ASV 202 this resulted in higher abundance beyond the supplementation period (**Supplementary Figure S5**), suggesting long-term colonization with this probiotic strain. In contrast, neither postmenstrual age nor probiotic-exposure status were associated with the abundance of *Lactobacillus*, similar to previous studies in preterm infants and adults (Costa et al., 2014; Abdulkadir et al., 2016). It should be noted, however, that stool analysis may underestimate probiotic *Lactobacillus* colonization, as *Lactobacillus* colonizes the small intestine (Hao and Lee, 2004) and attaches to colonic mucosae *in vivo* (Alander et al., 1999). While antibiotic exposure was not found to have a significant effect on the abundance of bifidobacteria; a negative and significant effect of exposure to antibiotics was observed for *Lactobacillus* in our study, which was similar to murine models of early-life antibiotic administration (Cox et al., 2014).

Probiotic exposure was shown to impact the microbial community structure within the preterm infant gut. No differences were observed in alpha diversity measures between probiotic-exposed and unexposed infants, however, beta diversity analysis indicated that samples from probiotic-exposed preterm infants at term age were found to cluster more closely with 10-day old full-term infants than did samples from unexposed preterm infants (**Figure 2A**). The clustering was not observed at later time points.

Our findings suggest that probiotic supplementation in preterm infants may promote an earlier convergence to an intestinal microbiome that is more similar to healthy, full-term infants; however, more research is needed to determine if probiotic strains of bacteria offer all of the same benefits as endogenous bacteria. More research is also needed to examine the dosage and the length of administration needed to achieve, or avoid, colonization in preterm infants. Furthermore, the influence of antibiotic exposure and breastfeeding on colonization needs to be studied. In our study, all infants were receiving breastmilk at the time of the introduction of the probiotic, with two infants being weaned from breastmilk while continuing to receive the probiotic (**Supplementary Figure S1**). Breastfeeding is important to the establishment of the microbiota in the infant gut (Bäckhed et al., 2015) and may be an important modifier to the establishment of probiotic strains in the gut. While we have demonstrated that probiotic administration had an effect on the preterm infant gut microbiome, it is still unclear whether probiotic strains colonized the preterm gut long-term. Better strain resolution, through longer read technology or cultured isolates, is needed to track the persistence of probiotic strains in the gut as preterm infants age. If probiotic bacteria can colonize the preterm infant gut, then questions remain about which strains are the most beneficial to infants during this critical stage in development (Ewaschuk et al., 2008).

Strengths of our study include the prospective design, high-resolution longitudinal collection of samples at frequent timepoints, and the quality of clinical data collected from our study population. The policy change within NICU to administer probiotics to all preterm infants born at less than 34 weeks gestation, instead of based on the clinical condition of the infants, created a natural experiment that was also a strength. Limitations of our study included: the small number of preterm infants and the variable timing of sample collection, a limited ability to explore the influence of antibiotic type and dosages on the microbiome., the lack of a placebo-control to account for the prebiotic effect of maltodextrin (Yeo and Liong, 2010) in the FloraBaby supplement, and the limited resolution of ASVs to discriminate probiotic strains of *B. longum*, *B. bifidum*, and *B. breve* from endogenous ones.

Early preterm infants are known to have delays in *Bifidobacterium* colonization compared to infants born full-term. Our results show that enteral administration of a multi-strain probiotic to early preterm infants during hospitalization results in the increased abundance of suspected probiotic bifidobacterial ASVs up to 5 months post-supplementation, and potential induction of probiotic colonization of the infant gut. This increase in *Bifidobacterium* may be related to the potential role of probiotics in reducing NEC development in preterm infants. Further research is needed to identify these probiotic strains and explore their functional role in microbiome development and infant health.

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DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to the potentially identifiable nature of the data and privacy concerns by study participants. Requests to access the datasets should be directed to JS, stearns@mcmaster.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hamilton Integrated Research Ethics Board, Hamilton Health Sciences Faculty of Health Sciences Research

Ethics Board, St. Joseph's Healthcare Hamilton Research Ethics Board, and Joseph Brant Hospital Research Ethics Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JS, EY, KM, and EH conceived the research objectives. JS, EY, KM, EH, JT, LG, and CS designed the methodology. MC, SD, SK, and EG recruited participants and collected samples and clinical information. EY and JS analyzed the data and wrote the manuscript. All authors edited the manuscript and approved the final draft.

FUNDING

This work was supported by a grant from the Canadian Institutes of Health Research and Joint Action Intestinal Microbiomics of the Joint Programming Initiative a Healthy Diet for a Healthy Life (IMG Grant – FRN 143923). Funding for the Baby & Mi pilot study was provided by Hamilton Academic Health Sciences Organization. JS is supported by a Farncombe Family Endowed Chair in Microbial Ecology and Bioinformatics. EY is supported by an Ontario Graduate Scholarship.

ACKNOWLEDGMENTS

We would like to thank Laura Rossi and Michelle Fontes who were instrumental to sample and data processing. In addition, Julia Simioni and Jenifer Li collected the Baby & Mi cohort clinical data and samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.574137/full#supplementary-material

REFERENCES

Abdulkadir, B., Nelson, A., Skeath, T., Marrs, E. C., Perry, J. D., Cummings, S. P., et al. (2016). Routine use of probiotics in preterm infants: longitudinal impact on the microbiome and metabolome. *Neonatology* 109, 239–247. doi: 10.1159/000442936

Aceti, A., Gori, D., Barone, G., Callegari, M. L., Di Mauro, A., Fantini, M. P., et al. (2015). Probiotics for prevention of necrotizing enterocolitis in preterm infants: systematic review and meta-analysis. *Ital. J. Pediatr.* 41:89.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., et al. (1999). Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. *Appl. Environ. Microbiol.* 65, 351–354. doi: 10.1128/aem.65.1.351-354.1999

AlFaleh, K., and Anabrees, J. (2014). Probiotics for prevention of necrotizing enterocolitis in preterm infants. *Evid. Based Child Health* 9, 584–671. doi: 10.1002/ebch.1976

American Academy of Pediatrics (1977). Committee on Nutrition: Nutritional needs of low-birth-weight infants. *Pediatrics* 60, 519–530.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17, 690–703.

Bartram, A. K., Lynch, M. D., Stearns, J. C., Moreno-Hagelsieb, G., and Neufeld, J. D. (2011). Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Appl. Environ. Microbiol.* 77, 3846–3852.

Bouhnik, Y., Pochart, P., Marteau, P., Arlet, G., Goderel, I., and Rambaud, J. C. (1992). Fecal recovery in humans of viable *Bifidobacterium* sp ingested in fermented milk. *Gastroenterology* 102, 875–878. doi: 10.1016/0016-5085(92)90172-u

Butcher, J., Unger, S., Li, J., Bando, N., Romain, G., Francis, J., et al. (2018). Independent of birth mode or gestational age, very-low-birth-weight infants fed their mothers' milk rapidly develop personalized microbiotas low in bifidobacterium. *J. Nutr.* 148, 326–335. doi: 10.1093/jn/nxx071

Butel, M. J., Suau, A., Campeotto, F., Magne, F., Aires, J., Ferraris, L., et al. (2007). Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. *J. Pediatr. Gastroenterol. Nutr.* 44, 577–582. doi: 10.1097/mpg.0b013e3180406b20

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* 13:581. doi: 10.1038/nmeth.3869

Chang, H.-Y., Chen, J.-H., Chang, J.-H., Lin, H.-C., Lin, C.-Y., and Peng, C.-C. (2017). Multiple strains probiotics appear to be the most effective probiotics in the prevention of necrotizing enterocolitis and mortality: an updated meta-analysis. *PLoS One* 12:e0171579. doi: 10.1371/journal.pone.0171579

Charbonneau, D., Gibb, R. D., and Quigley, E. M. (2013). Fecal excretion of *Bifidobacterium infantis* 35624 and changes in fecal microbiota after eight weeks of oral supplementation with encapsulated probiotic. *Gut Microbes* 4, 201–211. doi: 10.4161/gmic.24196

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., et al. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642.

Costa, G. N., Marcelino-Guimaraes, F. C., Vilas-Boas, G. T., Matsuo, T., and Miglioranza, L. H. (2014). Potential fate of ingested *Lactobacillus plantarum* and its occurrence in human feces. *Appl. Environ. Microbiol.* 80, 1013–1019. doi: 10.1128/aem.02588-13

Costeloe, K., Hardy, P., Juszczak, E., Wilks, M., and Millar, M. R. (2016). *Bifidobacterium breve* BBG-001 in very preterm infants: a randomised controlled phase 3 trial. *Lancet* 387, 649–660. doi: 10.1016/s0140-6736(15)01027-2

Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I., et al. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* 158, 705–721. doi: 10.1016/j.cell.2014.05.052

Deshpande, G., Rao, S., Patole, S., and Bulsara, M. (2010). Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Pediatrics* 125, 921–930. doi: 10.1542/peds.2009-1301

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Ewaschuk, J. B., Diaz, H., Meddings, L., Diederichs, B., Dmytrash, A., Backer, J., et al. (2008). Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. *Am. J. Physiol. Gastrointest Liver Physiol.* 295, G1025–G1034.

Groer, M. W., Luciano, A. A., Dishaw, L. J., Ashmeade, T. L., Miller, E., and Gilbert, J. A. (2014). Development of the preterm infant gut microbiome: a research priority. *Microbiome* 2:38. doi: 10.1186/2049-2618-2-38

Hao, W. L., and Lee, Y. K. (2004). Microflora of the gastrointestinal tract: a review. *Methods Mol. Biol.* 268, 491–502. doi: 10.1385/1-59259-766-1:491

Ho, T. T. B., Groer, M. W., Kane, B., Yee, A. L., Torres, B. A., Gilbert, J. A., et al. (2018). Dichotomous development of the gut microbiome in preterm infants. *Microbiome* 6:157.

Janvier, A., Malo, J., and Barrington, K. J. (2014). Cohort study of probiotics in a North American neonatal intensive care unit. *J. Pediatr.* 164, 980–985. doi: 10.1016/j.jpeds.2013.11.025

Kona, S. K., and Matlock, D. N. (2018). Probiotics, prebiotics, and synbiotics for preterm neonates. *NeoReviews* 19, e654–e663.

Korpela, K., Blakstad, E. W., Moltu, S. J., Strommen, K., Nakstad, B., Ronnestad, A. E., et al. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Sci. Rep.* 8:2453.

Kullen, M. J., Amann, M. M., O'Shaughnessy, M. J., O'Sullivan, D. J., Busta, F. F., and Brady, L. J. (1997). Differentiation of ingested and endogenous bifidobacteria by DNA fingerprinting demonstrates the survival of an unmodified strain in the gastrointestinal tract of humans. *J. Nutr.* 127, 89–94. doi: 10.1093/jn/127.1.89

Li, Y., Shimizu, T., Hosaka, A., Kaneko, N., Ohtsuka, Y., and Yamashiro, Y. (2004). Effects of bifidobacterium breve supplementation on intestinal flora of low birth weight infants. *Pediatr. Int.* 46, 509–515. doi: 10.1111/j.1442-200x.2004.01953.x

Mack, D. R., Michail, S., Wei, S., McDougall, L., and Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am. J. Physiol.* 276, G941–G950.

Mai, V., Torrazza, R. M., Ukhanova, M., Wang, X., Sun, Y., Li, N., et al. (2013). Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. *PLoS One* 8:e52876. doi: 10.1371/journal.pone.0052876

Maldonado-Gomez, M. X., Martinez, I., Bottacini, F., O'Callaghan, A., Ventura, M., van Sinderen, D., et al. (2016). Stable engraftment of *Bifidobacterium longum* AH1206 in the human gut depends on individualized features of the resident microbiome. *Cell Host Microbe* 20, 515–526. doi: 10.1016/j.chom.2016.09.001

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Mohan, R., Koebnick, C., Schildt, J., Schmidt, S., Mueller, M., Possner, M., et al. (2006). Effects of *Bifidobacterium lactis* Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study. *J. Clin. Microbiol.* 44, 4025–4031. doi: 10.1128/jcm.00767-06

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., O'hara, R., et al. (2018). *Package 'VEGAN'COMMUNITY Ecology Package*. Available online at: https://cran.r-project.org/web/packages/vegan/index.html (accessed September 1, 2019).

Olsen, R., Greisen, G., Schroder, M., and Brok, J. (2016). Prophylactic probiotics for preterm infants: a systematic review and meta-analysis of observational studies. *Neonatology* 109, 105–112. doi: 10.1159/000441274

Patel, A. L., Mutlu, E. A., Sun, Y., Koenig, L., Green, S., Jakubowicz, A., et al. (2016). Longitudinal survey of microbiota in hospitalized preterm very-low-birth-weight infants. *J. Pediatr. Gastroenterol. Nutr.* 62, 292–303. doi: 10.1097/mpg.0000000000000913

Perez-Muñoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., and Walter, J. (2017). A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* 5:48.

Plummer, E. L., Bulach, D. M., Murray, G. L., Jacobs, S. E., Tabrizi, S. N., Garland, S. M., et al. (2018). Gut microbiota of preterm infants supplemented with probiotics: sub-study of the ProPrems trial. *BMC Microbiol.* 18:184. doi: 10.1186/s12866-018-1326-1

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Sawh, S. C., Deshpande, S., Jansen, S., Reynaert, C. J., and Jones, P. M. (2016). Prevention of necrotizing enterocolitis with probiotics: a systematic review and meta-analysis. *PeerJ* 4:e2429. doi: 10.7717/peerj.2429

Stearns, J. C., Davidson, C. J., McKeon, S., Whelan, F. J., Fontes, M. E., Schryvers, A. B., et al. (2015). Culture and molecular-based profiles show shifts in bacterial communities of the upper respiratory tract that occur with age. *ISME J.* 9, 1246–1259. doi: 10.1038/ismej.2014.250

Stearns, J. C., Simioni, J., Gunn, E., McDonald, H., Holloway, A. C., Thabane, L., et al. (2017). Intrapartum antibiotics for GBS prophylaxis alter colonization patterns in the early infant gut microbiome of low risk infants. *Sci. Rep.* 7: 16527.

Stewart, C. J., Embleton, N. D., Marrs, E. C. L., Smith, D. P., Fofanova, T., Nelson, A., et al. (2017). Longitudinal development of the gut microbiome and metabolome in preterm neonates with late onset sepsis and healthy controls. *Microbiome* 5:75.

Stewart, C. J., Skeath, T., Nelson, A., Fernstad, S. J., Marrs, E. C., Perry, J. D., et al. (2015). Preterm gut microbiota and metabolome following discharge from intensive care. *Sci. Rep.* 5:17141.

Strus, M., Helwich, E., Lauterbach, R., Rzepecka-Weglarz, B., Nowicka, K., Wilinska, M., et al. (2018). Effects of oral probiotic supplementation on gut *Lactobacillus* and *Bifidobacterium* populations and the clinical status of low-birth-weight preterm neonates: a multicenter randomized, double-blind, placebo-controlled trial. *Infect Drug Resist.* 11, 1557–1571. doi: 10.2147/idr.s166348

Su, G. L., Ko, C. W., Bercik, P., Falck-Ytter, Y., Sultan, S., Weizman, A. V., et al. (2020). AGA clinical practice guidelines on the role of probiotics in the management of gastrointestinal disorders. *Gastroenterology* 159, 697–705. doi: 10.1053/j.gastro.2020.05.059

Watkins, C., Murphy, K., Dempsey, E. M., O'Shea, C. A., Murphy, B. P., O'Toole, P. W., et al. (2019). Dose-interval study of a dual probiotic in preterm infants. *Arch. Dis. Child Fetal Neonatal Ed.* 104, F159–F164.

Yassour, M., Vatanen, T., Siljander, H., Hamalainen, A. M., Harkonen, T., Ryhanen, S. J., et al. (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Sci. Transl. Med.* 8:343ra81.

Yeo, S. K., and Liong, M. T. (2010). Effect of prebiotics on viability and growth characteristics of probiotics in soymilk. *J. Sci. Food Agric.* 90, 267–275. doi: 10.1002/jsfa.3808

Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M., Bashiardes, S., et al. (2018). Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell* 174, 1388–405.e21.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 16 October 2020
doi: 10.3389/fmicb.2020.583254

Exopolysaccharides From *Lactobacillus paracasei* Isolated From Kefir as Potential Bioactive Compounds for Microbiota Modulation

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Edited by:
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Reviewed by:
Alex Galanis,
Democritus University of Thrace, Greece
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University of Chile, Chile

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *14 July 2020*
Accepted: *23 September 2020*
Published: *16 October 2020*

Citation:
Bengoa AA, Dardis C, Gagliarini N, Garrote GL and Abraham AG (2020) Exopolysaccharides From Lactobacillus paracasei Isolated From Kefir as Potential Bioactive Compounds for Microbiota Modulation. Front. Microbiol. 11:583254. doi: 10.3389/fmicb.2020.583254

Microbiota coexists in true symbiosis with the host playing pivotal roles as a key element for well-being and health. Exopolysaccharides from lactic acid bacteria are an alternative as novel potential prebiotics that increase microbiota diversity. Considering this, the aim of the present work was to evaluate the capacity of the EPS produced by two *L. paracasei* strains isolated from kefir grains, to be metabolized *in vitro* by fecal microbiota producing short chain fatty acids. For this purpose, fecal samples from healthy children were inoculated in a basal medium with EPS and incubated in anaerobiosis at 37°C for 24, 48, and 72 h. DGGE profiles and the production of SCFA after fermentation were analyzed. Additionally, three selected samples were sequenced by mass sequencing analysis using Ion Torrent PGM. EPS produced by *L. paracasei* CIDCA 8339 (EPS_{8339}) and CIDCA 83124 (EPS_{83124}) are metabolized by fecal microbiota producing a significant increase in SCFA. EPS_{8339} fermentation led to an increment of propionate and butyrate, while fermentation of EPS_{83124} increased butyrate levels. Both EPS led to a profile of SCFA different from the ones obtained with inulin or glucose fermentation. DGGE profiles of 72 h fermentation demonstrated that both EPS showed a different band profile when compared to the controls; EPS profiles grouped in a cluster that have only 65% similarity with glucose or inulin profiles. Mass sequencing analysis demonstrated that the fermentation of EPS_{8339} leads to an increase in the proportion of the genera *Victivallis*, *Acidaminococcus* and *Comamonas* and a significant drop in the proportion of enterobacteria. In the same direction, the fermentation of EPS_{83124} also resulted in a marked reduction of Enterobacteriaceae with a significant increase in the genus *Comamonas*. It was observed that the changes in fecal microbiota and SCFA profile exerted by both polymers are different probably due to

differences in their structural characteristics. It can be concluded that EPS synthesized by both *L. paracasei* strains, could be potentially used as bioactive compound that modify the microbiota increasing the production of propionic and butyric acid, two metabolites highly associated with beneficial effects both at the gastrointestinal and extra-intestinal level.

Keywords: prebiotics, probiotics, microbiota, short chain fatty acids, exopolysaccharide, lactic acid bacteria

INTRODUCTION

Microbiota coexists in true symbiosis with the host playing pivotal roles as a key element for well-being and health (Weiss and Hennet, 2017; Cani, 2018). The relevance of microbial ecology at the intestinal level on health status led the "International Scientific Association for Probiotics and Prebiotics" (ISAPP) to propose the concept of "normobiosis" to characterize a healthy microbiota where microorganisms with potential benefits for health predominate in number compared to potentially harmful ones, in contrast to "dysbiosis" in which one or a few potentially harmful microorganisms are dominant creating a disease situation (Roberfroid et al., 2010; Thursby and Juge, 2017). The intestinal microbiota responds to multiple stress factors such as diet, antibiotic use, inflammation of the intestinal tract and/or infection of the host with enteric pathogens (Conlon and Bird, 2015; Shen and Wong, 2016; Durack and Lynch, 2019). A stable microbiota with an adequate balance is necessary to maintain the integrity of the epithelial barrier, the immune balance and the physiological control of inflammatory processes. In turn, a "dysbiotic" microbial composition leads to an intestine with loss of integrity of the epithelial barrier which favors bacterial translocation and inflammation (Round and Mazmanian, 2009; Tsai et al., 2019). In this context, the use of diet as a basis for modifying the microbiota has re-emerged in recent years, validating the ancient concepts of the relevant role of nutrition in health (Requena et al., 2018). Therefore, the interest for probiotics or novel sources of prebiotic compounds is increasing all over the world (Ewaschuk and Dieleman, 2006; Gareau et al., 2010; Alagón Fernández del Campo et al., 2019; Venema et al., 2020).

Prebiotics has recently been defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit," expanding the concept of prebiotics to include non-carbohydrate substances with healthy effects even at distal sites (Gibson et al., 2017). Prebiotics has been studied for the modulation of infant microbiota on account of their long-lasting effects, extended even after the administration period. Otherwise, they have low risk of serious adverse effects and are easy to administrate in infant foods (Miqdady et al., 2020). The administration of prebiotics in children is associated with a number of beneficial health outcomes, such as reduced risk of some allergic reactions, reduced inflammation and risk of infections (Miqdady et al., 2020). Additionally, they may contribute to reduce the risk of development of dysbiosis associated chronic diseases like intestinal bowel disease, irritable bowel syndrome, and type 1 diabetes (Milani et al., 2017). Oligosaccharides present in human milk (HMO) as well as galactooligosacharides (GOS) and fructooligosaccharides (FOS) usually included in infant milk formulae are the most studied prebiotics used for children, which have been proved to induce specific changes in the composition and metabolic activity of the intestinal microbiota (Braegger et al., 2011; Parker et al., 2020). The end products of prebiotics fermentation are acetic, propionic and butyric acids, lactic acid, hydrogen, methane and carbon dioxide (Louis et al., 2014). Lactate and short chain fatty acids (SCFA) are used by host cells as an energy source. They participate as mediators of the host response since they are able to interact with G protein-coupled receptors (GPR43, GPR41, GPR81, and GPR109A) modulating positively or negatively the activity of enzymes that originate second messengers. Besides, some SCFA act as epigenetic regulators by inhibition of histone deacetylases (HDAC) (Kasubuchi et al., 2015). In addition, they can promote the integrity of the epithelial barrier function by reinforcing tight junctions (Morrison and Preston, 2016) and play a role in the regulation of inflammatory response mediated by inflammasome (Offermanns, 2014). They participate in the absorption of water and electrolytes (Vinolo et al., 2011; Koh et al., 2016) and are relevant not only in the context of gastrointestinal pathologies (Louis and Flint, 2009, 2017; Thorburn et al., 2014) but also of extraintestinal diseases (Durack and Lynch, 2019).

Fermented foods containing lactic acid bacteria, whether probiotic or not, are the main source of microorganisms that temporarily complete the microbial community of the gastrointestinal tract, constituting what is known as the transient microbiome (Koh et al., 2016) that can reach 10^{10}–10^{11} viable bacteria ingested per day, depending on the eating habits of each individual (Plé et al., 2015; Rezac et al., 2018). Some lactic acid bacteria produce exopolysaccharides (EPS) during fermentation, which, when ingested with the fermented product, can serve as a substrate for commensal bacteria (Ryan et al., 2015), stimulating the development of beneficial microorganisms at the intestinal level and the production of bioactive metabolites (Salazar et al., 2011; Hamet et al., 2016). Being selectively fermented by the microbiota, exopolysaccharides from lactic acid bacteria constitute an alternative as novel potential prebiotic compounds (Balzaretti et al., 2017; Lynch et al., 2018).

Kefir is an artisanal fermented food obtained by milk fermentation with the complex microbiota present in kefir grains. This fermented milk has a long tradition of offering health benefits such as antimicrobial activity, stimulation of immune system, anti-inflammatory, anti-obesity, cholesterol lowering and antioxidant effects, improvement of lactose tolerance, and enhancement of intestinal bacterial microbiota, among others. Lactic acid bacteria, yeast and acetic acid bacteria of different

genera, species and even strains coexist in this product and they and/or the metabolites synthesized by them during fermentation could contribute to beneficial health properties attributed to its consumption (Garrote et al., 2010; Bengoa et al., 2019b). *Lactobacillus paracasei* CIDCA 8339 and CIDCA 83124 are EPS-producing strains isolated from Argentine kefir grains (Hamet et al., 2013; Bengoa et al., 2018a) that have good technological properties and fulfill safe requirement for food application of Argentine and European regulation (Bengoa et al., 2019a). *L. paracasei* strains produce EPS both in milk (Hamet et al., 2015) and culture media (Bengoa et al., 2018a). Additionally, it has been demonstrated that these strains present potential probiotic properties like the adhesion ability to intestinal epithelial cells which is increased after passage through the gastrointestinal tract (Bengoa et al., 2018b) and the protective effect against *Salmonella* infection *in vitro* (Zavala et al., 2016).

Considering that microorganisms' metabolites may contribute to health properties of the fermented product, the aim of the present work was to evaluate the capacity of the EPS produced by *L. paracasei* CIDCA 8339 and CIDCA 83124 in milk to be metabolized *in vitro* by fecal microbiota producing short chain fatty acids.

MATERIALS AND METHODS

Microorganisms, Growth Conditions, and Fermented Milks Production

L. paracasei CIDCA 8339 and CIDCA 83124 were grown in MRS broth (Difco Laboratories, Detroit, MI, United States) under aerobic conditions at 30°C for 24 h. For fermented milks production, 10 mL of an active culture of the corresponding *L. paracasei* strain containing $\approx 1 \times 10^9$ CFU/mL were inoculated in 1,000 mL of UHT low-fat milk (La Serenísima, Mastellone Hnos S.A, Argentina) and then incubated in aerobic conditions at 30°C for 24 h.

Exopolysaccharide Obtainment

EPS extraction from the fermented milk was performed according to Rimada and Abraham (2003). Fermented milks (500 mL) were heated for 30 min at 100°C to promote the detachment and dissolution of the polysaccharide bound to the cells and the inactivation of enzymes that could hydrolyze EPS. Trichloroacetic acid 8% (Ciccarelli, Santa Fe, Argentina) was added to precipitate proteins and the samples were then centrifuged at 10,000 × g for 20 min at 20°C in an Avanti J25 centrifuge (Beckman Coulter Inc., Carlsbad, CA, United States). The EPS suspended in the supernatant was precipitated by adding two volumes of ethanol per volume of supernatant. Finally, the samples were dialyzed for 48 h at 4°C with stirring through a 1 kDa cut-off dialysis membrane (Spectra/Por, Spectrum laboratories, CA, United States) to remove lactose residues. In order to evaluate samples purity, the protein content was determined qualitatively by the Bradford method (Bradford, 1976). Thin layer chromatography (TLC) was used to determine the absence of lactose and other simple sugars in the EPS samples. EPS were finally lyophilized and preserved at room temperature until use. EPS extraction was performed from two independent cultures.

EPS Molecular Mass Determination

Average molecular weight (Mw) was determined by high-performance size exclusion chromatography using a OH-PAK SB-805HQ gel filtration column (SHODEX, Kawasaki, Japan) with refractive index (RI) detection system according to Piermaria et al. (2008). Samples were filtered through a 0.45 μm membrane (Millipore Corporation, Milford, MA, United States) and 50 μL of polysaccharide solutions (0.5 g/L in $NaNO_3$ 0.1 M) were injected for each run. Samples elution was performed at room temperature using $NaNO_3$ 0.1 M as mobile phase with a flow rate of 0.95 mL/min (pressure 120–130 psi). Dextrans with Mw ranging from 97,000 to 3,800,000 Da (ALO-2770, Phenomenex, Torrance, CA, United States) were used as standard.

In vitro EPS Fermentation by Human Fecal Microbiota

Fermentation assay was carried out using fecal samples from five healthy children aged between 8 months and 3 years old. The donors were selected taking into account that they had an optimal health state (normal anthropometric values, without overweight, and without previous pathologies), an omnivorous diet and that they had not consumed antibiotics in the last 6 months prior to the assay. In addition, a survey was conducted to the parents in relation to the aforementioned aspects (**Supplementary Figure S1**). Samples were collected by the parents according to the protocol and with the sterile materials provided and sent to the laboratory the same day, together with a note of informed consent in obedience to the protocol approved by the Central Bioethical Committee, National University of La Plata (May 2017). All of them were kept at 4°C and processed within 24 h after deposition to guarantee the viability of the microorganisms present.

For the fecal homogenate, equal amounts of the five samples (5 g) were suspended into sterile phosphate buffer saline (225 mL) and mixed to obtain a 1/10 diluted pool (Aguirre et al., 2014). Homogenates were inoculated to a carbohydrate-free basal medium (1/10) with the EPS under study as the only sugar source at a final concentration of 0.3% w/v. The carbohydrate-free basal medium was formulated according to Salazar et al. (2008): peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4$ 0.01 g/L, $CaCl_2$ $6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, cysteine HCl 2.5 g/L, bile salts 0.5 g/L, and tween 80 2 g/L. The medium was autoclaved and then 1 mL/L of hemin solution (50 mg/ml) and 10 μL/L of vitamin K previously sterilized by filtration were added.

Samples were incubated for 24, 48, or 72 h at 37°C in anaerobiosis using jars (AnaeroPack, Mitsubishi Gas Chemical Company, Japan) according to Salazar et al. (2008). Controls without sugar (BM), with glucose (GLU) (Britania, Buenos Aires, Argentina) and with inulin (INU) (Saporiti, Buenos Aires, Argentina) in the same concentration (0.3% w/v) were

included in the experiment. Each fermentation condition was performed in triplicate. After fermentation, the samples were centrifuged for 10 min at 10,000 xg, the supernatant was filtered through a 0.45 μm pore membrane and stored at −20°C for SCFA quantification by gas chromatography. The pellets were stored at −80°C for the subsequent characterization of microbial populations.

Determination of Organic Acids by Gas Chromatography

Chromatographic analysis was carried out using an Agilent 7890a GC system with a DB23 column (Agilent Technologies, Santa Clara, CA, United States) coupled to a flame detector (FID). The temperature at the injection port and at the FID was 250°C. Helium was used as carrier gas at a flow rate of 1.6 mL/min. For the run, 1 μL of the sample was injected with a 1:25 split and a temperature program that consisted of a ramp from 100 to 200°C at a speed of 8°C/min, keeping constant at 200°C for 3 min was used. Calibration curves with standards of glacial acetic acid (1–50 mM), propionic acid (0.5–20 mM), butyric acid (0.5–20 mM), iso-butyric acid (0.5–20 mM), and iso-valeric acid (0.5–20 mM) were prepared (Sigma-Aldrich, San Luis, MO, United States). Organic acids were identified by comparison to standard retention times and quantified with the corresponding peak area using the calibration curve. Differences were statistically tested using One-way analysis of variance (ANOVA) with Tukey's multiple comparison test ($p < 0.05$) conducted by the GraphPad Prism® software.

DNA Isolation

DNA extractions were performed by using a commercial QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and quality were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The DNA samples were used for DGGE analysis and mass sequencing. DNA extracted from pure cultures of *Lactobacillus casei, L. plantarum, L. kefiri,* and *Bifidobacterium adolescentis* were used as reference strains for DGGE analysis.

Microbiota Evolution Analysis by DGGE

The fecal microbiota evolution during batch fermentations was analyzed by partial amplification of the 16S rRNA gene using universal primers 518R and 338F-GC (**Table 1**). PCR amplification was performed using Taq polymerase Pegasus (PB-L Biological Products, Argentina) following the manufactures instruction and using 1 ng/μL of DNA template. The reaction was carried out in a T100 thermal cycler (Bio-rad laboratories, Irvine, CA, United States) with the following amplification program: 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 20 s; and a final extension step at 72°C for 1 min. The amplification products were analyzed by electrophoresis in 1% w/v agarose gels with ethidium bromide and revealed under UV light.

Denaturing-gradient-gel electrophoresis (DGGE) was performed in a DGGE-2401 analyzer (C.B.S. Scientific Co., Del Mar, CA, United States). The PCR products (15 μL) were seeded in 8 g/100 mL polyacrylamide gels (15 × 20 × 0.075 cm) in TAE buffer [50X TAE is 2 M Tris, 1 M acetic acid, and 50 mM EDTA (pH 8.0)]. A denaturing gradient of Urea-Formamide 40–60% (100% corresponds to Urea 7 M and Formamide 40% v/v) was used to achieve the optimal separation of the bands corresponding to Eubacteria. Electrophoresis was carried out at 90 V for 16 h at 60°C. Gels were then stained by immersion for 30 min in a 0.1 μL/mL Sybr-Gold solution (Invitrogen, United States) in TAE buffer and observed under UV light. Band patterns obtained for each sample were compered using the Bionumerics 6 program (Applied maths NV, Sint-Martens-Latem, Belgium). The percentage of similarity between the samples was calculated using the Dice Similarity Coefficient and the corresponding UPGMA dendrograms were constructed.

TABLE 1 | Primers used in this study.

Primer name	Sequence	References
338f-GC	GCclamp-ACTCCTACGGGAGGCAGCAG	Bakke et al., 2011
518r	ATTACCGCGGCTGCTG	
515	GTGYCAGCMGCCGCGGTAA	Caporaso et al., 2011
806	GGACTACNVGGGTWTCTAAT	

Mass Sequencing Analysis Using Ion Torrent PGM

The mass sequencing analysis was carried out in the MR DNA molecular research laboratory (TX, United States[1]), based on established and validated protocols[2]. Primers 515 and 806 that amplify the V4 variable region of the gene that codes for 16S rRNA were used (**Table 1**). For PCR, the HotStarTaq Plus Master Mix kit (Qiagen, Hilden, Germany) was used with an amplification program that consisted of 94°C 3 min, 30 cycles of 94°C 30 s, 53°C 40 s, 72°C 1 min and finally 72°C 5 min.

The mass sequencing analysis was performed using the Ion Torrent Personal Genome machine (PGM) system (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer's guidelines. The generated data was demultiplexed and analyzed using a pipeline developed in the MR DNA molecular research laboratory. Raw data sequencing reads were quality trimmed using the QIIME suite of tools. Sequences were depleted of barcodes and primers, followed by removal of short sequences (<150 bp), sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp. Noise from sequences and chimeras were also removed. Sequencing data were grouped into 3% divergence operating taxonomic units (OTUs) and taxonomically classified using the BLASTn.NET algorithm with the database derived from RDPII[3] and NCBI[4].

[1]www.mrdnalab.com, Shallowater,
[2]http://www.mrdnalab.com/
[3]http://rdp.cme.msu.edu
[4]http://www.ncbi.nlm.nih.gov

RESULTS

EPS Production by *L. paracasei* Strains in Milk

EPS production during milk fermentation at 30°C by *L. paracasei* CIDCA 8339 and CIDCA 83124 were about 130–145 and 140–160 mg of EPS per liter of fermented milk respectively. These values are within the expected range, since EPS yield by LAB is normally very low (Ruas-Madiedo et al., 2008; Llamas-Arriba et al., 2019). Crude EPS isolated from fermented milk with *L. paracasei* CIDCA 8339 (EPS_{8339}) and CIDCA 83124 (EPS_{83124}) were partially characterized by analyzing their molecular weight distribution (Mw) by gel permeation chromatography. EPS_{8339} consists of two fractions, a high Mw fraction of about 4 10^5 Da and a low Mw fraction of about 1 10^4 Da. On the other hand, EPS_{83124} presents four fractions: a low Mw fraction of 1 10^4 Da, an intermediate Mw fraction of 7 10^4 Da and a high Mw fraction constituted by two Mw distributions of 7 10^5 and 6 10^6 Da.

Evaluation of Fecal Microbiota Evolution During EPS Fermentation

PCR DGGE profiles were employed to monitor major qualitative changes in the compositions of microbial groups of fecal homogenates with and without sugar source added after 24, 48, and 72 h fermentation. DGGE profiles obtained were both time and sugar source dependent (**Figure 1A**). After 24 h of incubation, DGGE profiles of the homogenates fermented in the presence of EPS_{8339} and EPS_{83124} had a high similarity percentage (77–81%) with respect to the profile obtained for those grown in the carbohydrate-free basal medium (**Figure 1B**). In contrast, profiles of homogenates fermented with glucose or inulin presented less similarity when compared to the basal medium. However, after 72 h fermentation, the electrophoretic profile of the homogenates fermented in the presence of glucose and inulin resembled more the profile obtained in the basal medium, while those fermented in the presence of EPS_{8339} and EPS_{83124} showed a different band pattern, locating in a separated cluster and showing only 60% similarity with the two controls (GLU and INU). In the presence of glucose or inulin, changes in microbiota were observed after short fermentation times indicating the rapid use or assimilation of these sugars by fecal microorganisms. On the other hand, when EPS_{8339} and EPS_{83124} were added to basal medium, the changes in fecal microbiota occurred after long fermentation times, probably because the microorganisms need to adapt to this new carbon source.

Dendrogram comparing V3 DGGE profiles obtained with all growth media assayed at different fermentation times is shown in **Figure 2**. Two main clusters that have a similarity value lower than 48% were observed. One of them joined the samples corresponding to the 72 h of fermentation in the media containing EPS_{8339} and EPS_{83124} and the other cluster joined the rest samples grouped in two subclusters. It can be observed that all the samples obtained after 24 h fermentation joined in the same subcluster with 71% similarity. The other subcluster grouped DGGE profiles of the rest of the samples obtained after 48 and 72 h fermentation (61% of similarity). Within this second subcluster inulin and glucose fermentation after 48 and 72 h grouped together with 72% of similarity.

Since fecal homogenates fermented during 72 h with EPS_{8339} and EPS_{83124} presented the higher differences in the DGGE band pattern compared to basal medium control, we proceed to perform a mass sequencing analysis of these samples using Ion Torrent PGM. Sequence depth performed in the analysis was adequate since rarefaction curves reached a plateau for all samples (**Supplementary Figure S2**). The analysis of alpha diversity across the samples gave Shannon diversity indices of 3.79, 3.23, and 3.30 for fermentation in carbohydrate-free basal medium, basal medium with EPS_{8339} and basal medium with EP_{83124}, respectively, indicating high diversity in the three samples.

Figure 3A shows the distribution of the populations present in homogenates samples after 72 h fermentation at the phylum level. The main phyla present in the sample from basal medium (control) were *Proteobacteria* (58%), *Bacteroidetes* (19%), *Firmicutes* (17%), and *Actinobacteria* (2%). Fermentation of EPS_{8339} led to an increase in the relative proportion of *Firmicutes* (29%) and *Lentisphaerae* (32%), accompanied by the decrease in *Actinobacteria* (0.5%), *Proteobacteria* (27%) and *Bacteroidetes* (8%). On the other hand, fermentation of EPS_{83124} caused a reduction of the *Actinobacteria* (0.7%) and *Bacteroidetes* (9%) phyla with an increase in the proportion of *Proteobacteria* (73%).

At the genus level, it was observed that, regardless of the EPS used during the fermentation, the proportion of *Klebsiella* and *Escherichia* (γ-*Proteobacteria*) were reduced in 6 and 28% respectively while *Bacteroides* (*Bacteroidetes*) dropped in about 11–12% (**Figure 3B**). Nonetheless, the genera that showed an increment in their relative abundance after fermentation were different in both EPS samples, indicating that EPS_{8339} and EPS_{83124} are selectively used by different microorganisms present in fecal microbiota. The proportion of *Comamonas* (β-*Proteobacteria*) increased 6% for EPS_{8339} and 52% for EPS_{83124}. This substantial rise of *Comamonas* genera explains the difference in the proportion of *Proteobacteria* phylum previously mentioned. Furthermore, fermentation of EPS_{8339} increased the proportion of the genera *Victivallis* (33%) and *Acidaminococcus* (15%) which correspond to almost the total rise in *Lentisphaerae* and *Firmicutes* phyla evidenced. However, fermentation of both EPS did not induce major changes in the population of *Lactobacillus* and *Bifidobacterium*.

SCFA Production During EPS Fermentation by Fecal Microbiota in Batch Cultures

When analyzing the SCFA levels in the supernatant of fermented samples, it was evidenced that EPS_{8339} and EPS_{83124} are metabolized by the fecal microbiota producing, consequently, a significant increase in organic acids with recognized biological activity (propionate and butyrate) compared to basal medium. As an example, chromatograms obtained with fecal homogenates with EPS_{83124} after different fermentation times are shown in **Supplementary Figure S3**. Total SCFA levels increased during

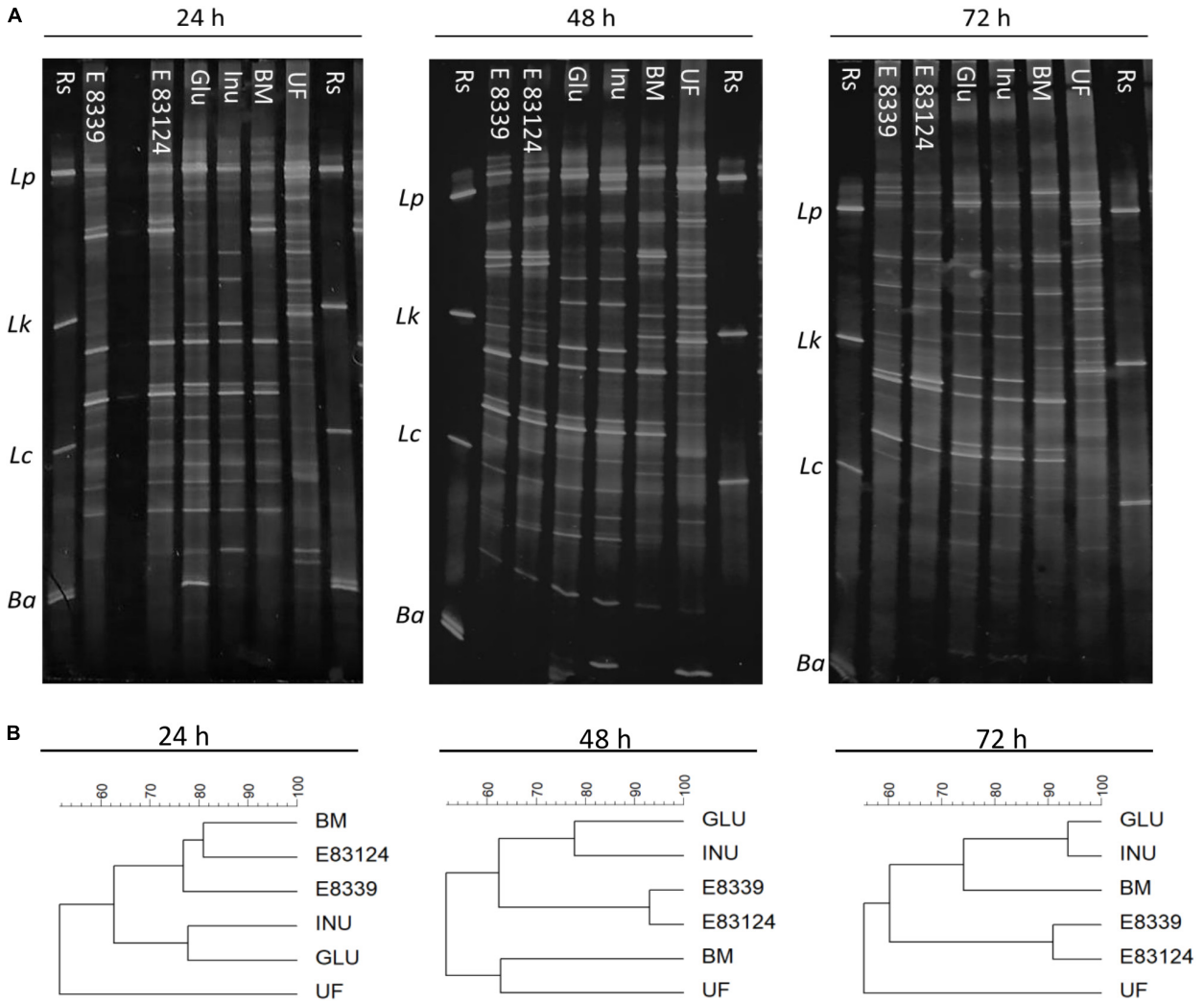

FIGURE 1 | PCR-based DGGE profiles **(A)** and the respective dendrograms **(B)** of fecal homogenates after 24, 48, and 72 h fermentation with EPS_{8339} (E 8339), EPS_{83124} (E 83124), glucose (Glu), inulin (Inu) or without extra sugar added (BM). UF profile corresponds to the one obtained with the unfermented fecal homogenate and Rs corresponds to reference strains. Lp, *Lactobacillus. plantarum*; Lk, *Lactobacillus kefiri;* Lc, *Lactobacillus casei;* Ba, *Bifidobacterium adolescentis.* Dendrograms were obtained by similarity analysis of DGGE profiles using Dice similarity coefficient and UPGMA cluster analysis.

the first 24 and 48 h of incubation with and without external carbon sources while no changes or a decrease in total SCFA were observed after 72 h depending on the sugar source. Fermentation of EPS_{8339} showed a pattern similar to glucose, where the greatest increase in SCFA was evidenced at 48 and 72 h. The fermentation of EPS_{83124} showed a significant increase after 24 h followed by a drop after 72 h of fermentation. This reduction could be due to the fact that some SCFA produced can be then consumed by some microbial population whose activity was selectively stimulated by this EPS (**Figure 4A**).

On account of the levels of each individual SCFA with biological activity, it was observed that neither EPS_{8339} nor EPS_{83124} significantly increased acetate levels. However, in the case of EPS_{83124} a reduction in acetate levels was observed after 72 h fermentation similar to the results obtained with carbohydrate-free basal medium (**Figure 4B**). Regarding propionate, EPS_{8339} and glucose fermentation led to a significant increase of this organic acid at 48 and 72 h, while fermentation of EPS_{83124} produced a significant increase in propionate only at 48 h (**Figure 4C**). However, the levels of propionate achieved in the presence of EPS are low compared to inulin fermentation, since this prebiotic was metabolized by fecal microbiota producing propionate in concentrations of about 100 mM (**Table 2**), 10 times higher than those obtained with EPS_{8339}, EPS_{83124} or glucose. Furthermore, it is noteworthy that fermentation of both EPS led to a significant increase in butyrate at all fermentation times, unlike the results observed with glucose and inulin where no increment in butyrate was observed (**Figure 4D** and **Table 2**).

These results show that EPS_{8339} fermentation led to an increment of propionate and butyrate, while fermentation of EPS_{83124} increased butyrate levels. Noteworthy, both EPS

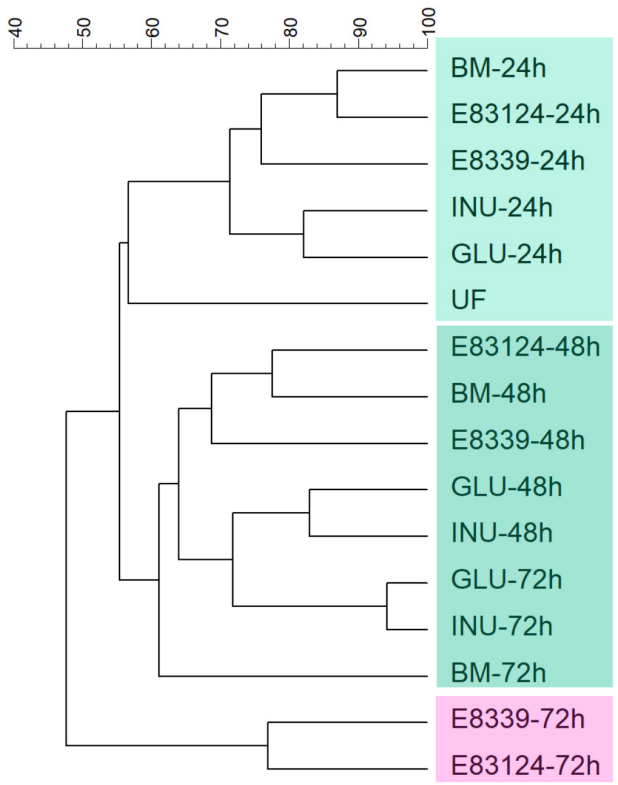

FIGURE 2 | UPGMA dendrogram of DGGE profiles of fecal homogenates microbiota after different fermentation times with EPS_{8339} (E 8339), EPS_{83124} (E 83124), glucose (GLU), inulin (INU) or without sugar source added (BM). UF corresponds to the unfermented fecal homogenate. Dendrogram was obtained by similarity analysis of DGGE profiles using Dice similarity coefficient and UPGMA cluster analysis.

generated a different pattern of SCFA than inulin fermentation, which significantly increases propionate but does not modify butyrate levels.

In addition, two peaks that elute at 2.63 and 3.25 min and that were identified as isobutyric and isovaleric acid respectively, progressively increased with fermentation time when both bacterial EPS were used as sugar sources (**Table 3**). In contrast, these organic acids were not detected when inulin or glucose were present.

DISCUSSION

The understanding of the mechanisms by which a balanced microbiome contributes to health has been considerably expanded in the last years, being the role of the metabolites one of the focus of research. In particular, SCFA results of great interest because of their wide variety of health benefit effects at both intestinal and extra-intestinal level. The homeostasis of the intestinal microbiota and its corresponding metabolome depends on the characteristics of the host (age, sex, genetic background) and on environmental conditions (stress, medications, gastrointestinal surgery, infectious, and toxic agents) (Conlon and Bird, 2015). Although microbiota composition is diverse between individuals in terms of genus and species, it is dominated by four main phyla: *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria* (Hollister et al., 2014; Hugon et al., 2015) as it was described in the present work. It is worth to note that the percentage of each phylum described herein was in concordance to those described previously (Milani et al., 2017). The early colonization process is crucial for long-term health benefits having the infant gut microbiota a main role in modulating risk factors related to adult health conditions (Milani et al., 2017). It was observed that gut bacterial microbiome rapidly diversifies over the first years of life in healthy children while is less diverse in those who develop allergy or asthma or who are malnourished (Durack and Lynch, 2019). As the composition of the infant's microbiome can have a profound effect on adult life, the search of new compounds that contribute to microbiota modulation for their inclusion in infant diet results of relevance. In the present work, the changes in infant fecal microbiota and SCFA levels induced by EPS_{8339} and EPS_{83124} fermentation were studied. For that purpose, fecal homogenates prepared in a basal medium were added with different sugars including glucose and inulin that were used as controls.

Lactic acid bacteria exopolysaccharides are widely studied for its contribution to food texture. Still, they can also act as bioactive compounds that are able to exert their effect by direct interaction to the epithelial cells or indirectly by inducing specific changes in the composition and metabolic activity of the intestinal microbiota (Gibson et al., 2017). They are highly diverse in structure and sugar composition and it has been evidenced that the biological activity attributed to each biopolymer, such as the prebiotic potential, is mostly dependent on its molecular characteristics (Salazar et al., 2016). The fermentation of EPS synthetized by *L. paracasei* CIDCA 8339 and CIDCA 83124 led to changes in fecal microbiota as well as in SCFA profile. These results indicate in first place, that both EPS are fermentable by fecal microbiota. However, EPS synthetized by *L. paracasei* CIDCA 8339 and CIDCA 83124 in milk showed different molecular weight distribution. Thus, it is not surprising that the changes in the fecal microbiota and in SCFA profile exerted by both polymers are different.

When analyzing the mass sequencing data from 72 h fermentation samples, it was evidenced that neither EPS_{8339} nor EPS_{83124} favored the growth of the genera commonly consider as beneficial such as *Lactobacillus* and *Bifidobacterium*. Moreover, despite the production of propionate and butyrate evidenced in these samples, none of the microorganisms generally associated to the production of those SCFA including *Faecalibacterium prausnitzii, Eubacterium rectale, Eubacterium hallii, Ruminococcus bromii, Akkermansia municiphilla*, and *Roseburia intestinalis* (Morrison and Preston, 2016) were increased after 72 h fermentation when compared to basal medium. It has been reported that species from the genera *Victivallis, Acidaminococcus* and *Comamonas*, the most favored by EPS_{8339} and EPS_{83124} fermentation, are part of the human gastrointestinal tract (Jumas-Bilak et al., 2007; Segata et al., 2012; Samb-Ba et al., 2014; Ricaboni et al., 2017). However, they

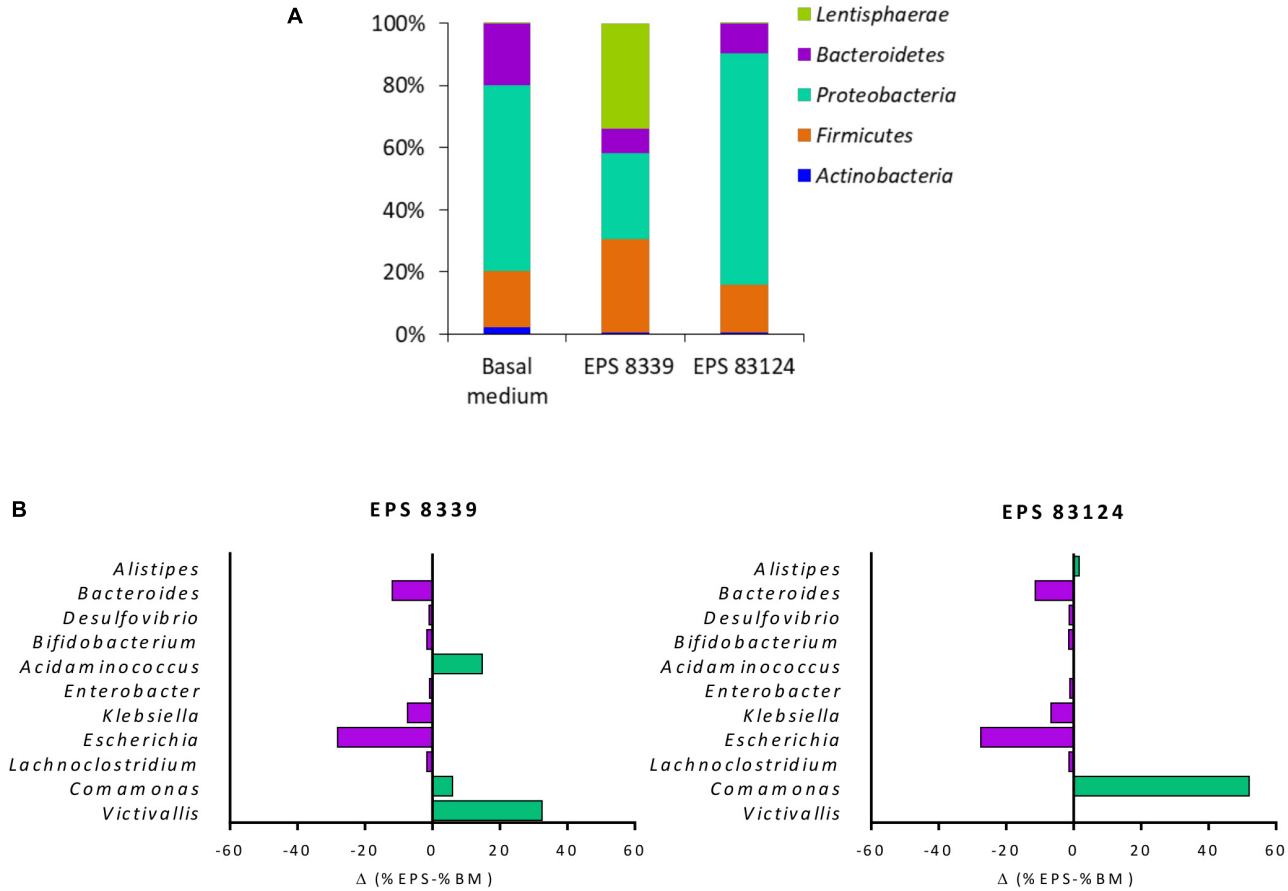

FIGURE 3 | Relative abundance at the phylum level of fecal homogenates fermented for 72 h in the presence EPS_{8339}, EPS_{83124} or without extra sugar added (BM). Only phyla with relative abundance higher than 1% in at least one sample were included **(A)**. Differences in bacterial relative abundance at the genus level in fecal homogenates fermented for 72 h with EPS_{8339} and EPS_{83124} in comparison with homogenates fermented 72 h without extra sugar added (BM). Only genera that showed differences higher than 1% in at least one sample were included **(B)**.

have not been widely studied and their role in the intestinal microbiota is not clearly yet. Although they do not correspond to any of the genera commonly associated with the production of SCFA, it has been reported in the literature that *Acidaminococcus* species are capable of producing acetate, propionate and butyrate (Jumas-Bilak et al., 2007). Furthermore, the genus *Victivallis* also contributes to acetate production (Zoetendal et al., 2003). Despite no significant increase in acetate levels were observed in the samples fermented in the presence of EPS, it must be considered that acetate can be used by many gut commensals to produce propionate and butyrate in a growth-promoting cross-feeding process (Verkhnyatskaya et al., 2019). Considering this, the production of acetate by *Acidaminococcus* and *Victivallis* could indirectly promote the production of other SCFA, such as butyrate through the butyryl-CoA: acetate-CoA transferase pathway present in some microorganisms of the microbiota (Louis and Flint, 2017). In this way, these populations could be directly or indirectly responsible for the significant increase in butyrate observed during fermentation of EPS_{8339}. The fermentation of EPS_{83124} results in an increase of species of the genera *Comamonas* such as *C. aquatica* and *C. kerstersii* (data not shown) that do not produce SCFA but instead are able to consume them (Wauters et al., 2003). Therefore, the pronounced increase in *Comamonas* due to EPS_{83124} fermentation could explain the significant drop in acetate and butyrate evidenced after 72 h fermentation. It has been evidenced that the presence of this genus, that is normally located in the Lieberkühn crypts, is beneficial since it participates in the maintenance of local homeostasis that is essential for epithelial regeneration (Pédron et al., 2012). Similarly, the consumption of milk containing *L. casei* BL23 in Balb/c mice also generated a significant increase in *Comamonas* (Yin et al., 2014). Moreover, the significant decrease in the *Enterobacteriaceae* family, that include genera usually associated with pathogens (*Klebsiella* and *Escherichia*), evidenced with both EPS also contributes to a more anti-inflammatory and healthy gut state.

It can also be highlighted that results obtained with these EPS differ from the results obtained with inulin that was included as a positive prebiotic control. Fermentation of both EPS conducted to a different SCFA profile and led to greater changes in the microbial population after 72 h fermentation. In the DGGE profiles obtained for glucose (a sugar easily fermentable by

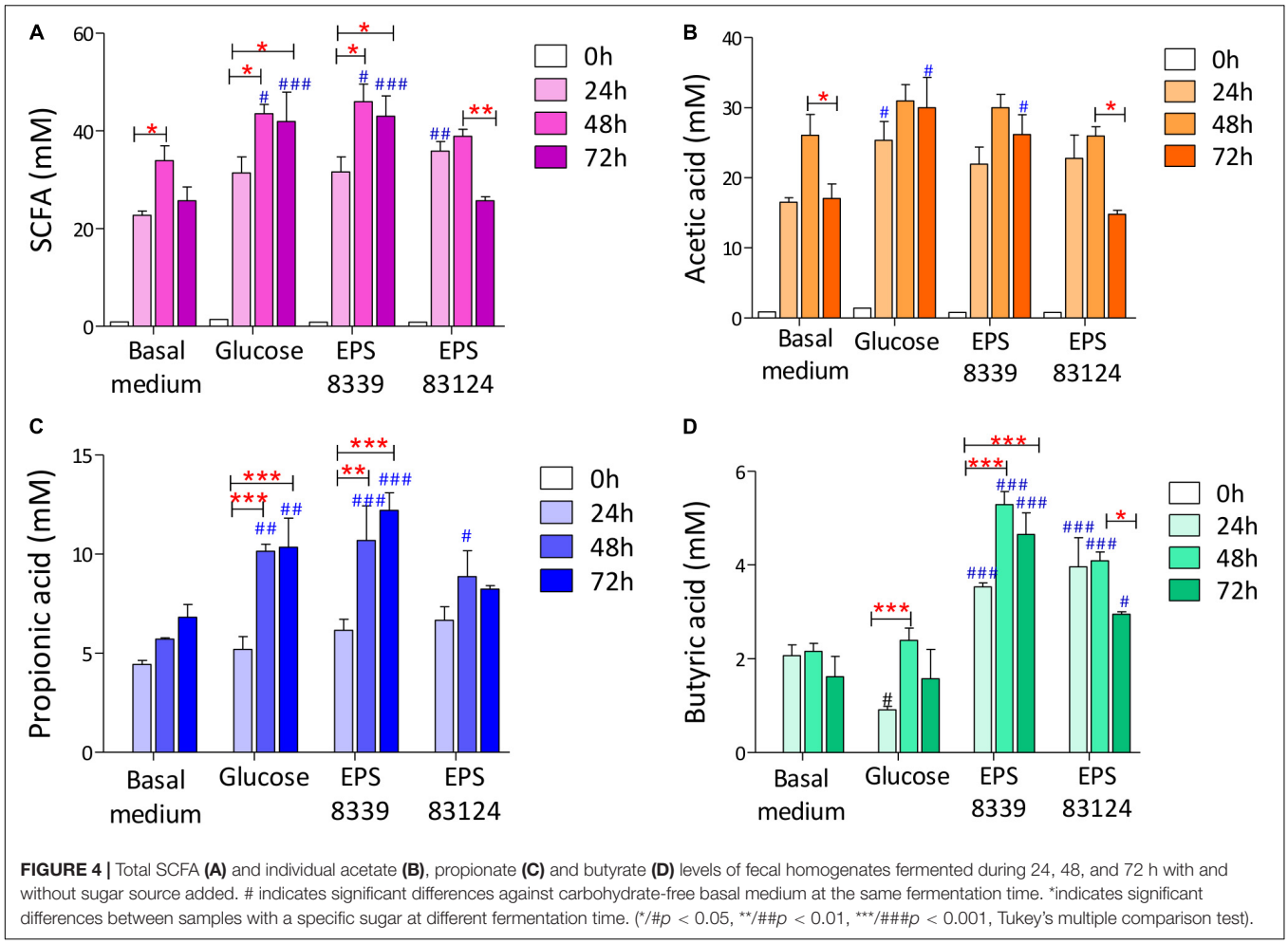

FIGURE 4 | Total SCFA **(A)** and individual acetate **(B)**, propionate **(C)** and butyrate **(D)** levels of fecal homogenates fermented during 24, 48, and 72 h with and without sugar source added. # indicates significant differences against carbohydrate-free basal medium at the same fermentation time. *indicates significant differences between samples with a specific sugar at different fermentation time. (*/#$p < 0.05$, **/##$p < 0.01$, ***/###$p < 0.001$, Tukey's multiple comparison test).

TABLE 2 | Short chain fatty acids (SCFA) concentration of the homogenates fermented in basal medium with inulin at different times.

	Fermentation time		
	24 h	**48 h**	**72 h**
Total SCFA (mM)	131.90 ± 0.67	150.25 ± 34.20	135.00 ± 12.51
Acetic acid (mM)	27.25 ± 1.33	31.41 ± 6.52	31.63 ± 2.91
Propionic acid (mM)	102.29 ± 0.41	116.21 ± 27.17	101.78 ± 9.55
Butyric acid (mM)	1.59 ± 0.21	2.63 ± 0.51	1.59 ± 0.17

Significant differences ($p < 0.05$, Tukey's multiple comparison test) were no detected at different fermentation times.

TABLE 3 | Isobutyric and isovaleric levels from homogenates fermented in basal medium with or without EPS at different incubation times.

	Fermentation time (h)	**Isobutyric acid (mM)**	**Isovaleric acid (mM)**
Basal medium	24	0.37 ± 0.03 ^{a}	0.24 ± 0.17 ^{a}
	48	0.79 ± 0.13^{ab}	0.45 ± 0.06^{a}
	72	1.15 ± 0.50^{b}	0.90 ± 0.55^{a}
Basal medium with EPS_{8339}	24	0.37 ± 0.12 ^{a}	0.20 ± 0.13^{a}
	48	1.28 ± 0.45 ^{b}	2.86 ± 1.74 ^{b}
	72	3.03 ± 0.20 ^{c}	6.01 ± 0.65 ^{c}
Basal medium with EPS_{38124}	24	0.30 ± 0.09 ^{a}	ND
	48	1.30 ± 0.51 ^{b}	2.59 ± 1.26 ^{b}
	72	2.27 ± 0.01 ^{d}	4.00 ± 0.012 ^{b}

ND, non-detected. Different letters indicate significant differences ($p < 0.05$, Tukey's multiple comparison test).

microorganisms that was included as control of bacterial growth and activity) and inulin it can be observed a high percentage of similarity between both sugars at 48 and 72 h fermentation with practically identical band patterns. These similar patterns obtained with inulin and glucose could be attributed to the fact that the donors of fecal samples regularly consumed formula milk with inulin, FOS or GOS, so their microbiota is probably adapted to inulin-type prebiotics. These results highlight the relevance of finding new compounds that can exert a beneficial effect on the intestinal microbiota different from those observed with the prebiotics commonly used in food in order to contribute to establish a widely diverse intestinal microbiota usually associated with a healthy state (Durack and Lynch, 2019). In this context, the EPS produced by *L. paracasei* CIDCA 8339 and CIDCA 83124 emerge as alternative potential prebiotics that can be fermented by the fecal microbiota *in vitro*, producing modifications in

the DGGE microbiological profile after 72 h that differs from the changes induced by inulin. Additionally, in contrast to inulin that induced mainly the production of propionate, the fermentation of EPS_{8339} and EPS_{83124} led to a significant increase in butyrate levels, a bioactive metabolite with several beneficial effects at the intestinal level. It is remarkable that, even though bacterial EPS_{8339} and $EPS8_{3124}$ required 72 h fermentation to induce changes in microbial population, they rapidly modified microbiota activity as can be evidenced by the increase in SCFA after 24 h. Considering this, both EPS could be used as complementary prebiotics that contribute to consumers' health inducing favorable changes at the intestinal microbiota that are different from the ones induced by inulin.

The increase of butyrate as a consequence of EPS_{8339} and EPS_{83124} fermentation may bring a wide range of health benefits, particularly at the intestinal level. Butyrate can be used as an energy source by enterocytes (van der Beek et al., 2015). Moreover, it contributes to strengthen the intestinal epithelial barrier through a mechanism that involves the induction of tight junctions' proteins expression such as Claudin 1 and ZO-1 and their redistribution in the membrane (Morrison and Preston, 2016). The loss of integrity of the intestinal barrier and the consequent increase in its permeability is generally associated with an increase in bacterial translocation and/or its wall components, which results in a mild chronic inflammatory state that has been associated with pathologies such as obesity, insulin resistance and diabetes type 2 (Cani et al., 2008; Qin et al., 2012). Furthermore, propionate production would also be beneficial in people that suffer of obesity since it inhibits cholesterol synthesis at the liver, regulates lipogenesis in adipose tissue (Ríos-Covián et al., 2016; Tsai et al., 2019) and regulates appetite through the expression of leptin, PYY and GLP-1 (Chambers et al., 2015). Thus, the use of prebiotics that leads to the production of butyrate and/or propionate at the intestinal level results interesting in individuals with these kinds of metabolic disorders. On the other hand, SCFA regulate the immune response at the intestinal level, contributing to the healthy state in patients suffering from inflammatory bowel diseases. Butyrate, for instance, exerts an anti-inflammatory effect by inhibiting the activation of the transcription factor NFκB in macrophages and the expression of proinflammatory cytokines (IL-6 and IL-12) in dendritic cells. Moreover, butyrate and propionate are able to regulate the production and function of regulatory T cells by inhibiting histone deacetylases (Morrison and Preston, 2016; Requena et al., 2018). Dysbiosis observed in inflammatory bowel diseases, including ulcerative colitis and Crohn's disease, is generally associated with a reduction in SCFA levels (Alagón Fernández del Campo et al., 2019) and an increase in species of the *Enterobacteriaceae* family and other opportunistic pathogens (Gonçalves et al., 2018; Uchiyama et al., 2019). The microbiota modulation favoring the production of SCFA during infancy is relevant to reduce the risk of disease development in the future. In this context, Roduit et al. (2019) studied the role of SCFA in the prevention of allergy and asthma by analyzing SCFA levels in 1-year-old children fecal samples and correlating them with the development of disease when those children were 6 years old. The authors evidenced that children that presented the highest levels of propionate and/or butyrate when they were 1 year old, were less likely to develop asthma, food allergy and allergic rhinitis when they grew up. In the same way, it has been suggested that butyrate-producing bacteria play a key role in reducing the risk of developing type 1 diabetes in children between 1 and 5 years old (de Goffau et al., 2014).

This study revealed that the EPS produced by *L. paracasei* CIDCA 8339 and CIDCA 83124 isolated from kefir induce substantial distinct effects on fecal microbiota activity and composition of healthy children leading to selective enrichments of those microorganisms that possess the ability to adapt their growth to the respective substrates. Mass sequencing analysis demonstrated that the fermentation of EPS_{8339} leads to an increase in the proportion of the genera *Victivallis*, *Acidaminococcus,* and *Comamonas* and a significant drop in the proportion of enterobacteria. In the same direction, the fermentation of the EPS_{83124} also resulted in a marked reduction in the population of Enterobacteriaceae with a significant increase in the genus *Comamonas.* These responses were linked to directed changes in SCFA toward butyrate production in higher concentration than controls. It was observed that both EPS presented a different fermentation profile probably due to differences in their structural characteristics. EPS_{8339} fermentation led to an increment of propionate and butyrate, while fermentation of EPS_{83124} increased mainly butyrate levels. These increase in the production of propionate and/or butyrate, accompanied by a decrease in the population of Enterobacteriaceae allowed us to hypothesize that the consumption of both EPS could contribute to reduce the inflammation at the intestinal level.

Although fecal microbiota composition partially correlates with gut microbiota, these results are a first step in the knowledge of the ability of two EPS from *L. paracasei* strains isolated from kefir to be fermented by human microbiota. It can be concluded that the EPS synthesized by *L. paracasei* $CIDCA_{8339}$ and $CIDCA_{83124}$ in milk can be considered bioactive compounds that modify the microbiota increasing the production of propionic and/or butyric acid, two metabolites highly associated with beneficial effects both at the gastrointestinal and extra-intestinal level.

DATA AVAILABILITY STATEMENT

SRA data generated in this study was uploaded in NCBI database. Project number PRJNA665182. BioSample accessions SAMN16245014, SAMN16245015, and SAMN16245016.

AUTHOR CONTRIBUTIONS

AB contributed in study design and conception, and performed experimental work, data interpretation, and manuscript writing. CD contributed with DGGE experiments, and contributed to

microbiota data interpretation and writing the manuscript. NG contributed to acid organic determination and DNA extraction and revised the manuscript. GG participated in study design and conception, funding, and manuscript revising. AA participated in study design and conception, funding, and manuscript revising. All authors contributed to the article and approved the submitted version.

FUNDING

The present work was supported by CONICET, Universidad Nacional de La Plata (UNLP 18/X813, 2018-2021) and ANPCyT (PICT 2014-3137 and PICT 2016-0639).

ACKNOWLEDGMENTS

AB and NG are fellows of Consejo Nacional de Ciencia y Tecnologia (CONICET). CD is fellow of Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT). GG and AA are members of Scientific Career of CONICET. We thank M. S. Sinkec and C. Reyes Cantera for chromatographic technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.583254/full#supplementary-material

REFERENCES

Aguirre, M., Ramiro-Garcia, J., Koenen, M. E., and Venema, K. (2014). To pool or not to pool? Impact of the use of individual and pooled fecal samples for in vitro fermentation studies. *J. Microbiol. Methods* 107, 1–7. doi: 10.1016/j.mimet.2014.08.022

Alagón Fernández del Campo, P., De Orta Pando, A., Straface, J. I., López Vega, J. R., Toledo Plata, D., Niezen Lugo, S. F., et al. (2019). The use of probiotic therapy to modulate the gut microbiota and dendritic cell responses in inflammatory bowel diseases. *Med. Sci.* 7, 1–17. doi: 10.3390/medsci7020033

Bakke, I., De Schryver, P., Boon, N., and Vadstein, O. (2011). PCR-based community structure studies of Bacteria associated with eukaryotic organisms: a simple PCR strategy to avoid co-amplification of eukaryotic DNA. *J. Microbiol. Methods* 84, 349–351. doi: 10.1016/j.mimet.2010.12.015

Balzaretti, S., Taverniti, V., Guglielmetti, S., Fiore, W., Minuzzo, M., Ngo, H. N., et al. (2017). A novel rhamnose-rich hetero-exopolysaccharide isolated from *Lactobacillus paracasei* DG activates THP-1 human monocytic cells. *Appl. Environ. Microbiol.* 83, e2702–e2716. doi: 10.1128/aem.02702-16

Bengoa, A. A., Iraporda, C., Acurcio, L. B., de Cicco Sandes, S. H., Costa, K., Moreira Guimarães, G., et al. (2019a). Physicochemical, immunomodulatory and safety aspects of milks fermented with *Lactobacillus paracasei* isolated from kefir. *Food Res. Int.* 123, 48–55. doi: 10.1016/j.foodres.2019.04.041

Bengoa, A. A., Iraporda, C., Garrote, G. L., and Abraham, A. G. (2019b). Kefir micro-organisms: their role in grain assembly and health properties of fermented milk. *J. Appl. Microbiol.* 126, 686–700. doi: 10.1111/jam.14107

Bengoa, A. A., Llamas, M. G., Iraporda, C., Dueñas, M. T., Abraham, A. G., and Garrote, G. L. (2018a). Impact of growth temperature on exopolysaccharide production and probiotic properties of *Lactobacillus paracasei* strains isolated from kefir grains. *Food Microbiol.* 69, 212–218. doi: 10.1016/j.fm.2017.08.012

Bengoa, A. A., Zavala, L., Carasi, P., Trejo, S. A., Bronsoms, S., Serradell, M., et al. (2018b). Simulated gastrointestinal conditions increase adhesion ability of *Lactobacillus paracasei* strains isolated from kefir to Caco-2 cells and mucin. *Food Res. Int.* 103, 462–467. doi: 10.1016/j.foodres.2017.09.093

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L., et al. (2011). Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. *J. Pediatr. Gastroenterol. Nutr.* 52, 238–250. doi: 10.1097/MPG.0b013e3181fb9e80

Cani, P. D. (2018). Human gut microbiome: hopes, threats and promises. *Gut* 67, 1716–1725. doi: 10.1136/gutjnl-2018-316723

Cani, P. D., Bibiloni, R., Knauf, C., Neyrinck, A. M., and Delzenne, N. M. (2008). Changes in gut microbiota control metabolic diet-induced obesity and diabetes in mice. *Diabetes Metab. Res. Rev.* 57, 1470–1481. doi: 10.2337/db07-1403

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Chambers, E. S., Viardot, A., Psichas, A., Morrison, D. J., Murphy, K. G., Zac-Varghese, S. E. K., et al. (2015). Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. *Gut* 64, 1744–1754. doi: 10.1136/gutjnl-2014-307913

Conlon, M., and Bird, A. (2015). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* 7, 17–44. doi: 10.3390/nu7010017

de Goffau, M. C., Fuentes, S., van den Bogert, B., Honkanen, H., de Vos, W. M., Welling, G. W., et al. (2014). Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. *Diabetologia* 57, 1569–1577. doi: 10.1007/s00125-014-3274-0

Durack, J., and Lynch, S. V. (2019). The gut microbiome: relationships with disease and opportunities for therapy. *J. Exp. Med.* 216, 20–40. doi: 10.1084/jem.20180448

Ewaschuk, J. B., and Dieleman, L. A. (2006). Probiotics and prebiotics in chronic inflammatory bowel diseases. *World J. Gastroenterol.* 12, 5941–5950. doi: 10.3748/wjg.v12.i37.5941

Gareau, M. G., Sherman, P. M., and Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nat. Rev. Gastroenterol. Hepatol.* 7, 503–514. doi: 10.1038/nrgastro.2010.117

Garrote, G. L., Abraham, A. G., and De Antoni, G. L. (2010). "Microbial interactions in kefir: a natural probiotic drink," in *Biotechnology of Lactic Acid Bacteria*, eds F. Mozzi, R. R. Raya, and G. M. Vignolo (Ames, IO: Wiley-Blackwell), 327–340.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14, 491–502. doi: 10.1038/nrgastro.2017.75

Gonçalves, P., Araújo, J. R., and Di Santo, J. P. (2018). A cross-talk between microbiota-derived short-chain fatty acids and the host mucosal immune system regulates intestinal homeostasis and inflammatory bowel disease. *Inflamm. Bowel Dis.* 24, 558–572. doi: 10.1093/ibd/izx029

Hamet, M. F., Londero, A., Medrano, M., Vercammen, E., Van Hoorde, K., Garrote, G. L., et al. (2013). Application of culture-dependent and culture-independent methods for the identification of *Lactobacillus kefiranofaciens* in microbial consortia present in kefir grains. *Food Microbiol.* 36, 327–334. doi: 10.1016/j.fm.2013.06.022

Hamet, M. F., Medrano, M., Pérez, P. F., and Abraham, A. G. (2016). Oral administration of kefiran exerts a bifidogenic effect on BALB/c mice intestinal microbiota. *Benef. Microb.* 7, 237–246. doi: 10.3920/BM2015.0103

Hamet, M. F., Piermaria, J. A., and Abraham, A. G. (2015). Selection of EPS-producing *Lactobacillus strains* isolated from kefir grains and rheological characterization of the fermented milks. *LWT Food Sci. Technol.* 63, 129–135. doi: 10.1016/j.lwt.2015.03.097

Hollister, E. B., Gao, C., and Versalovic, J. (2014). Compositional and functional features of the gastrointestinal microbiome and their effects on human health. *Gastroenterology* 146, 1449–1458. doi: 10.1053/j.gastro.2014.01.052

Hugon, P., Dufour, J.-C., Colson, P., Fournier, P.-E., Sallah, K., and Raoult, D. (2015). A comprehensive repertoire of prokaryotic species identifi ed in human beings. *Lancet Infect. Dis.* 15, 1211–1230. doi: 10.1016/S1473-3099(15)00293-5

Jumas-Bilak, E., Carlier, J. P., Jean-Pierre, H., Mory, F., Teyssier, C., Gay, B., et al. (2007). *Acidaminococcus intestini* sp. nov., isolated from human clinical samples. *Int. J. Syst. Evol. Microbiol.* 57, 2314–2319. doi: 10.1099/ijs.0.64883-0

Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A., and Kimura, I. (2015). Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients* 7, 2839–2849. doi: 10.3390/nu7042839

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Llamas-Arriba, M. G., Hernández-Alcántara, A. M., Yépez, A., Aznar, R., Dueñas, M. T., and López, P. (2019). Functional and nutritious beverages produced by lactic acid bacteria. *Nutr. Beverag.* 12, 419–465. doi: 10.1016/b978-0-12-816842-4.00012-5

Louis, P., and Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* 294, 1–8. doi: 10.1111/j.1574-6968.2009.01514.x

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* 19, 29–41. doi: 10.1111/1462-2920.13589

Louis, P., Hold, G. L., and Flint, H. J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. *Nat. Rev. Microbiol.* 12, 661–672. doi: 10.1038/nrmicro3344

Lynch, K. M., Zannini, E., Coffey, A., and Arendt, E. K. (2018). Lactic acid bacteria exopolysaccharides in foods and beverages: isolation, properties, characterization, and health benefits. *Annu. Rev. Food Sci. Technol.* 9, 155–176.

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017). The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol. Mol. Biol. Rev.* 81:e0036-17. doi: 10.1128/mmbr.00036-17

Miqdady, M., Al Mistarihi, J., Azaz, A., and Rawat, D. (2020). Prebiotics in the infant microbiome: the past, present, and future. *Pediatr. Gastroenterol. Hepatol. Nutr.* 23, 1–14. doi: 10.5223/pghn.2020.23.1.1

Morrison, D. J., and Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microb.* 7, 189–200. doi: 10.1080/19490976.2015.1134082

Offermanns, S. (2014). Free fatty acid (FFA) and hydroxy carboxylic acid (HCA) receptors. *Annu. Rev. Pharmacol. Toxicol.* 54, 407–434. doi: 10.1146/annurev-pharmtox-011613-135945

Parker, A., Fonseca, S., and Carding, S. R. (2020). Gut microbes and metabolites as modulators of blood-brain barrier integrity and brain health. *Gut Microb.* 11, 135–157. doi: 10.1080/19490976.2019.1638722

Pédron, T., Mulet, C., Dauga, C., Frangeul, L., Chervaux, C., Grompone, G., et al. (2012). A crypt-specific core microbiota resides in the mouse colon. *mBio* 3:e00116-12. doi: 10.1128/mBio.00116-12

Piermaria, J. A., de la Canal, M. L., and Abraham, A. G. (2008). Gelling properties of kefiran, a food-grade polysaccharide obtained from kefir grain. *Food Hydrocoll.* 22, 1520–1527. doi: 10.1016/j.foodhyd.2007.10.005

Plé, C., Breton, J., Daniel, C., and Foligné, B. (2015). Maintaining gut ecosystems for health: are transitory food bugs stowaways or part of the crew? *Int. J. Food Microbiol.* 213, 139–143. doi: 10.1016/j.ijfoodmicro.2015.03.015

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490, 55–60. doi: 10.1038/nature11450

Requena, T., Martínez-Cuesta, M. C., and Peláez, C. (2018). Diet and microbiota linked in health and disease. *Food Funct.* 9, 688–704.

Rezac, S., Kok, C. R., Heermann, M., and Hutkins, R. (2018). Fermented foods as a dietary source of live organisms. *Front. Microbiol.* 9:1785. doi: 10.3389/fmicb.2018.01785

Ricaboni, D., Mailhe, M., Benezech, A., Cadoret, F., Fournier, P. E., and Raoult, D. (2017). '*Acidaminococcus timonensis*' sp. nov. and '*Acidaminococcus massiliensis*' sp. nov. isolated from human gut. *New Microb. New Infect.* 15, 46–48. doi: 10.1016/j.nmni.2016.11.010

Rimada, P. S., and Abraham, A. G. (2003). Comparative study of different methodologies to determine the exopolysaccharide produced by kefir grains in milk and whey. *Lait* 83, 79–87. doi: 10.1051/lait:2002051

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., De los Reyes-Gavilán, C. G., and Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* 7:185. doi: 10.3389/fmicb.2016.00185

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., et al. (2010). Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* 104, S1–S63. doi: 10.1017/S0007114510003363

Roduit, C., Frei, R., Ferstl, R., Loeliger, S., Westermann, P., Rhyner, C., et al. (2019). High levels of butyrate and propionate in early life are associated with protection against atopy. *Allergy* 74, 799–809. doi: 10.1111/all.13660

Round, J. L., and Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9, 313–323. doi: 10.1038/nri2515

Ruas-Madiedo, P., Abraham, A. G., Mozzi, F., and de los Reyes-Gavilán, C. G. (2008). "Functionality of exopolysaccharides produced by lactic acid bacteria," in *Molecular Aspects of Lactic Acid Bacteria for Traditional and New Applications*, eds B. Mayo, P. López, and G. Pérez-Martínez (Kerala: Research Signpost), 137–166.

Ryan, P. M., Ross, R. P., Fitzgerald, G. F., Caplice, N. M., and Stanton, C. (2015). Sugar-coated: Exopolysaccharide producing lactic acid bacteria for food and human health applications. *Food Funct.* 6, 679–693. doi: 10.1039/c4fo00529e

Salazar, N., Binetti, A., Gueimonde, M., Alonso, A., Garrido, P., González del Rey, C., et al. (2011). Safety and intestinal microbiota modulation by the exopolysaccharide-producing strains *Bifidobacterium animalis* IPLA R1 and *Bifidobacterium longum* IPLA E44 orally administered to Wistar rats. *Int. J. Food Microbiol.* 144, 342–351. doi: 10.1016/j.ijfoodmicro.2010.10.016

Salazar, N., Gueimonde, M., Gonzalez de los Reyes-Gavilán, C., and Ruas-Madiedo, P. (2016). Exopolysaccharides produced by lactic acid bacteria and *Bifidobacteria* as fermentable substrates by the intestinal microbiota. *Crit. Rev. Food Sci. Nutr.* 56, 1440–1453. doi: 10.1080/10408398.2013.770728

Salazar, N., Gueimonde, M., Hernández-Barranco, A. M., Ruas-Madiedo, P., and Gonzalez de los Reyes-Gavilán, C. (2008). Exopolysaccharides produced by intestinal *Bifidobacterium* strains act as fermentable substrates for human intestinal bacteria. *Appl. Environ. Microbiol.* 74, 4737–4745. doi: 10.1128/AEM.00325-08

Samb-Ba, B., Mazenot, C., Gassama-Sow, A., Gory Dubourg, G., Richet, H., Hugon, P., et al. (2014). MALDI-TOF identification of the human gut microbiome in people with and without diarrhea in senegal. *PLoS One* 9:e87419. doi: 10.1371/journal.pone.0087419

Segata, N., Haake, S. K., Mannon, P., Lemon, K. P., Waldron, L., Gevers, D., et al. (2012). Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol.* 13, 1–18. doi: 10.1186/gb-2012-13-6-r42

Shen, S., and Wong, C. H. (2016). Bugging inflammation: role of the gut microbiota. *Clin. Transl. Immunol.* 5:e72. doi: 10.1038/cti.2016.12

Thorburn, A. N., Macia, L., and Mackay, C. R. (2014). Diet, metabolites, and "Western-Lifestyle" inflammatory diseases. *Immunity* 40, 833–842. doi: 10.1016/j.immuni.2014.05.014

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem. J.* 474, 1823–1836. doi: 10.1042/BCJ20160510

Tsai, Y.-L., Lin, T.-L., Chang, C.-J., Wu, T.-R., Lai, W.-F., Lu, C.-C., et al. (2019). Probiotics, prebiotics and amelioration of diseases. *J. Biomed. Sci.* 26:3. doi: 10.1186/s12929-018-0493-6

Uchiyama, K., Naito, Y., and Takagi, T. (2019). Intestinal microbiome as a novel therapeutic target for local and systemic inflammation. *Pharmacol. Ther.* 199, 164–172. doi: 10.1016/j.pharmthera.2019.03.006

van der Beek, C. M., Bloemen, J. G., van den Broek, M. A., Lenaerts, K., Venema, K., Buurman, W. A., et al. (2015). Hepatic uptake of rectally administered butyrate prevents an increase in systemic butyrate concentrations in humans. *J. Nutr.* 145, 2019–2024. doi: 10.3945/jn.115.211193

Venema, K., Verhoeven, J., Verbruggen, S., and Keller, D. (2020). Xylo-oligosaccharides from sugarcane show prebiotic potential in a dynamic computer-controlled in vitro model of the adult human large intestine. *Benef. Microb.* 11, 191–200. doi: 10.3920/BM2019.0159

Verkhnyatskaya, S., Ferrari, M., De Vos, P., and Walvoort, M. T. C. (2019). Shaping the infant microbiome with non-digestible carbohydrates. *Front. Microbiol.* 10:343. doi: 10.3389/fmicb.2019.00343

Vinolo, M. A. R., Rodrigues, H. G., Hatanaka, E., Sato, F. T., Sampaio, S. C., and Curi, R. (2011). Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J. Nutr. Biochem.* 22, 849–855. doi: 10.1016/j.jnutbio.2010.07.009

Wauters, G., De Baere, T., Willems, A., Falsen, E., and Vaneechoutte, M. (2003). Description of *Comamonas aquatica* comb. nov. and *Comamonas kerstersii* sp. nov. for two subgroups of *Comamonas terrigena* and emended description of *Comamonas terrigena*. *Int. J. Syst. Evol. Microbiol.* 53, 859–862. doi: 10.1099/ijs.0.02450-0

Weiss, G. A., and Hennet, T. (2017). Mechanisms and consequences of intestinal dysbiosis. *Cell. Mol. Life Sci.* 74, 2959–2977. doi: 10.1007/s00018-017-2509-x

Yin, X., Yan, Y., Kim, E. B., Lee, B., and Marco, M. L. (2014). Short communication: effect of milk and milk containing *Lactobacillus casei* on the intestinal microbiota of mice. *J. Dairy Sci.* 97, 2049–2055. doi: 10.3168/jds.2013-7477

Zavala, L., Golowczyc, M. A., Van Hoorde, K., Medrano, M., Huys, G., Vandamme, P., et al. (2016). Selected *Lactobacillus strains* isolated from sugary and milk kefir reduce *Salmonella* infection of epithelial cells in vitro. *Benef. Microb.* 7, 585–595. doi: 10.3920/BM2015.0196

Zoetendal, E. G., Plugge, C. M., Akkermans, A. D., and de Vos, W. M. (2003). *Victivallis vadensis* gen. nov., sp. nov., a sugar-fermenting anaerobe from human faeces. *Int. J. Syst. Evol. Microbiol.* 53, 211–215.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 30 October 2020
doi: 10.3389/fmicb.2020.569700

Effects of Different Human Milk Oligosaccharides on Growth of *Bifidobacteria* in Monoculture and Co-culture With *Faecalibacterium prausnitzii*

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Edited by:
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Federal University of Paraíba, Brazil

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *04 June 2020*
Accepted: *02 October 2020*
Published: *30 October 2020*

Citation:
Cheng L, Kiewiet MBG, Logtenberg MJ, Groeneveld A, Nauta A, Schols HA, Walvoort MTC, Harmsen HJM and de Vos P (2020) Effects of Different Human Milk Oligosaccharides on Growth of Bifidobacteria in Monoculture and Co-culture With Faecalibacterium prausnitzii. Front. Microbiol. 11:569700. doi: 10.3389/fmicb.2020.569700

Human milk oligosaccharides (hMOs) are important bioactive components in mother's milk contributing to infant health by supporting colonization and growth of gut microbes. In particular, *Bifidobacterium* genus is considered to be supported by hMOs. Approximately 200 different hMOs have been discovered and characterized, but only a few abundant hMOs can be produced in sufficient amounts to be applied in infant formula. These hMOs are usually supplied in infant formula as single molecule, and it is unknown which and how individual hMOs support growth of individual gut bacteria. To investigate how individual hMOs influence growth of several relevant intestinal bacteria species, we studied the effects of three hMOs (2′-fucosyllactose, 3-fucosyllactose, and 6′-sialyllactose) and an hMO acid hydrolysate (lacto-N-triose) on three *Bifidobacteria* and one *Faecalibacterium* and introduced a co-culture system of two bacterial strains to study possible cross-feeding in presence and absence of hMOs. We observed that in monoculture, *Bifidobacterium longum* subsp. *infantis* could grow well on all hMOs but in a structure-dependent way. *Faecalibacterium prausnitzii* reached a lower cell density on the hMOs in stationary phase compared to glucose, while *B. longum* subsp. *longum* and *Bifidobacterium adolescentis* were not able to grow on the tested hMOs. In a co-culture of *B. longum* subsp. *infantis* with *F. prausnitzii*, different effects were observed with the different hMOs; 6′-sialyllactose, rather than 2′-fucosyllactose, 3-fucosyllactose, and lacto-N-triose, was able to promote the growth of *B. longum* subsp. *infantis*. Our observations demonstrate that effects of hMOs on the tested gut microbiota are hMO-specific and provide new means to support growth of these specific beneficial microorganisms in the intestine.

Keywords: human milk oligosaccharides, co-culture, *Bifidobacterium longum* subsp. *infantis*, *Faecalibacterium prausnitzii*, hMO structure-specific

INTRODUCTION

It is widely accepted that breastfeeding is the gold standard for infant nutrition, which offers complete nutrition for the newborn. Mother's milk contains bioactive components that contribute to the healthy development of the newborn (Le Doare et al., 2018). For these reasons, the World Health Organization (WHO) recommends to feed infants for at least 6 months exclusively with breastfeeding (World Health Organization [WHO], 2002; Walker, 2010). However, for a variety of reasons, there are still over 70% of the infants that cannot be exclusively breastfed (Walker, 2010; Heymann et al., 2013). These non-breastfed infants are most often fed with cow-derived infant formula (Coulet et al., 2014; Aly et al., 2018). Up to now, these cow-milk derived infant formula lack human milk oligosaccharides (hMOs), which are one of the most important bioactive components of mother's milk. These hMOs are unique to humans and provide numerous health-promoting effects (Bode, 2015; Triantis et al., 2018). Until recently, it was not possible to produce hMOs in large amounts for the application in infant formula, but this lately changed. Major advances have been made in large-scale production of hMOs allowing application of a few abundant hMOs in infant formula (Vandenplas et al., 2018).

The beneficial effects of hMOs in human milk are well-established and numerous (Bode, 2015; Cheng et al., 2019, 2020; Kong et al., 2019). One important effect of hMOs is considered to be the support of growth of beneficial gut bacteria (Thomson et al., 2018). The first year of a baby's life is critical for the establishment of the intestinal microbiome, and hMOs are an important factor in shaping the gut microbiome in the first year of life (Goldsmith et al., 2015). It is, however, still unclear which and how individual hMOs, which are already applied or considered for infant formula, support growth of individual gut bacteria. *Bifidobacterium* is one of the dominant species in the intestine of healthy breastfed infants, and can represent up to 90% of the total microbiome (Moore and Townsend, 2019). hMOs are specifically known to support the growth of *Bifidobacterium* genus (Thomson et al., 2018), e.g., *Bifidobacterium longum* subsp. *infantis*, a strong hMO user that grows well when cultured with hMOs isolated from human milk as the sole carbohydrate source (Underwood et al., 2015). However, whether individual hMOs currently developed for infant formula can also influence the growth of *B. longum* subsp. *infantis* is unknown.

The infant intestine needs, however, fast colonization not only by *Bifidobacterium* genus but also by other species that contribute to making fermentation products such as short-chain fatty acids (SCFAs) that support metabolism and immunity (Conlon and Bird, 2015). Some bacteria ferment hMO and other carbohydrates to produce SCFAs such as acetate, propionate, and butyrate, which are an important energy source for intestinal epithelium, and modulate epithelial integrity (McLeod et al., 2019). A potent SCFA producer is, for example, *Faecalibacterium prausnitzii*, which produces butyrate. This bacterium colonizes the gut during late infancy and is one of the most dominant bacterial species in the large intestine of healthy adults (Laursen et al., 2017). Although its importance for a healthy gut is broadly recognized, it is unknown how *F. prausnitzii* behaves when exposed to hMOs. It is also not known how *F. prausnitzii* is influenced by already present *Bifidobacterium* genus, and whether it benefits from cross-feeding of hMO fermented by *Bifidobacterium* genus.

To gain more insight into how currently applied or proposed hMOs for infant formula influence growth of several relevant intestinal species, we studied the effects of three hMOs 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), 6′-sialyllactose (6′-SL) and one hMO's acid hydrolysate lacto-N-triose (LNT2) on *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *Bifidobacterium adolescentis*, and *F. prausnitzii*. We first used individual hMOs as the only carbohydrate source for different bacterial strains in monoculture to investigate whether individual hMOs can modulate single-strain growth. Then, *B. longum* subsp. *infantis* and *F. prausnitzii* were brought into co-culture to study the possible interaction between these two bacteria strains. The fermentation products of *B. longum* subsp. *infantis* and *F. prausnitzii* as well as glycosidic degradation of effective hMOs under mono- and co-culture systems were analyzed.

RESULTS

Effects on Bacterial Growth of 2′-FL, 3-FL, 6′-SL, and LNT2 Were Bacterial Strain Dependent

Figure 1 shows the growth curves of *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. adolescentis*, and *F. prausnitzii* in YCFA broth with glucose, 2′-FL, 3-FL, 6′-SL, or LNT2 as the single carbon source. We found that all bacterial strains were able to grow on glucose, and the effects of 2′-FL, 3-FL, 6′-SL, and LNT2 were variations with the tested strain. *B. longum* subsp. *infantis* could grow on all the substrates we provided, but the effects were hMO structure-dependent (**Figure 1A**). When grown in the presence of 2′-FL, *B. longum* subsp. *infantis* could reach OD_{600} of 3.6 and reach stationary phase after 56 h of culture. On 3-FL, it grew to an OD_{600} of 1.7 and reached stationary phase after 32 h of culture, which was 24 h earlier than on 2′-FL. With 6′-SL, the growth of *B. longum* subsp. *infantis* started at 48 h of culture, which is much slower than with the other substrates, and reached an OD_{600} of 1.6 after 64 h of culture. On LNT2, *B. longum* subsp. *infantis* grew to an OD_{600} of 1.2 and reached stationary phase after 32 h of culture. In the presence of 2′-FL, the OD_{600} of 3.6 of *B. longum* subsp. *infantis* in stationary phase was significantly higher than with 3-FL, 6′-SL, and LNT2 as the carbon source ($p < 0.0001$, **Supplementary Figure S1**). In contrast, *B. longum* subsp. *longum* only reached a high cell density ($OD_{600} > 1$) on glucose, where the OD_{600} was 2.8 after 32 h of culture. None of the tested hMOs was able to support the growth of *B. longum* subsp. *longum* (**Figure 1B**). The growth behavior of *B. adolescentis* was similar to that observed for *B. longum* subsp. *longum*. On glucose, *B. adolescentis* grew to a high OD_{600} of 3.9 after 32 h of culture, but was not able to grow on the different hMOs and on the hMOs acid hydrolysis product LNT2 (**Figure 1C**). *F. prausnitzii* reached a lower cell density on the hMOs compared to glucose, and the growth pattern was hMO

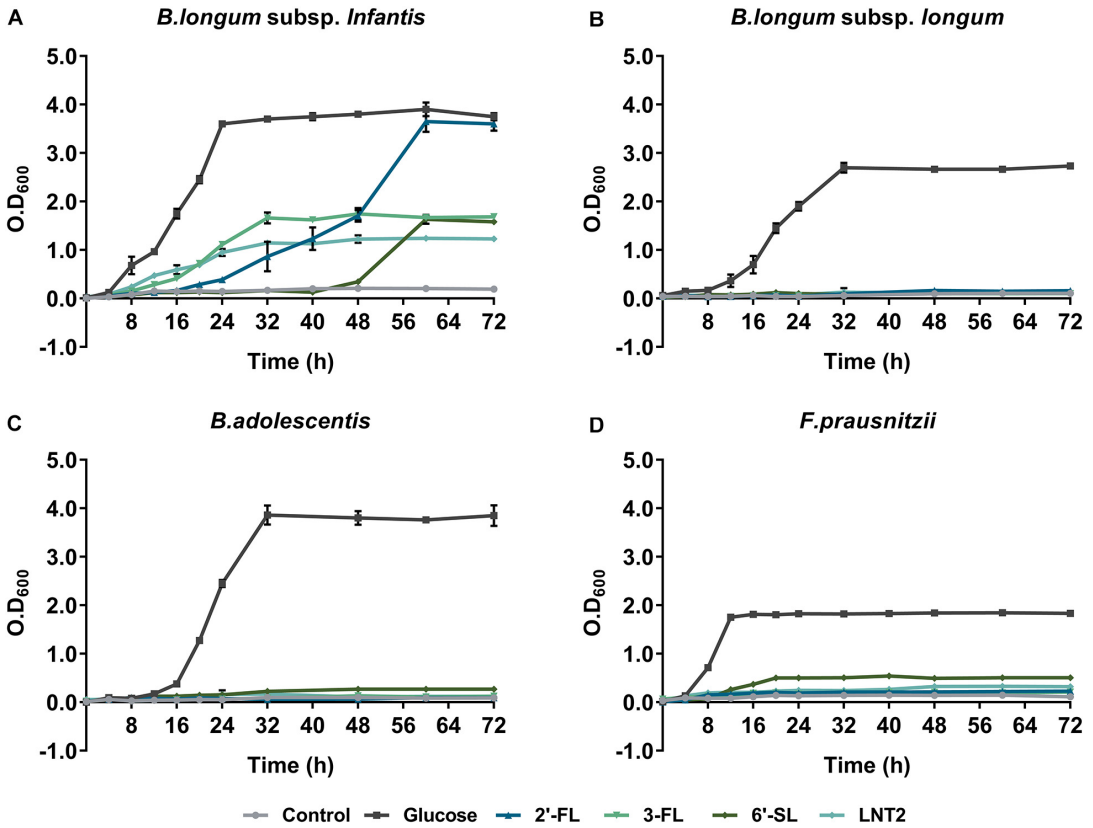

FIGURE 1 | The growth curve of **(A)** *B. longum* subsp. *infantis,* **(B)** *B. longum* subsp. *longum,* **(C)** *B. adolescentis*, and **(D)** *F. prausnitzii* in monoculture, determined by OD_{600}. Glucose, 2′-FL, 3-FL, 6′-SL, and LNT2 were included as a sole carbon source as indicated. Basal broth with no carbohydrates added was used as negative control. The assays were carried out three times in duplicate. A representative curve for each condition is shown.

structure-dependent (**Figure 1D**). *F. prausnitzii* quickly reached a final cell density at OD_{600} of 1.8 after 12 h of culture on glucose. On 2′-FL and 3-FL, it reached OD_{600} 0.2 in the stationary phase, while with LNT2, it grew to OD_{600} 0.3 at the endpoint. With 6′-SL, the OD_{600} reached 0.5 at the endpoint, which was higher than on 2′-FL, 3-FL, and LNT2, but still significantly lower than on glucose ($p < 0.0001$, **Supplementary Figure S1**). For the subsequent cross-feeding studies, we selected two bacterial strains to determine whether and which hMOs may impact the growth of the two beneficial bacteria. We chose two essential early life colonizing bacteria; *B. longum* subsp. *infantis*, which is a potent hMO user, and *F. prausnitzii*, which is a major anti-inflammatory commensal bacterium in early life in the gut (Sokol et al., 2008) and a less capable utilizer of hMOs.

The Co-culture System Can Promote the Growth of the Bacteria

We found that co-culture of *B. longum* subsp. *infantis* and *F. prausnitzii* resulted in different growth rates compared to monoculture and that it was influenced by the type of hMO. In the presence of 2′-FL, co-culture and *B. longum* subsp. *infantis* monoculture cell densities reached similar values (**Figure 2A**). However, bacteria grew significantly faster in co-cultures than in *B. longum* subsp. *infantis* monocultures, as co-cultures reached stationary phase after 32 h compared to 56 h for the monoculture of *B. longum* subsp. *infantis* (**Figure 2A**). In the presence of 3-FL, co-culture and *B. longum* subsp. *infantis* monoculture reached a similar cell density, 1.80 and 1.75 at OD_{600}, respectively, while the *F. prausnitzii* monoculture reached OD_{600} 0.2 at the endpoint (**Figure 2B**). Interestingly, in the presence of 6′-SL, the growth curves showed a similar trend as observed with 2′-FL, with the co-culture growing faster than the *B. longum* subsp. *infantis* monoculture, albeit at an overall delayed time. The *B. longum* subsp. *infantis* and *F. prausnitzii* co-culture reached stationary phase after 48 h culturing, while the *B. longum* subsp. *infantis* monoculture just started to grow at this time point. It took 16 additional hours before the *B. longum* subsp. *infantis* monoculture reached the stationary phase. At this faster growth, the OD_{600} in stationary phase was 1.8 in co-culture, 1.6 in *B. longum* subsp. *infantis* monoculture, and 0.5 in *F. prausnitzii* monoculture (**Figure 2C**). With LNT2 as carbon source, co-cultures and monocultures of *B. longum* subsp. *infantis* reached stationary phases on similar time, but in co-culture, it reached a higher cell density in stationary phase of 1.5 at OD_{600}, while in the monocultures of *B. longum* subsp. *infantis* and *F. prausnitzii*, the cell density was only 1.15 and 0.3 at OD_{600}, respectively, in the stationary phase (**Figure 2D**). The different growth rates

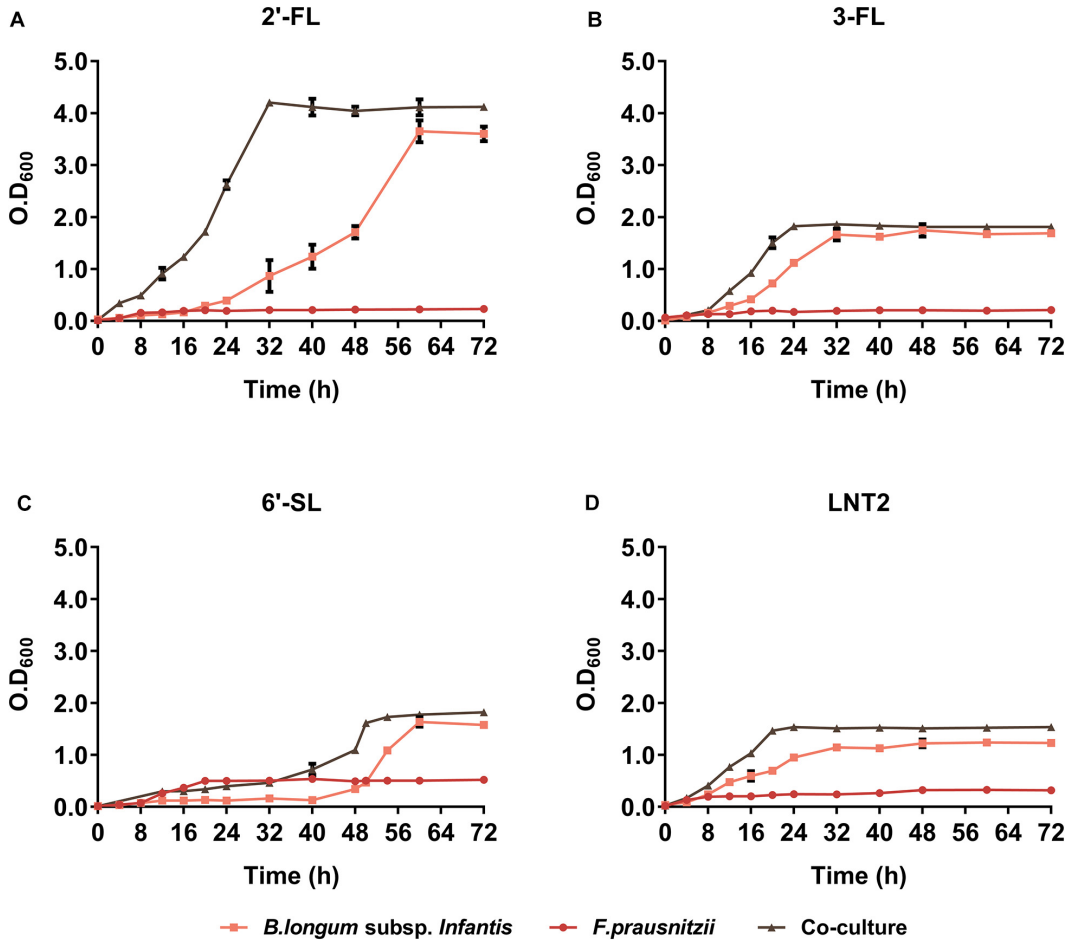

FIGURE 2 | The growth curve of *B. longum* subsp. *infantis* and *F. prausnitzii* in monoculture and co-cultures, determined at OD_{600}. **(A)** 2′-FL, **(B)** 3-FL, **(C)** 6′-SL, and **(D)** LNT2 were included as carbon source. The fermentations were carried out three times in duplicate. A representative curve for each condition is shown.

and final cell density differences in co-cultures and monocultures containing 2′-FL, 3-FL, 6′-SL, and LNT2 as carbon source indicate that the bacteria influence each other's growth pattern and promote the growth of the bacteria in an hMO structure-dependent way.

2′-FL, 3-FL, and LNT2 Do Not Enhance SCFA Production in Co-cultures While 6′-SL Promotes Acetate Production

As *B. longum* subsp. *infantis* ferments the hMOs (Ward et al., 2007), we also investigated the production of SCFAs in the mono- and co-cultures (**Figures 3**, **4**). SCFAs are one of the most important metabolic products of the bacteria and reflects activity of the metabolic processes. To this end, we quantified the SCFAs acetate, propionic acid, and butyrate after 48 h and 72 h of monoculture and co-culture. The concentrations of the produced metabolites were calculated by subtracting the initial values at 0 h.

Bifidobacterium longum subsp. *infantis* in monoculture with glucose as carbon source (control) produced a high concentration of acetic acid and minor amounts of propionic acid and butyric acid at both 48 h and 72 h. Results were different when 2′-FL, 3-FL, 6′-SL, and LNT2 were used as carbon sources. With 2′-FL, 3-FL, and LNT2 as carbon source in monoculture with *B. longum* subsp. *infantis*, we observed that no significant differences were detected compared to glucose at 48 and 72 h (**Figures 3A**, **4A**). While on 6′-SL, no acetate was detected after 48 h monoculture, and 7.31 μmol/mg acetate was produced after 72 h (**Figures 3A**, **4A**). With *F. prausnitzii*, grown on glucose as carbon source, *F. prausnitzii* consumed acetate in the broth and produced butyric acid and propionic acid in monoculture (**Figures 3**, **4**). With 2′-FL, 3-FL, 6′-SL, and LNT2, only a small amount of butyrate was produced in monoculture. This SCFA production was always significantly lower than on glucose ($p < 0.0001$), but no significant differences between hMOs were observed on both 48- and 72-h cultures (**Figures 3C**, **4C**).

When co-cultures of *B. longum* subsp. *infantis* and *F. prausnitzii* were incubated with the different substrates, high levels of acetate were observed (**Figures 3A**, **4A**). With regard to butyrate production, only a small amount of butyrate

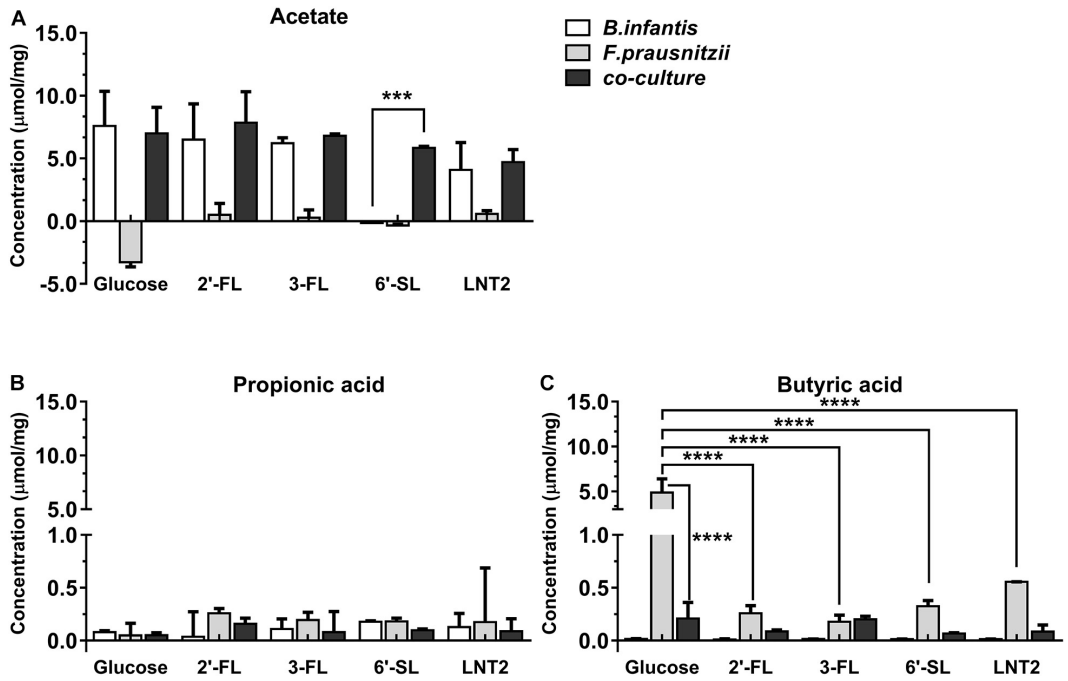

FIGURE 3 | SCFA production of glucose, 2′-FL, 3-FL, 6′-SL, and LNT2 in monoculture and co-cultures of *B. longum* subsp. *infantis* and *F. prausnitzii* after 48-h cultures. The acetate **(A)**, propionic acid **(B)**, and butyrate **(C)** products of *B. longum* subsp. *infantis* and *F. prausnitzii* were measured after 48 h of monoculture and co-cultures when having either 2′-FL, 3-FL, 6′-SL, and LNT2 as carbon source. Glucose served as positive control. Values are changes in concentrations calculated by subtracting the initial values from 0 h. Data are presented as median ± range ($n = 3$). Statistical significance was measured using Kruskal–Wallis test followed by the Dunn's test and indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, or by $^{****}p < 0.0001$.

was detected with 2′-FL, 3-FL, 6′-SL, or LNT2 after 48 h as well as after 72 h of culture. On glucose, we found similar production rates of acetate when comparing *B. longum* subsp. *infantis* monoculture and co-cultures. However, the butyrate production of the co-culture was significantly lower when compared to *F. prausnitzii* monoculture ($p < 0.0001$) at 48 and 72 h. For acetate production, in the presence of 2′-FL, 3-FL, or LNT2, no differences were found between *B. longum* subsp. *infantis* monocultures and co-cultures. Interestingly, a different result was obtained with 6′-SL. We found that in co-cultures, 5.84 μmol/mg acetate was produced, which was significantly higher than in monoculture of *B. longum* subsp. *infantis* ($p < 0.0001$, **Figure 3A**), and no acetate was detected in the monoculture of *F. prausnitzii* after 48-h cultures, and the differences between co-culture and monoculture disappeared after 72-h cultures (**Figure 4A**). With 2′-FL, 3-FL, 6′-SL, and LNT2, only small amounts of butyrate were detected in co-culture, and no significant differences were detected between the groups at 48- and 72-h cultures.

Co-culture Promotes the Utilization of 6′-SL

As 6′-SL was differently stimulating the production of SCFAs in co-cultures compared to monoculture of *B. longum* subsp. *infantis*, we decided to study and compare the degradation profile of 6′-SL in *B. longum* subsp. *infantis* monocultures and *B. longum* subsp. *infantis* and *F. prausnitzii* co-cultures. To this end, we took samples at 48 and 72 h from the *B. longum* subsp. *infantis* monocultures and *B. longum* subsp. *infantis* and *F. prausnitzii* co-cultures and studied glycosidic degradation in the samples as a measure for carbohydrate utilization.

As shown in **Figure 5**, we observed that only small amounts of 6′-SL were utilized in the monocultures of *B. longum* subsp. *infantis* after 48 h, and the peak of 6′-SL was not decreased in high-performance anion exchange chromatography (HPAEC) chromatogram profiles. In contrast, a clear degradation of 6′-SL in the *B. longum* subsp. *infantis* and *F. prausnitzii* co-culture was observed after 48 h (**Figure 5A**). The quantification results also showed that only 5.4% of the 6′-SL was utilized in the monocultures of *B. longum* subsp. *infantis* after 48 h, while 65.1% of 6′-SL in the *B. longum* subsp. *infantis* and *F. prausnitzii* co-culture was consumed (**Figure 5B**). After 72 h, 6′-SL 74.0% and 84.7% was used in *B. longum* subsp. *infantis* monoculture and in the co-culture system, respectively.

DISCUSSION

Human milk oligosaccharides are specifically known to support the growth of beneficial microorganisms in the infant gut (Jost et al., 2015). In particular, *Bifidobacterium* genus (Kirmiz et al., 2018) is acknowledged for that, but which individual hMO and how specific hMOs modulate this process is still unclear. In the present study, our results show that the modulatory effects

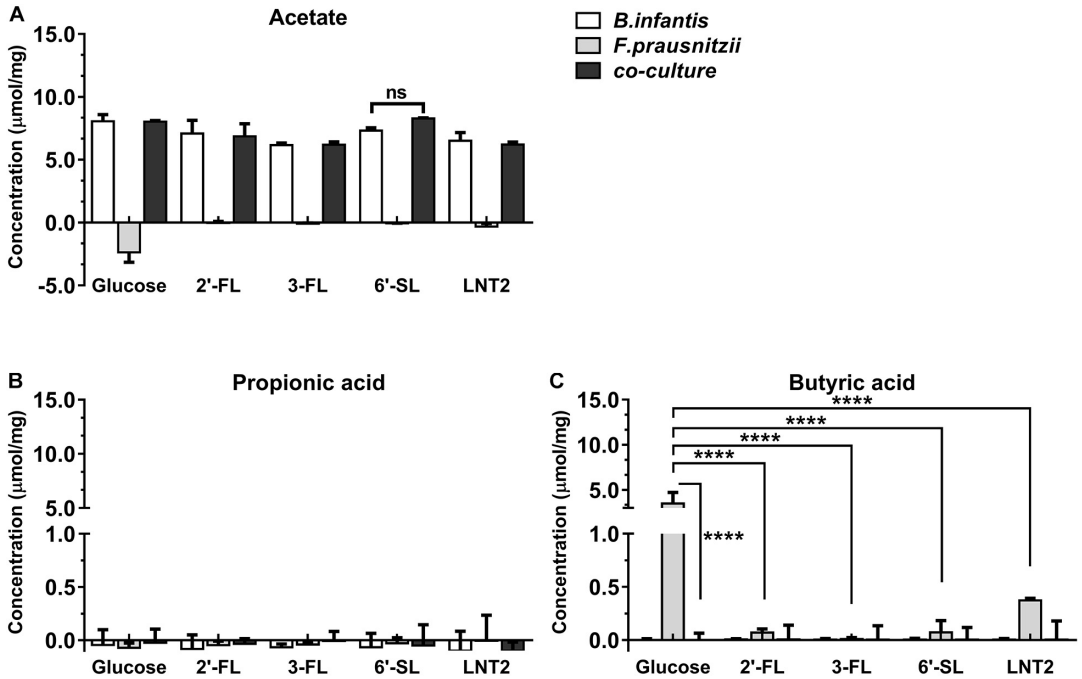

FIGURE 4 | SCFA production of glucose, 2′-FL, 3-FL, 6′-SL, and LNT2 in monoculture and co-cultures of *B. longum* subsp. *infantis* and *F. prausnitzii* after 72-h cultures. The acetate **(A)**, propionic acid **(B)**, and butyrate **(C)** products of *B. longum* subsp. *infantis* and *F. prausnitzii* were measured after 72 h of monoculture and co-cultures when having either 2′-FL, 3-FL, 6′-SL, and LNT2 as carbon source. Glucose served as positive control. Values are changes in concentrations calculated by subtracting the initial values from 0 h. Data are presented as median ± range ($n = 3$). Statistical significance was measured using Kruskal–Wallis test followed by the Dunn's test and indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, or by $^{****}p < 0.0001$.

of individual hMOs on bacterial growth are strongly structure-dependent in both monoculture and co-cultures.

In monoculture, the effects of 2′-FL, 3-FL, 6′-SL, and LNT2 on bacterial growth are bacterial strain- as well as hMO structure-dependent. Different growth patterns were observed for different bacteria strains when exposed to the same hMO. The growth of bacteria on 2′-FL, 3-FL, 6′-SL, and LNT2 was hMO structure-dependent. As shown in **Figure 6**, hMOs are composed of five monomers: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid (Sia) (**Figure 6A**). All hMOs are synthesized from lactose (Galβ1-4Glc), which can be further extended and form more than 200 different structures of hMOs (**Figure 6B**; Bode, 2015; Thurl et al., 2017). Several studies have demonstrated that the individual hMOs have different effects and that the final outcome of a specific health benefit depends on the composition of individual hMOs (Cheng et al., 2019, 2020; Kong et al., 2019). In the current study, we found that *B. longum* subsp. *infantis* grew faster on 3-FL as carbon source and reached higher cell densities on 2′-FL. 2′-FL and 3-FL are both trisaccharide hMOs that are formed by fucosylation of lactose. The molecules have the same molecular composition and only differ in the attachment position of L-fucose (Fuc) residues on the lactose core region (**Figure 6C**).

The fermentation of both 2′-FL and 3-FL by bacteria occurs via bacterial α-fucosidase and β-galactosidase enzymes (Thomson et al., 2018), and we observed that the growth rate was higher with 3-FL, and the final cell density was higher with 2′-FL. This indicates different kinetics of fermentation of 2′-FL and 3-FL by *B. longum* subsp. *infantis*. Different enzymes are needed to ferment 6′-SL and LNT2. For fermenting 6′-SL, the bacteria need β-galactosidase and α-sialidase, and fermentation of LNT2 requires β-galactosidase and β-hexosaminidase (Thomson et al., 2018). The different growth patterns of *B. longum* subsp. *infantis* on individual hMOs might be impacted by the catalytic ability of the enzymes. The observation that *F. prausnitzii* reaches a higher final OD_{600} on 6′-SL than when grown with 2′-FL, 3-FL, or LNT2 suggests that *F. prausnitzii* has a higher α-sialidase activity and less α-fucosidase and β-hexosaminidase activity.

Bifidobacterium longum subsp. *infantis* produce a high concentration of acetic acid, with minor amounts of propionic acid and butyric acid, while *F. prausnitzii* used acetate in the broth and produced butyric acid (Moens et al., 2016). In the co-culture system, the growth rate was higher than in monoculture of the individual strains. Therefore, we decided to investigate whether the acetate supplied by *B. longum* subsp. *infantis* would stimulate the metabolic activity of *F. prausnitzii* in co-culture. Hence, the SCFA production was quantified and compared between monoculture and co-cultures of *B. longum* subsp. *infantis* and *F. prausnitzii*. Interestingly, under all co-culture conditions tested, no butyrate was detected after 48 h of culture, which suggests that *F. prausnitzii*, while being a potent butyrate producer, was not able to grow in co-culture. This might be associated with the hMO utilization strategy of *B. longum* subsp. *infantis* (Thomson et al., 2018). The utilization of hMOs

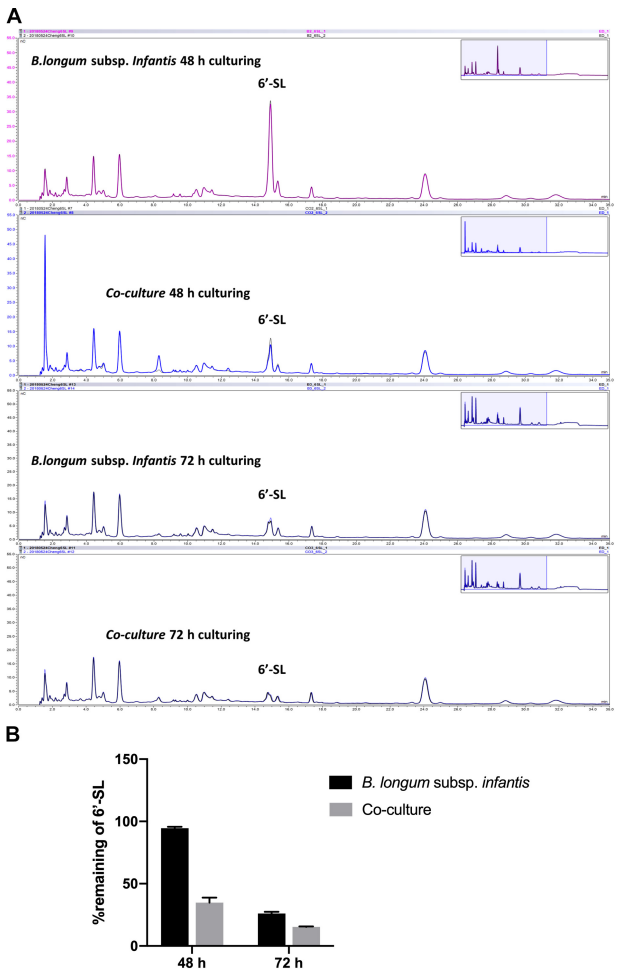

FIGURE 5 | Degradation of 6′-SL after 48-h and 72-h fermentation by *B. longum* subsp. *infantis* and *F. prausnitzii* in mono- and co-cultures. **(A)** HPAEC chromatogram profiles of 6′-SL when *B. longum* subsp. *infantis* in monoculture or co-culture with *F. prausnitzii* after 48 h and 72 h. **(B)** The percentage of remaining 6′-SL in the broth when *B. longum* subsp. *infantis* was in monoculture or co-culture with *F. prausnitzii* after 48 h and 72 h.

by *B. longum* subsp. *infantis* is based on the uptake of intact hMOs inside the bacteria, where it is intracellularly degraded (Garrido et al., 2011).

Although *F. prausnitzii* was not able to grow in the co-cultures, we still observed that with 6′-SL as carbon source, *B. longum* subsp. *infantis* and *F. prausnitzii* co-cultures had higher growth rates than in the monocultures (**Figure 2C**). This indicated that the presence of *F. prausnitzii* may promote the growth of *B. longum* subsp. *infantis* on 6′-SL in co-culture. Since only a small amount of butyrate was produced in *F. prausnitzii* monoculture as well as co-culture (**Figures 3**, **4**), and since there were no statistically significant difference between *F. prausnitzii* monoculture and co-culture, we concluded that 6′-SL was not substantially fermented by *F. prausnitzii*. Therefore the degradation of 6′-SL in *F. prausnitzii* monoculture was not included. This is in accordance with the SCFA production and carbohydrate degradation results, which showed that *B. longum* subsp. *infantis* and *F. prausnitzii* co-culture promotes acetate production and 6′-SL utilization. However, this promoting effect of *F. prausnitzii* was only observed on 6′-SL, and not on 2′-FL, 3-FL, and LNT2. As only the utilization of 6′-SL involves a sialidase (Thomson et al., 2018), we hypothesized that the co-culture of *B. longum* subsp. *infantis* and *F. prausnitzii* might enhance sialidase expression, as only 6′-SL promoted the growth and metabolism of *B. longum* subsp. *infantis*. However, in the current study, only one sialylated hMO was included. Hence, in order to confirm this hypothesis, more sialylated hMOs, such as 3′-sialyllactose (3′-SL), are needed. Unfortunately, due to the technical limitations, pure 3′-SL is not available yet, but would be of great value to test our hypothesis.

In conclusion, we demonstrate that the utilization of individual hMOs as sole carbohydrate sources is bacterial strain- and hMO structure-dependent. Our results show that hMOs currently applied or developed to be applied in infant formula are able to modulate the growth of *B. longum* subsp. *infantis* in a structure-dependent way and stimulate further growth of *B. longum* subsp. *infantis* during co-cultures. In particular, 6′-SL, which can promote the growth of *B. longum* subsp. *infantis* in *B. longum* subsp. *infantis* and *F. prausnitzii* co-culture, showed promising results. Again, we demonstrate that the effects of individual hMOs are highly structure-dependent (Cheng et al., 2019; Kong et al., 2019). Small differences in the molecular structure of hMOs can have significant impact on their biological efficacy. Follow-up studies are needed to identify the specific structure responsible for modulation of bacterial growth, e.g., impact of other sialylated hMOs, which might provide new effective and targeted ways of supporting the growth of beneficial microorganisms in the infant intestine.

MATERIALS AND METHODS

Components

In the present study, 2′-FL (provided by FrieslandCampina Domo, Amersfoort, Netherlands), 3-FL, 6′-SL, and LNT2 (provided by Glycosyn LLC, Woburn, MA, United States) were tested. An overview of the structures of these components are shown in **Table 1**.

Bacterial Strains

Faecalibacterium prausnitzii A2-165 (DSM 17677) was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ, Göttingen, Germany). *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* ATCC 15697, and *B. adolescentis* (collection strain, NIZO B659) were kindly provided by HH, Department of Medical Microbiology, University Medical Center Groningen (UMCG, Groningen, Netherlands).

Growth Broth, Monoculture, and Co-culture Conditions

All strains were grown and maintained in yeast extract, casitone, and fatty acid broth (YCFA) (Lopez-Siles et al., 2012). First, the

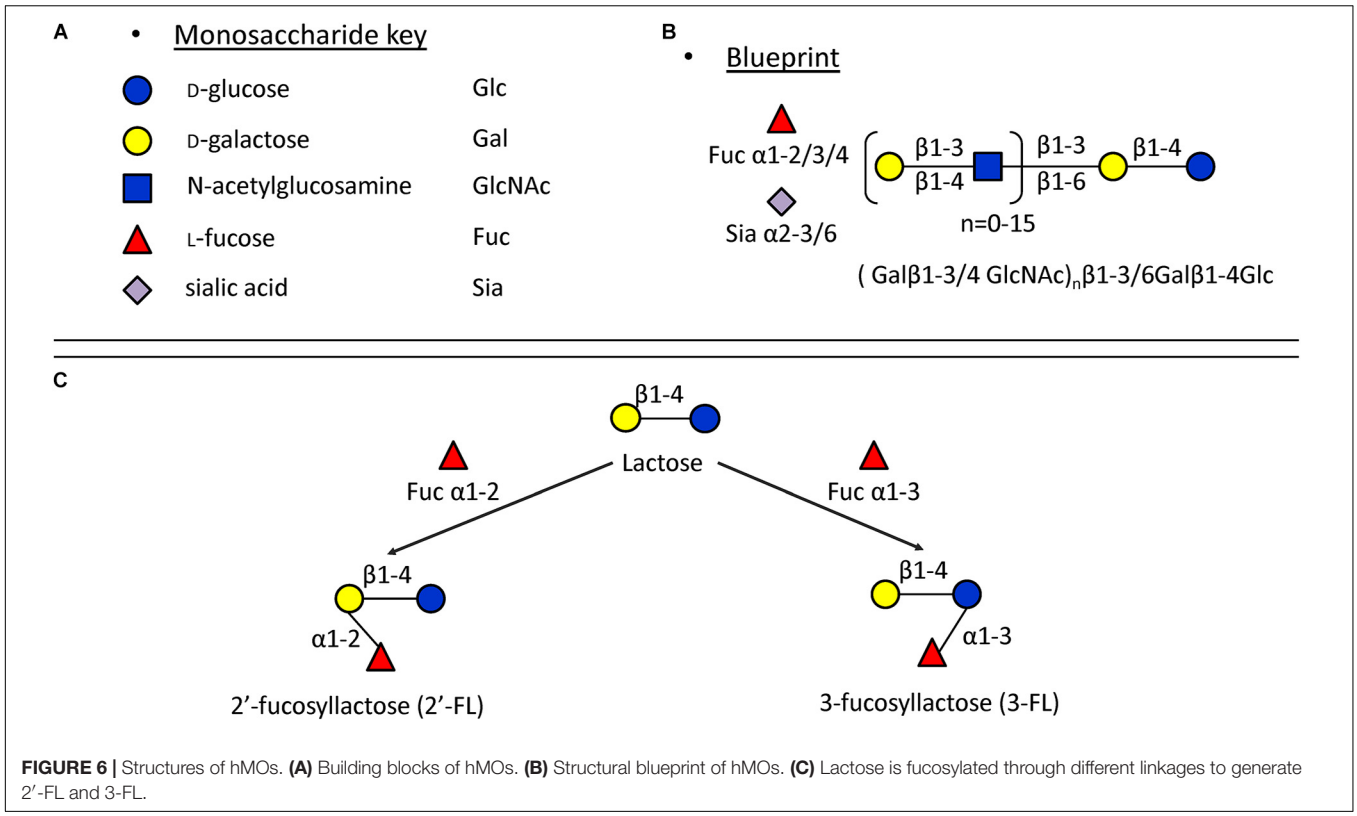

FIGURE 6 | Structures of hMOs. **(A)** Building blocks of hMOs. **(B)** Structural blueprint of hMOs. **(C)** Lactose is fucosylated through different linkages to generate 2′-FL and 3-FL.

growth and substrate fermentation capacities of the individual strains on different hMO sources were studied. In order to do this, various hMOs were added to the broth at a concentration of 5 mg/ml. YCFA containing 5 mg/ml glucose (Thermo Scientific, Breda, Netherlands) was used as a positive control, while media without carbohydrate served as a negative control. Broth was autoclaved at 121°C for 20 min first, after which the carbohydrate source was added to the cooled broth after sterile filtration. The final pH of the broth was adjusted to 6.5 ± 0.1 by using sterile HCl.

All strains were inoculated in 5 ml of YCFA broth supplemented with glucose as the sole energy source (YCFAG) and incubated anaerobically at 37°C for 24 h. Subsequently, the strains were propagated to 5 ml of YCFAG broth culture overnight as pre-culture; after that, different strains were added to the tubes of YCFA broth supplemented with the different carbon sources at a ratio of 1:100 in duplicate at 37°C. Growth in cultures was monitored spectrophotometrically every 4 h from 0- to 72-h culture by measuring the OD_{600} by using Ultrospec 10 cell density meter (Amersham Biosciences GmbH, Germany).

To study the interaction between *B. longum* subsp. *infantis* and *F. prausnitzii*, co-culture fermentations were performed in YCFA with 5 mg/ml of the different carbon sources. *B. longum* subsp. *infantis* and *F. prausnitzii* cells were inoculated as described above. Subsequently, the strains were propagated to 5 ml of YCFAG media and were pre-cultured separately, and the cell density was monitored spectrophotometrically by measuring the OD_{600} using Ultrospec 10 cell density meter. When OD_{600} reached 1.0 in the pre-culture, *B. longum* subsp. *infantis* and *F. prausnitzii* were added to the same tube of YCFA broth supplemented with the different carbon sources at a ratio of 1:100. Each batch of experiments was made with the same inocula for both *B. longum* subsp. *infantis* and *F. prausnitzii*.

TABLE 1 | Overview of the structures of selected hMOs.

Name (abbreviated)	Structure	Schematic diagram
2′-FL	Fucα1-2Galβ1-4Glc	
3-FL	Galβ1-4Glc Fucα1-3/	
6′-SL	NeuNAcα2-6Galβ1-4Glc	
LNT2	GlcNAcβ1-3Galβ1-4Glc	

Glucose; Galactose; Fucose; Sialic Acid

SCFA Production

Samples for the SCFA analyses were taken at 48 h and 72 h of monoculture and co-culture incubation. The fermentation digest (0.5 ml) was heated at 100°C for 5 min and then centrifuged at 13,200 × *g* for 10 min at room temperature. Analysis of SCFAs (acetate, propionate, and butyrate) by gas chromatography (GC)

was done as described previously by Gu et al. (2018). A 250-µl aliquot of the fivefold diluted supernatant of the fermentation product was mixed with 125 µl of a solution containing oxalic acid (0.09 M), HCl (0.3 M), and internal standard 2-ethyl butyric acid (0.45 mg/ml). Afterward, the mixture was allowed to stand at room temperature for 30 min. The temperature profile during GC analysis was as follows: 100°C, maintained for 0.5 min; raised to 180°C at 8°C/min, maintained for 1 min; raised to 200°C at 20°C/min, maintained for 5 min. Xcalibur software (Thermo Scientific, Breda, Netherlands) was used to process data from GC.

Glycosidic Degradation

Samples for the degradation analysis were taken at 48 and 72 h of *B. longum* subsp. *infantis* monoculture and co-culture. HPAEC was used to measure the carbohydrate degradation as a measure for utilization of carbohydrates by the microorganisms (Cardarelli et al., 2016). An ISC 3000 (Dionex, Sunnyvale, CA, United States), equipped with a 2 × 250 mm Dionex CarboPac PA-1 column and a 2 × 50 mm CarboPac PA-1 guard column, was used for quantification (Albrecht et al., 2009). Briefly, samples were adjusted to a final concentration of 0.05 mg of the substrate per milliliter, and 10 µl of the samples was injected using a Dionex ISC3000 autosampler. The oligosaccharides were eluted (0.3 ml/min) by a gradient of 0–350 mM sodium acetate in 100 mM NaOH for 35 min. Each elution was followed by a washing step with 1 M NaOAc in 100 mM NaOH for 5 min and an equilibration step with 100 mM NaOH for 15 min. A Dionex ED40 detector in pulsed amperometric detection mode was used for detection. 6′-SL at final concentrations of 0.05 mg/ml were used as standards. Chromeleon software Version 6.70 (Dionex) was used for the integration and evaluation of the chromatograms obtained.

Statistics

The results were analyzed using GraphPad Prism. The normality of distribution of the data was tested by using the Kolmogorov–Smirnov test. Values are expressed as median ± range. Statistical comparisons were performed using the Kruskal–Wallis test followed by the Dunn's test. $p < 0.05$ was considered as statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LC and PV conceived and designed the experiments. LC and ML performed the experiments and analyzed the data. AG supplied hMOs. LC, MK, ML, AG, AN, and PV participated in the discussion. LC, MK, ML, AG, AN, HS, MW, HH, and PV wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This project was financially supported by the China Scholarship Council (CSC). This research was performed within the framework of the Sino-Dutch Doctoral Program on Sustainable Dairy coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.569700/full#supplementary-material

Supplementary Figure S1 | The endpoint OD600 of *B. longum* subsp. infantis, *B. longum* subsp. *longum*, *B. adolescentis* and *F. prausnitzii* in mono-culture. Glucose, 2′-FL, 3-FL, 6′-SL, and LNT2 were included as carbon source. The assays were carried out 3 times in duplicate. Values are expressed as median ± range. Statistical significance was measured using Kruskal-Wallis test followed by the Dunn's test and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

REFERENCES

Albrecht, S., van Muiswinkel, G. C. J., Schols, H. A., Voragen, A. G. J., and Gruppen, H. (2009). Introducing Capillary Electrophoresis with Laser-Induced Fluorescence Detection (CE-LIF) for the Characterization of Konjac Glucomannan Oligosaccharides and Their in Vitro Fermentation Behavior. *J. Agric. Food Chem.* 57, 3867–3876. doi: 10.1021/jf8038956

Aly, E., Ali Darwish, A., Lopez-Nicolas, R., Frontela-Saseta, C., and Ros-Berruezo, G. (2018). "Bioactive components of human milk: similarities and differences between human milk and infant formula," in *Selected Topics in Breastfeeding*, ed. R. Mauricio Barría (London: IntechOpen). doi: 10.5772/intechopen.73074

Bode, L. (2015). The functional biology of human milk oligosaccharides. *Early Hum. Dev.* 91, 619–622. doi: 10.1016/j.earlhumdev.2015.09.001

Cardarelli, H. R., Martinez, R. C. R., Albrecht, S., Schols, H., Franco, B. D. G. M., Saad, S. M. I., et al. (2016). *In vitro* fermentation of prebiotic carbohydrates by intestinal microbiota in the presence of *Lactobacillus amylovorus* DSM 16998. *Benef. Microbes* 7, 119–133. doi: 10.3920/BM2014.0151

Cheng, L., Kiewiet, M. B. G., Groeneveld, A., Nauta, A., and de Vos, P. (2019). Human milk oligosaccharides and its acid hydrolysate LNT2 show immunomodulatory effects via TLRs in a dose and structure-dependent way. *J. Funct. Foods* 59, 174–184. doi: 10.1016/j.jff.2019.05.023

Cheng, L., Kong, C., Walvoort, M. T. C., Faas, M. M., and de Vos, P. (2020). Human milk oligosaccharides differently modulate goblet cells under homeostatic, proinflammatory conditions and ER stress. *Mol. Nutr. Food Res.* 64:1900976. doi: 10.1002/mnfr.201900976

Conlon, M. A., and Bird, A. R. (2015). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* 7, 17–44. doi: 10.3390/nu7010017

Coulet, M., Phothirath, P., Allais, L., and Schilter, B. (2014). Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'FL). *Regul. Toxicol. Pharmacol.* 68, 59–69. doi: 10.1016/j.yrtph.2013.11.005

Garrido, D., Kim, J. H., German, J. B., Raybould, H. E., and Mills, D. A. (2011). Oligosaccharide binding proteins from *Bifidobacterium longum* subsp. *infantis* reveal a preference for host glycans. *PLoS One* 6:e17315. doi: 10.1371/journal.pone.0017315

Goldsmith, F., O'Sullivan, A., Smilowitz, J. T., and Freeman, S. L. (2015). Lactation and intestinal microbiota: how early diet shapes the infant gut. *J. Mammary Gland Biol. Neoplasia* 20, 149–158. doi: 10.1007/s10911-015-9335-2

Gu, F., Borewicz, K., Richter, B., van der Zaal, P. H., Smidt, H., Buwalda, P. L., et al. (2018). *In vitro* fermentation behavior of isomalto/malto-polysaccharides using human fecal inoculum indicates prebiotic potential. *Mol. Nutr. Food Res.* 62:1800232. doi: 10.1002/mnfr.201800232

Heymann, J., Raub, A., and Earle, A. (2013). Breastfeeding policy: a globally comparative analysis. *Bull. World Health Organ.* 91, 398–406. doi: 10.2471/BLT.12.109363

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. *Nutr. Rev.* 73, 426–437. doi: 10.1093/nutrit/nuu016

Kirmiz, N., Robinson, R. C., Shah, I. M., Barile, D., and Mills, D. A. (2018). Milk glycans and their interaction with the infant-gut microbiota. *Annu. Rev. Food Sci. Technol.* 9, 429–450. doi: 10.1146/annurev-food-030216-030207

Kong, C., Elderman, M., Cheng, L., de Haan, B. J., Nauta, A., and de Vos, P. (2019). Modulation of intestinal epithelial glycocalyx development by human milk oligosaccharides and non-digestible carbohydrates. *Mol. Nutr. Food Res.* 63:1900303. doi: 10.1002/mnfr.201900303

Laursen, M. F., Laursen, R. P., Larnkjær, A., Mølgaard, C., Michaelsen, K. F., Frøkiær, H., et al. (2017). Faecalibacterium Gut Colonization Is Accelerated by Presence of Older Siblings. *mSphere* 2:e00448-17. doi: 10.1128/msphere.00448-17

Le Doare, K., Holder, B., Bassett, A., and Pannaraj, P. S. (2018). Mother's Milk: a purposeful contribution to the development of the infant microbiota and immunity. *Front. Immunol.* 9:361. doi: 10.3389/fimmu.2018.00361

Lopez-Siles, M., Khan, T. M., Duncan, S. H., Harmsen, H. J. M., Garcia-Gil, L. J., and Flint, H. J. (2012). Cultured representatives of two major phylogroups of human colonic *Faecalibacterium prausnitzii* can utilize pectin, uronic acids, and host-derived substrates for growth. *Appl. Environ. Microbiol.* 78, 420–428. doi: 10.1128/AEM.06858-11

McLeod, K. H., Richards, J. L., Yap, Y. A., and Mariño, E. (2019). "Dietary short chain fatty acids: how the gut microbiota fight against autoimmune and inflammatory diseases," in *Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases*, eds R. Watson and V. Preedy (Cambridge, MA: Academic Press), 139–159. doi: 10.1016/b978-0-12-813820-5.00007-6

Moens, F., Weckx, S., and De Vuyst, L. (2016). Bifidobacterial inulin-type fructan degradation capacity determines cross-feeding interactions between bifidobacteria and *Faecalibacterium prausnitzii*. *Int. J. Food Microbiol.* 231, 76–85. doi: 10.1016/j.ijfoodmicro.2016.05.015

Moore, R. E., and Townsend, S. D. (2019). Temporal development of the infant gut microbiome. *Open Biol.* 9:190128. doi: 10.1098/rsob.190128

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadoux, J.-J., et al. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16731–16736. doi: 10.1073/pnas.0804812105

Thomson, P., Medina, D. A., and Garrido, D. (2018). Human milk oligosaccharides and infant gut bifidobacteria: molecular strategies for their utilization. *Food Microbiol.* 75, 37–46. doi: 10.1016/j.fm.2017.09.001

Thurl, S., Munzert, M., Boehm, G., Matthews, C., and Stahl, B. (2017). Systematic review of the concentrations of oligosaccharides in human milk. *Nutr. Rev.* 75, 920–933. doi: 10.1093/nutrit/nux044

Triantis, V., Bode, L., and van Neerven, J. R. J. (2018). Immunological effects of human milk oligosaccharides. *Front. Pediatr.* 6:190. doi: 10.3389/fped.2018.00190

Underwood, M. A., German, J. B., Lebrilla, C. B., and Mills, D. A. (2015). *Bifidobacterium longum* subspecies *infantis*: champion colonizer of the infant gut. *Pediatr. Res.* 77, 229–235. doi: 10.1038/pr.2014.156

Vandenplas, Y., Berger, B., Carnielli, V. P., Ksiazyk, J., Lagström, H., Luna, M. S., et al. (2018). Human milk oligosaccharides: 2'-fucosyllactose (2'-FL) and lacto-n-neotetraose (LNnT) in infant formula. *Nutrients* 10:1161. doi: 10.3390/nu10091161

Walker, A. (2010). Breast milk as the gold standard for protective nutrients. *J. Pediatr.* 156, S3–S7. doi: 10.1016/j.jpeds.2009.11.021

Ward, R. E., Niñonuevo, M., Mills, D. A., Lebrilla, C. B., and German, J. B. (2007). In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria. *Mol. Nutr. Food Res.* 51, 1398–1405. doi: 10.1002/mnfr.200700150

World Health Organization [WHO] (2002). Infant and young child nutrition: global strategy on infant and young child feeding. *Fifty Fifth World Heal. Assem.* 53, 1–18.

Conflict of Interest: AG and AN are employed by the company FrieslandCampina.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 05 November 2020
doi: 10.3389/fmicb.2020.591568

Molecular Insights Into *O*-Linked Glycan Utilization by Gut Microbes

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O-linked glycosylation is a post-translational modification found mainly in eukaryotic cells, which covalently attaches oligosaccharides to secreted proteins in certain threonine or serine residues. Most of *O*-glycans have *N*-acetylgalactosamine (GalNAc) as a common core. Several glycoproteins, such as mucins (MUCs), immunoglobulins, and caseins are examples of *O*-glycosylated structures. These glycans are further elongated with other monosaccharides and sulfate groups. Some of them could be found in dairy foods, while others are produced endogenously, in both cases interacting with the gut microbiota. Interestingly, certain gut microbes can access, release, and consume *O*-linked glycans as a carbon source. Among these, *Akkermansia muciniphila*, *Bifidobacterium bifidum*, and *Bacteroides thetaiotaomicron* are prominent *O*-linked glycan utilizers. Their consumption strategies include specialized α-fucosidases and α-sialidases, in addition to endo-α-*N*-acetylgalactosaminidases that release galacto-*N*-biose (GNB) from peptides backbones. *O*-linked glycan utilization by certain gut microbes represents an important niche that allows them to predominate and modulate host responses such as inflammation. Here, we focus on the distinct molecular mechanisms of consumption of *O*-linked GalNAc glycans by prominent gut microbes, especially from mucin and casein glycomacropeptide (GMP), highlighting the potential of these structures as emerging prebiotics.

Keywords: glycans, prebiotics, GMP, mucin, microbiota, glycoprotein

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Edited by:
Sonia G. Sáyago-Ayerdi, Instituto Tecnológico de Tepic, Mexico

Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *04 August 2020*
Accepted: *20 October 2020*
Published: *05 November 2020*

Citation:
González-Morelo KJ, Vega-Sagardía M and Garrido D (2020) Molecular Insights Into O-Linked Glycan Utilization by Gut Microbes. Front. Microbiol. 11:591568. doi: 10.3389/fmicb.2020.591568

INTRODUCTION

There is a great interest regarding the impact and modulation of the gut microbiota through our diet. Among several dietary interventions, consumption of fibers and prebiotics has mainly been considered as positive for our health (Sawicki et al., 2017). A recent definition of prebiotic corresponds to a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). These benefits include a reduced microbial load of pathogens (Gibson et al., 2005), stimulation of the immune system (Shokryazdan et al., 2017), and lower allergy rates (Brosseau et al., 2019). These effects are attributed in part to the ability of prebiotics to be fermented by healthy microorganisms and stimulate the production of certain short-chain fatty acids (SCFA), especially butyrate (Rivière et al., 2016).

Traditionally, carbohydrates such as inulin and fructo-oligosaccharides (FOS) have been studied as prebiotics (Vandeputte et al., 2017; Wilson and Whelan, 2017; BeMiller, 2019).

In general, these plant-derived prebiotics have a simple structure containing one monosaccharide and repeats of one linkage. In contrast, host-derived glycans such as human milk oligosaccharides (HMO) and those found in glycoproteins, are structurally more complex and thought to be more suitable and selective toward beneficial members of the gut microbiota. HMO are complex glycans that promote the growth and activity of beneficial gut microbes such as infant-gut associated bifidobacteria, among other effects (Thompson et al., 2018). Several advances have been made to synthesize HMO at the industrial level, and they are currently being added as functional ingredients to infant formula (Puccio et al., 2017).

Besides, some human and bovine milk proteins possess similar glycans to HMO in their structures. Glycosylation is a post-translational modification where oligosaccharides are covalently bound to asparagine (*N*-glycans), or serine or threonine (Ser/Thr; *O*-glycans; Vliegenthart, 2017). These glycans serve as signaling molecules for secretion and other cellular processes, providing increased resistance to proteolysis (Baum and Cobb, 2016). These glycans are being proposed as emerging prebiotics due to their similarity to host-derived glycans compared to plant-derived prebiotics and their enrichment of dominant and health-promoting gut microbes, such as *Bacteroides* spp. (*Ba.*), *Bifidobacterium* spp. (*Bi.*), and *Akkermansia muciniphila* (Bergstrom and Xia, 2013; Kirmiz et al., 2018). Several molecular adaptations for accessing and consuming *N*- and *O*-glycans have been described in gut microorganisms, indicating that the gut microbiota is quite adapted for metabolizing these oligosaccharides. Importantly, some of these bioactive glycans could be also be found in dairy streams, warranting a wide availability for potential prebiotic use for the food industry.

N-linked glycans are complex oligosaccharides that possess *N*-Acetylglucosamine (GlcNAc) as a common core, conjugated with an additional GlcNAc and three mannose (Man) residues, forming a $Man_3GlcNAc_2$ motif found in all *N*-glycans. *N*-glycans could be further modified by extensive mannosylation (high-mannose *N*-glycans) or by lactosamine chains (Galβ1-4GlcNAc; complex *N*-glycans). Terminal fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc) modifications are commonly added to complex *N*-glycans (Stanley et al., 2017). *N*-glycans are characteristic of immunoglobulins and lactoferrin in milk (Le Parc et al., 2015; Davis et al., 2016). *N*-glycans are an example of host-derived oligosaccharides that can be used by beneficial microbes. *Bifidobacterium longum* subsp. *infantis* ATCC 15697 is a dominant beneficial infant gut microbe that has been shown to access *N*-glycans *in vitro* by a specialized endo-β-*N*-acetylglucosaminidase (Garrido et al., 2012b). It also shows vigorous growth *in vitro* using *N*-glycans (Karav et al., 2016). The released *N*-glycans from milk glycoproteins such as lactoferrin and immunoglobulins have been well characterized (Le Parc et al., 2015). These results, while promising, are yet to be tested *in vivo* for claiming any prebiotic effect.

Less attention has been paid to *O*-linked glycans. These are characterized by an *N*-acetylgalactosamine (GalNAc) residue linked to Ser/Thr as a common core. GalNAc is usually bound to Galβ1-3, forming galacto-*N*-biose (GNB) as a building block in Core 1 and Core 2, which could be further extended forming larger chains similar to *N*-glycans. *O*-linked glycans are a significant component of mucins (MUCs; **Figure 1**), also found in immunoglobulins and κ-caseins (Magnelli et al., 2011). *O*-glycosylated proteins have a higher resistance to proteolysis (Boutrou et al., 2008; Kesimer and Sheehan, 2012), probably reaching lower sections of the gut and interacting the gut microbiota. Therefore, it is not surprising that gut microbes have evolved strategies to utilize *O*-linked glycans as a carbon source.

Prominent gut microbes such as *A. muciniphila*, *Bifidobacterium bifidum*, and *Bacteroides thetaiotaomicron* are representative species capable of *O*-linked glycan consumption. However, the mechanisms involved and the consequences of this process for the host are not fully known. This review aims to summarize and discuss current research regarding the structures of *O*-linked glycans, their potential prebiotic activity, and consumption by gut microorganisms.

STRUCTURES OF *O*-LINKED GLYCANS

The elongation of *O*-linked glycans and their attachment to secreted proteins begins in the Golgi apparatus (Brockhausen and Stanley, 2017). A polypeptide-*N*-acetyl-galactosaminyltransferase (ppGalNAcT) catalyzes the addition of a GalNAcα1 to a Ser/Thr available as a glycosylation site (Varki and Lowe, 2009). Several glycosyltransferases act in conjunction to attach sugar residues in single *O*-glycans. The Core 1 *O*-glycan (Galβ1-3GalNAcα1-Ser/Thr) is the first synthesized, and then the Core 2 unit [Galβ1-3(GlcNAcβ1,6) GalNAcα1-Ser/Thr; Itano, 2019].

Here, we will focus on two model glycoproteins containing *O*-glycans: MUC and glycomacropeptide (GMP). The wide availability of mucins from animal sources makes them reference glycoproteins for the study of their contribution to host processes and their interaction with the gut microbiota. Similarly, GMP is a glycopeptide derived from cheesemaking, available in large quantities. It contains neutral and acidic *O*-glycans, which are shorter and simpler compared to mucin *O*-glycans. Both types of glycans have been shown to interact with the gut microbiota and could be considered promising prebiotics considering their stimulation of beneficial gut microbes.

O-Linked Glycans From Mucin

Mucins are highly glycosylated (up to 90%), high-molecular-weight (200 kDa–200 MDa), and large (Rg 10–300 nm) extracellular glycoproteins that serve as a dense barrier between the intestinal lumen and epithelium (Kesimer and Sheehan, 2012). They are generally found in epithelial tissues in the gastrointestinal tract and certain secretions. They provide a crucial role in forming a physicochemical barrier against the luminal compartment through their gel-forming properties (Bansil and Turner, 2018). Interestingly, mucin serves as a scaffold for the attachment and colonization by certain microorganisms (Ringot-Destrez et al., 2017). Two different mucus layers have been identified: an outer mucus layer (containing 10^6 microbial cells/g) and a tight inner mucus layer (10^5 microbial cells/g; Atuma et al., 2001). Mucins can be found in different parts of the human body: kidneys

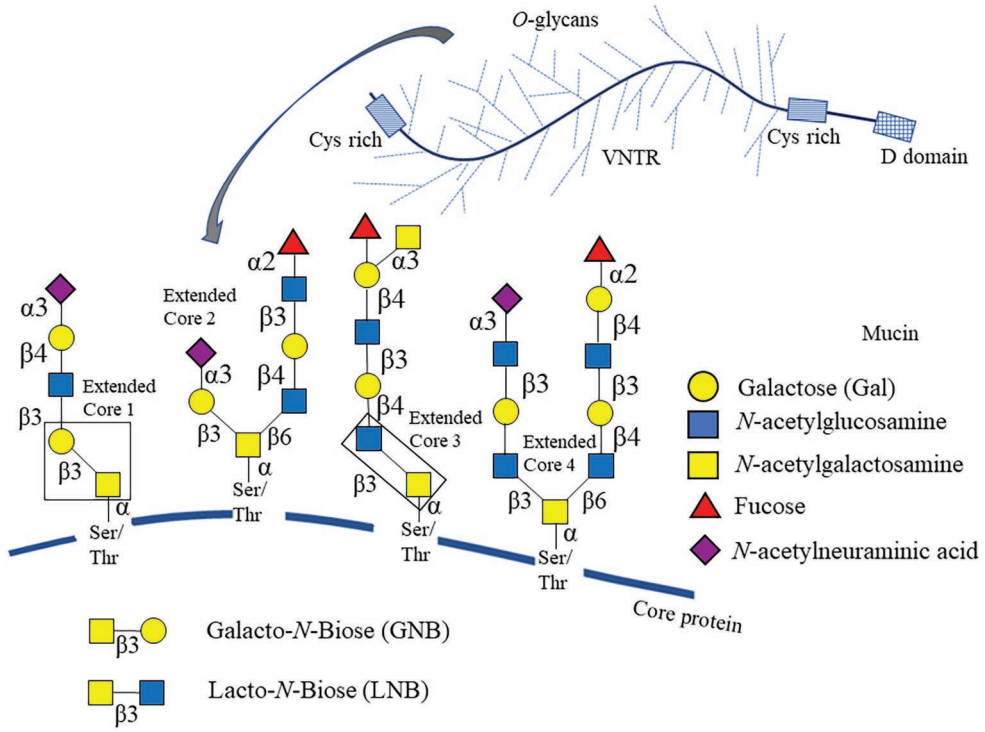

FIGURE 1 | Schematic representation of *O*-glycan cores linked to mucin. The *O*-glycans structures linked to Ser/Thr residues from mucin (MUC) core protein are composed of four dominant extensible cores (Cores 1–4), with the addition of residues such as Gal, *N*-acetylgalactosamine (GlcNAc), fucose (Fuc), and *N*-acetylneuraminic acid (NeuAc). Carbohydrate legends are shown to the right. The distribution of variable number of tandem repeats (VNTR) and other domains are also shown.

and bladder, respiratory and urinary tracts, among others (Atuma et al., 2001). They are produced by goblet cells, specialized secretory cells found in the epithelial layer (Lamacchia et al., 2018).

There are at least 20 different MUC genes, whose products are classified into two families: secreted and transmembrane mucins (Corfield, 2015; Dhanisha et al., 2018). Secreted mucins can be small and soluble, or large gel-forming mucins. The latter are able to cross-link, forming complex networks and contributing to the viscosity of mucins. MUC5AC is among the most studied mucin being secreted by epithelial cells in the stomach, while MUC6 is secreted in the deep gastric glands in the stomach and ileum (Magalhães et al., 2016). MUC2 is the most abundant secreted mucin in the small intestine and the colon (Corfield, 2000; Arike et al., 2017). Transmembrane mucins participate primarily in cellular adhesion, while secreted mucins are responsible for viscoelasticity for both the inner and outer mucus layers (Lillehoj et al., 2013; Demouveaux et al., 2018). All these differences between secreted and membrane-bound mucins contribute to the dynamic properties of the mucus layer across the GI tract.

Mucins could also be found in secretions and milk. MUC1 is a transmembrane mucin expressed on the apical surface of most epithelial cells (Ross et al., 2015). This type of mucin can be found in breast milk, which is transferred to the milk fat globule membrane upon its secretion from the cells. MUC15 is also a mucin-type isolated from bovine milk fat globule membranes (Bernard et al., 2018). The function of milk mucins has not been extensively studied, but it is suggested to be mostly structural (Donovan, 2019).

Eight cores of *O*-glycan structures have been identified in mucins, four of them being the most predominant (Core 1–4; **Figure 1**). These *O*-glycans are found in the region known as the variable number of tandem repeats (VNTR; Arike and Hansson, 2016). The VNTR section is rich in Ser/Thr, which can make up to 80% of the weight of the mucin. In MUC2, VNTRs are accompanied by two cysteine-rich regions at their ends and a D domain involved in mucin polymerization (Arike and Hansson, 2016). Depending on the tissue, MUC5AC and MUC6 could be glycosylated with predominant Core 1–2 structures (Jin et al., 2017). The *O*-linked glycans of colonic MUC2 are predominantly Core 1–3 structures (Bergstrom et al., 2017).

Mucin *O*-glycans can be either branched or linear in structure, depending on their Core (e.g., Core 1 glycans are linear, and Core 2 glycans are branched; Podolsky, 1985; Li and Chai, 2019). *O*-glycans have been found to contain up to 20 residues and may include blood group determinants of ABO, Lewis groups, and glycan epitopes such as the linear antigen *i* (Galβ1-4GlcNAcβ1-3Gal; Vliegenthart, 2017). Fuc and NeuAc are monosaccharides decorating *O*-glycans in terminal α-linkages, and usually mucins contain sulfate groups. The latter two confer

a negative charge on the mucin structure, crucial for selective mucin permeability and its rheological properties (De Weirdt and Van de Wiele, 2015). An acidic gradient, based on increasing amounts of NeuAc in mucins, has been shown in the gastrointestinal tract (Robbe et al., 2003).

Several studies have shown how alterations in mucin *O*-glycosylation patterns participate in disease. In cystic fibrosis patients, the respiratory epithelium shows higher degradation rates for MUC5B and MUC5AC, in addition to reduced sulfation, higher sialylation, and lower fucosylation (Schulz et al., 2007). This disease is characterized by chronic pulmonary infection and severe inflammation, and the changes mentioned above could be used as biomarkers. In the stomach, a broad diversity of *O*-glycans has been observed, but specific epitopes, such as Lewis b and α1-4GlcNAc, were found to promote adhesion of *Helicobacter pylori* to the mucus layer (Rossez et al., 2012; Jin et al., 2017). This pathogen is a direct cause of gastrointestinal ulcers and gastric cancer.

Similarly to mucins, glycoproteins in cancer cells show alterations in their *O*-glycosylation profiles. Truncated *O*-glycans such as a GalNAc residue with no further glycosylation (GalNAcα-Ser/Thr; Tn antigen) is considered a tumor marker given its presence is abnormal in glycoproteins (Itzkowitz et al., 1989). Sialylated Tn is also a common feature of cancer cells. An increase in core fucosylation is also observed in these cells. Consequently, these changes interfere with cell-cell adhesion processes, promoting tumor cell invasion (Pinho and Reis, 2015).

Importantly, *O*-linked glycans from human gastric mucin are structurally similar to those from the porcine stomach mucin. The oligosaccharides in both glycoproteins are mainly extended with Core 1 and Core 2 structures. The availability of porcine stomach mucin has facilitated the study of mucin function and its interactions with gut microorganisms. However, oligosaccharides in pig mucins have unique characteristics, like that they could contain extended Core 3 and Core 4 glycans (Padra et al., 2018). Pig gastric mucins are highly sulfated, usually terminating with galactose residues and have lower sialylation (Quintana-Hayashi et al., 2018).

Mucin glycosylation is in part mediated by gut microbiota. Using germ-free (GF) mice as control, it has been shown that the presence of certain members of gut microbiota is critical for the expression of glycosyltransferases participating in mucin *O*-glycosylation for example, ppGalNAcT, Core 1 β1,3-Galactosyltransferase (C1GALT1), and Fucosyltransferase (FUT2; Johansson et al., 2015). MUC2 *O*-linked glycans from colonic tissues of conventionally raised animals were more sialylated, fucosylated, and longer than GF mice (Arike et al., 2017). MUC2 from GF mice showed a reduced abundance of β1,6-*N*-acetyl glucosaminyltransferases, enzymes responsible for the formation of Core 2 and Core 4 *O*-glycans. These GF animals tended to produce shorter *O*-glycans (Arike et al., 2017). This result suggests that the gut microbiota modulates the *O*-glycosylation patterns of mucins, which in turn influence the composition the gut microbiota.

Mucin utilization by the gut microbiota is important for host health. It is known that animals that are fed mucin show higher fecal butyrate levels. This compound is an anti-inflammatory SCFA considered positive for health and significantly decreased in inflammatory diseases such as Ulcerative Colitis (UC; Chen et al., 2018; Yamada et al., 2019). In UC subjects, mucin *O*-glycan abundance correlates negatively with butyrate production in feces, showing a reduced utilization of *O*-glycans by the gut microbiota (Yamada et al., 2019). This study presented important *in vivo* evidence linking the foraging of host-derived glycans, the action of the gut microbiota, and inflammatory bowel diseases (IBDs). In a different context, mucin *O*-glycan consumption by gut microbes could be considered unfavorable for the host. Animals deprived of fiber in their diets show the promotion of gut inflammation and colitis. This was explained partly by their gut microbiota turning into endogenous mucin resources as the last carbon source available. Accessing the mucin layer by mucin degraders permits the colonization by *Citrobacter rodentium*, a mucosal pathogen of mice sharing several pathogenic features with human gastrointestinal pathogens such as enteropathogenic *Escherichia coli* (Collins et al., 2014; Desai et al., 2016).

Mucins and their *O*-glycans are essential in the gut barrier function. Modifications of their expression, organization, or glycosylation are likely to prevent this effect, as observed in IBD. The altered balance between pro-inflammatory cytokines (TNF, IL-1b, IL-8, and IL-17), anti-inflammatory cytokines (IL-4 and IL-13), and immuno-regulatory cytokines described in IBD is likely to modify mucin expression and glycosylation (Guan and Zhang, 2017). Furthermore, altered glycosylation and sulfation of colonic mucins in IBD subjects could alter the protective role of the colonic mucus barrier (Groux-Degroote et al., 2020).

O-Linked Glycans From GMP

Glycomacropeptide is a constituent of whey (20–25% of the protein moiety), derived from κ-casein after chymosin treatment during cheesemaking (Manso and López-Fandiño, 2004; Sunds et al., 2019). GMP is a hydrophilic, negatively charged, 64 amino acid glycopeptide. The molecular weight of GMP ranges from 6.7 to 8 kDa (Córdova-Dávalos et al., 2019). GMP is available in large quantities in dairy streams (Rojas and Torres, 2013).

The glycan portion is simpler compared to mucin oligosaccharides, containing only NeuAc, GalNAc, and Gal. GMP has five distinct *O*-linked glycans bound to Ser/Thr (**Figure 2**), corresponding to monosaccharides (0.8%), disaccharides (6.3%), trisaccharides (36.5%), and tetrasaccharides (56%; Saito and Itoh, 1992). The disaccharide GNB (**Figure 2**) is a building block found in GMP (Fiat and Jollès, 1989). Different *O*-glycosylation sites have been proposed in GMP, as determined by 2D gel electrophoresis and tandem mass spectrometry (**Figure 2**; Neelima et al., 2013). Thr in positions 121, 131, 133, 136, and 142 appear to be used as glycosylation sites, and positions 165, 135, and 141 have been proposed as potential sites (Thomä-Worringer et al., 2006). As expected, the functional and adhesion properties of GMP will depend on the position where *O*-glycans are attached, especially the sialylated. NeuAc is an acid sugar easily recognizable by mucin-degrading bacteria (Neelima et al., 2013).

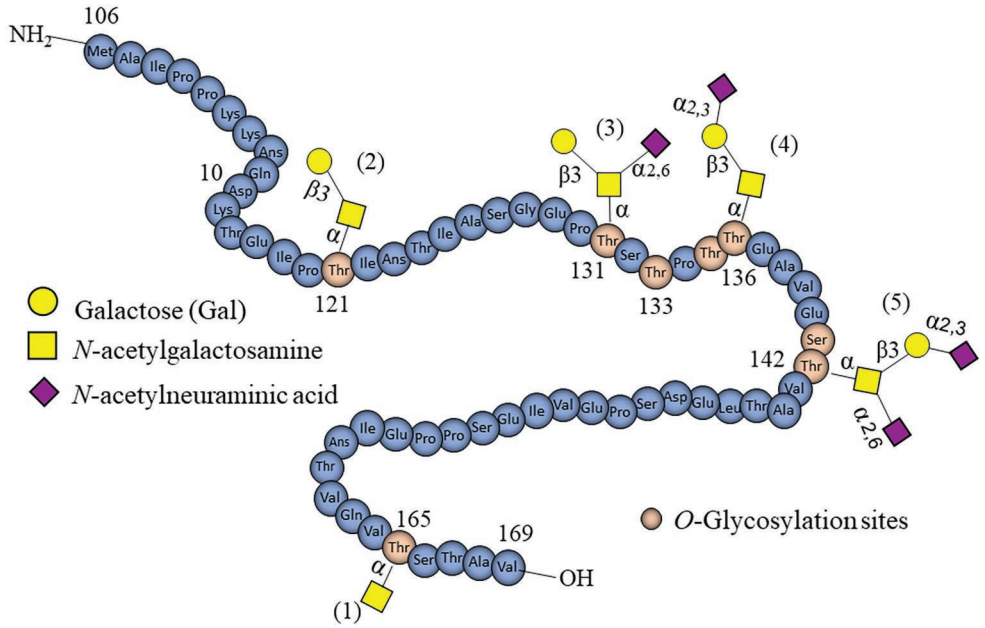

FIGURE 2 | Schematic representation of *O*-linked glycans found in casein glycomacropeptide (GMP). Within the GMP structure, there are five identified *O*-glycans containing Gal, GalNAc, and NeuAc (named 1–5). Threonine residues serving as accepting glycosylation sites are also marked in brown.

UTILIZATION OF MUCIN *O*-GLYCANS BY GUT MICROBES

While certain gut bacteria use mucin glycan as attachment sites, a few others go beyond and additionally deploy enzymatic activities to use mucin *O*-glycans as a nutrient (Tailford et al., 2015). Interestingly, this enzymatic capability is distributed in members across the four most dominant phyla in the gut microbiota (*Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia*), suggesting it is an important trait for the gut microbiota.

Mucin *O*-glycan degradation by intestinal microorganisms requires an extensive array of glycosyl hydrolases (GHs), as expected from the complex structures, compositions, and diverse linkages found (Tailford et al., 2015). Most mucin-degrading bacteria base their strategies on exo-acting enzymes, including sialidases (GH33), fucosidases (GH29 and GH95), exo- and endo-*β*-*N*-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, and GH42), among others. Their activities imply sequential degradation. Other enzymes required for mucin utilization are *α*-*N*-acetylglucosaminidases (GH89) and α-*N*-acetylgalactosaminidases (GH101, GH129), which cleave a monosaccharide-peptide linkage after glycosidase activity. One potential exception to this exo-acting mechanism is a GH16 endoactive *O*-glycanase with mucin breakdown activities (Crouch et al., 2020). These activities release mono or disaccharides, which could be imported inside the bacterial cell or serve as cross-feeding metabolites (Turroni et al., 2018). Several enzymes related to mucin deglycosylation have been described, but the full mechanisms by which gut microbes utilize these glycans are unknown.

Utilization of *O*-Linked Glycans by *Bacteroidetes* spp.

The *Bacteroides* genus is predominant in the adult gut microbiota, being generally considered as commensals. Genomic and functional studies in animals show that these species show a preference for utilizing complex carbohydrates (e.g., HMO), rather than simple carbohydrates (Wexler and Goodman, 2017). *Bacteroides* devote a large part of their genomes to polysaccharide utilization loci (PULs), which correlates with their broad diverse polysaccharide utilization. These gene clusters encode multiple extracellular GHs enzymes, oligosaccharide transporters, and binding proteins.

Early works studied the molecular system of complex glycan degradation in starch utilization in these species (Anderson and Salyers, 1989). *Bacteroides thetaiotaomicron* VPI-5482 is a model bacterium for mucin *O*-glycan utilization. The microorganism shows a remarkable growth in porcine mucin glycans (Martens et al., 2008). These oligosaccharides can induce the expression of at least 16 PULs in *Ba. thetaiotaomicron*, aiding in identifying loci involved in *O*-glycan utilization. These genes encode for putative glycolytic enzymes, including an α-L-fucosidase, endo-β-GlcNAc-ase, endo-*β*-galactosidase, α-GalNAc-ase, in addition to proteases, neuraminidase, and a sulfatase. The enzymes are suggested to play an orchestrated degradation of *O*-linked glycans (Sicard et al., 2017; Luis et al., 2018). The relevance of these genetic determinants has been confirmed *in vivo* experiments in GF mice, where mutants for these loci show reduced gut colonization.

These enzymes are part of PULs also known as Starch utilization system (Sus), an operon of eight genes (SusRABCDEFG). Their products are predicted to locate in

the periplasm or the outer membrane (**Figure 3**). Carbohydrates are metabolized in response to the SusR signal, which triggers the expression of hydrolyzing proteins. The processed oligosaccharides are transported by a TonB protein and are metabolized inside the cells (Cockburn and Koropatkin, 2016). SusD homologs possess a binding site for *O*-glycans (Tailford et al., 2015) and have been implicated in the utilization of *O*-linked mucin glycans. Interestingly, the transcriptional response mounted to utilize *O*-glycans is similar to that required for HMO consumption (Marcobal et al., 2011). Four PULs in *Ba. thetaiotaomicron* were found to be induced in HMO but not in the presence of mucin glycans, indicating that this microorganism can respond to building blocks in HMO not found in mucin. Regardless, the deletion of these PULs did not affect the ability of *Ba. thetaiotaomicron* to consume HMO. Apparently, for this strain *O*-glycans are not a premium substrate since several mono or simple carbohydrates repressed these genes (Pudlo et al., 2015). This was not observed for other *Bacteroides* species, indicating the complex regulation of glycan utilization and diversity on preference for carbon sources even at the species level (Pudlo et al., 2015).

Monocolonization of GF mice with *Ba. thetaiotaomicron* modulates cellular responses in mucin-producing goblet cells, increasing their differentiation and synthesis of mucins, changing their glycosylation patterns with a higher NeuAc content (Wrzosek et al., 2013). In the presence of *Faecalibacterium prausnitzii*, a keystone microbe and butyrate producer, these cellular responses were attenuated, probably maintaining epithelial homeostasis between cell lines.

Bacteroides fragilis is a commensal species, but certain strains could be enterotoxigenic causing serious diseases (Purcell et al., 2017; Chung et al., 2018). This species also has a PUL involved in the consumption of host *O*-glycans. This PUL is known as the commensal colonization factor (CCF; Lee et al., 2013). The genes within the CCF are homologous to *Ba. thetaiotaomicron* PULs, which are up-regulated possibly producing extracellular enzymes to sense and mediate *O*-glycan processing. These enzymes allow the commensal colonization of mucus, specifically in the crypts of the colon. Some of these have been characterized biochemically (Praharaj et al., 2018; Yamamoto et al., 2018).

Utilization of *O*-Linked Glycans in *Firmicutes*

This phylum contains a broad diversity of genera, including commensal *Clostridium, Eubacterium*, and *Lactobacillus* species. In general, most *Firmicutes* in the gut microbiota prefer the assimilation of smaller rather than complex carbohydrates (Cockburn and Koropatkin, 2016; Ravcheev and Thiele, 2017). Several *Lactobacillus* species are endowed with mucin binding proteins (van Tassell and Miller, 2011), but they apparently lack any mucin utilization mechanism. However, within the *Firmicutes* a few members have acquired the ability to gain access to *O*-linked glycans, deploying several extracellular GHs.

A few pathogens in this group have evolved mechanisms for *O*-glycan utilization. *Clostridium perfringens*, an opportunistic pathogenic bacterium, can consume *N*-glycans and *O*-glycans from intestinal mucin by releasing extracellular glycosidases such as sialidases (Koutsioulis et al., 2008; Pluvinage et al., 2019). *Enterococcus faecalis* strains possess genes encoding endo-*β*-*N*-acetylglucosaminidase and endo-*α*-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase) that release *N*-glycans. However, this pathobiont appears not to be able to utilize mucin *O*-linked glycans (Roberts et al., 2000; Morio et al., 2019). These microorganisms are not usually

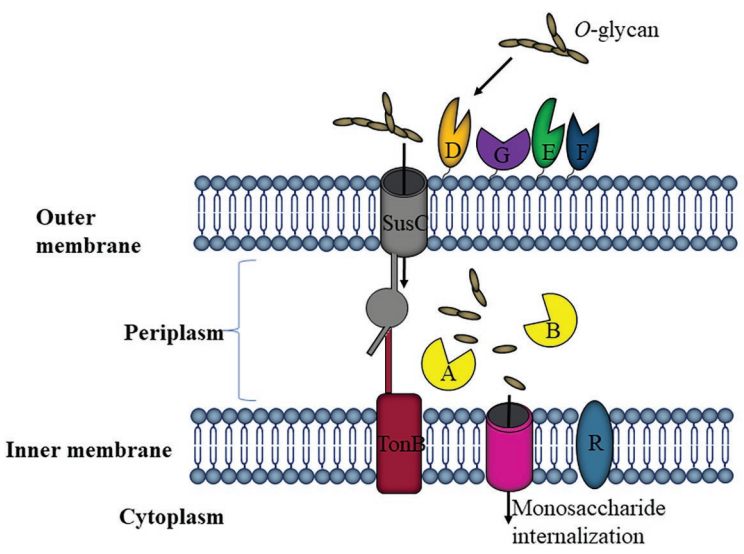

FIGURE 3 | Representation of *O*-linked glycan utilization by *Bacteroides thetaiotaomicron*. The starch utilization system (Sus) is the machinery best described in *Ba. thetaiotaomicron* for the utilization of mucin-type *O*-glycans. SusDEF are recognition and binding sites for *O*-glycans, SusC is a TonB-dependent transporter for sugar importation, SusR is a predicted membrane-spanning regulator, and SusAB are glycosyl hydrolases (GHs; Zhang et al., 2018).

dominant in the gut microbiota, probably due to the barrier effect mounted by this community.

Commensal Firmicutes such as *Ruminococcus torques* and *Ruminococcus gnavus*, belonging to the *Lachnospiraceae* family, can also access the *O*-linked glycans from mucin, especially MUC2. *Ruminococcus torques* is endowed with an *α*-sialidase (GH33), α-fucosidase (GH29), *β*-galactosidase, β-*N*-acetylgalactosaminidase, β-*N*-acetylglucosaminidase, sialate *O*-acetylesterase, and glycosulfatase activities. Mucin deglycosylation is carried out from a mixture of exo and endo activities (Crost et al., 2016). *Ruminococcus gnavus* contains a similar enzymatic arsenal, but its strategy involves the chemical modification of NeuAc by a trans-sialidase, releasing 2,7-anhydro-Neu5Ac (Bell et al., 2019). Interestingly, this modification allows this microorganism to use this carbohydrate, making it inaccessible for others gut microbes (Bell et al., 2019). This strategy has proven essential for proper colonization of *R. gnavus* in mice.

Ruminococcus gnavus is part of the healthy gut microbiota but appears to be increased in IBDs (Hall et al., 2017). Similar to *Ba. thetaiotaomicron*, *R. gnavus* has the ability to modulate host cellular responses, especially genes encoding MUC2 and certain glycosyltransferases (Graziani et al., 2016). However, both *R. gnavus* and *R. torques* are increased several-fold in the intestinal mucosa of UC and Crohn's disease subjects, suggesting that their mucolytic activities contribute to disease progression (Png et al., 2010).

Sheridan et al. (2016) used *in vitro* growth assays and comparative genomics to identify genes involved in general carbohydrate metabolism in 11 *Roseburia* spp. and *Eubacterium rectale* strains. These bacteria have been suggested next-generation probiotics (Lordan et al., 2019), considered desirable for the host due to the production of butyrate. Two *Roseburia inulinivorans* strains were found to contain a Gram-positive PUL (gpPUL) with putative mucin glycan degradation genes such as a desulfatase, four glycosidases, and an ATP-binding cassettes (ABC) transporter. While this evidence suggests certain butyrate-producers could use mucin *O*-glycans as a carbon source, none of the strains of this study was able to grow using type 2 or type 3 porcine-derived mucin. Further experiments are required to clarify the ability of these microorganisms to target mucin as carbon source and the role of the gpPUL.

Utilization of *O*-Linked Glycans by *Bifidobacterium*

A few members of the phylum Actinobacteria can utilize glycans found in mucin, especially *Bifidobacterium* (Turroni et al., 2010; Katoh et al., 2017). This genus contains mostly commensal or beneficial microorganisms, dominant in the infant's gut.

Milani et al. (2016) showed that *Bi. bifidum* PRL2010 has a set of chromosomal loci that allows both *N*-glycans and *O*-glycan utilization. Seventy-seven genes, including GHs, glycosyltransferases, and glycosyl esterases were identified. *Bifidobacterium bifidum* contains a complete set of extracellular GHs, including *exo*-*α*-sialidases, 1,2-α-L-fucosidase, 1,3/4-α-L-fucosidase, *N*-acetyl-β-hexosaminidases, and four β-galactosidases (**Figure 4A**). Several of these enzymes have been biochemically characterized, showing that they are membrane-bound and essential for mucin *O*-glycan assimilation (Shimada et al., 2015).

The consumption strategy of *Bi. bifidum* is mostly extracellular, where all these enzymes contain transmembrane domains and participate in mucin deglycosylation rendering GNB as a result. Apparently, *Bi. bifidum* does not utilize Fuc or NeuAc from complex glycans (Garrido et al., 2015). However, sialidase activity in this bacterium appears to promote bacterial adhesion to the epithelium (Nishiyama et al., 2017). This consumption mechanism allows the resulting mono and disaccharides to participate in cross-feeding with other microorganisms, for example, with *Bifidobacterium breve* and *Eubacterium hallii* (Egan et al., 2014; Bunesova et al., 2018).

The Lacto-N-Biose (LNB)/GNB cluster present in most bifidobacteria allows these microorganisms to consume Lacto-*N*-Tetraose from HMO and GNB resulting from *O*-glycan degradation. This cluster is present in several infant and adult-associated bifidobacteria. It contains an ABC transporter specific for these two disaccharides and intracellular enzymes for LNB/GNB utilization in the bifid shunt (Thompson et al., 2018).

A few enzymes in this genus have been characterized by their ability to target mucin glycans. Earlier works described an endo-α-GalNAc-ase in *Bi. longum* subsp. *longum* JCM 1217 (EngBF), which is a conserved extracellular GH101 enzyme in this subspecies, being highly specific for Core 1 *O*-glycans (Fujita et al., 2005). An α-acetylgalactosaminidase (NagBb) from *Bi. bifidum* JCM 1254 is a GH101 enzyme that hydrolyzes *O*-glycans from Core 1 and Core 3 from mucin MUC2 (Kiyohara et al., 2012). The enzyme shows a high affinity for the Tn antigen (GalNAcα-Ser/Thr). Finally, a GH89 from the same microorganism shows affinity for GlcNAcα1-4Gal, an epitope commonly found in terminal positions in mucin glycans (Shimada et al., 2015). All these enzymes have great potential as a tool in glycobiology studies, and the obtention of prebiotics from complex mucin sources. Finally, evidence regarding the utilization of mucin *O*-glycans by *Bifidobacterium* has primarily been studied *in vitro*, and further studies are required to understand the implications of these strategies for the host. *Bi. longum*, as well as inulin, show protective effects against mucus deterioration caused by a mucin-eroding gut microbiota (Schroeder et al., 2018).

Utilization of *O*-Linked Glycans by *Verrucomicrobia*

In recent years, *A. muciniphila* has received considerable attention for its associations with health. Its abundance inversely correlates with the onset of IBDs and metabolic disorders (Dao et al., 2016; Lopez-Siles et al., 2018; Yassour et al., 2018), and it has been suggested to play an anti-inflammatory role (Cani and de Vos, 2017). It has also been proposed as a candidate next-generation probiotic, which in addition to the modulation of host responses possesses several technological properties compatible with scale-up processes (Cani and de Vos, 2017). Recently, the administration of pasteurized doses of

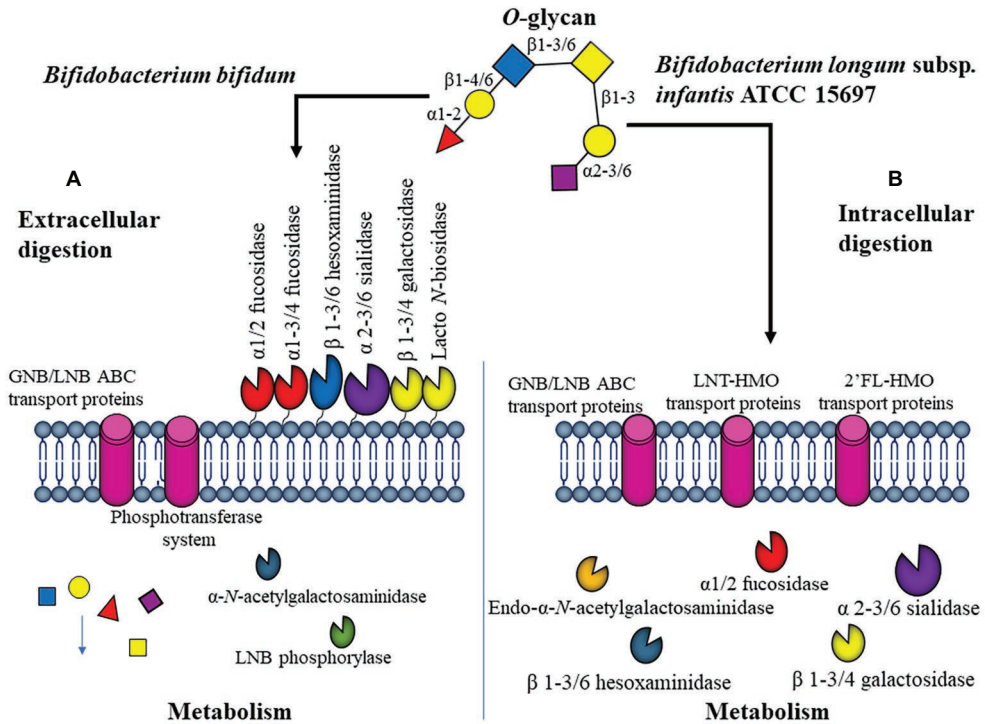

FIGURE 4 | Representation of *O*-linked glycan utilization by *Bifidobacterium*: **(A)** *Bifidobacterium bifidum*, **(B)** *Bifidobacterium infantis*. Two major mechanisms of digestion of *O*-glycan utilization are presented, either extracellular to the left, or intracellular to the right (Garrido et al., 2012a).

A. muciniphila to overweight individuals improved certain inflammation and metabolic markers, being well-tolerated and safe (Depommier et al., 2019).

Akkermansia muciniphila is a Gram-negative bacterium that specializes in using mucins as nitrogen and carbon sources. This contrasts with other generalist gut microbes such as *Bacteroides* spp., which can target multiple sources for growth. Its specificity for mucin also presents a remarkable example of microbial adaptations to host conditions. *Akkermansia muciniphila* is the only member of the phylum *Verrucomicrobia* cultured and found in the gut microbiota, representing the 3% of intestinal bacteria detected in adult feces (Everard et al., 2014). Despite being considered a strict anaerobic microbe, recent studies have determined that it colonizes the mucus layer near epithelial cells, characterized by microaerophilic levels of oxygen (Ouwerkerk et al., 2016). *Akkermansia muciniphila* cannot synthesize Thr; however, it obtains this amino acid from mucin, which is one of the most abundant amino acids in its structure (van der Ark et al., 2018).

This microorganism is underrepresented in IBD, especially UC patients. Yamada et al. (2019) reported that fermentation of mucin *O*-glycans is associated with the production of butyrate in healthy subjects. However, subjects with UC show altered mucin *O*-glycosylation patterns, and the abundance of *A. muciniphila* was inversely proportional to the markers of inflammation (Earley et al., 2019; Yamada et al., 2019). This suggests that the *O*-glycosylation patterns present in mucins influence the anti-inflammatory activity of certain commensal microorganisms.

Studies show that the mucolytic activity and metabolite production by *A. muciniphila* promotes beneficial microbial networks (Belzer et al., 2017). Mucin degradation by gut microorganisms might be considered a pathogenic trait because it reduces the protective layer of the complex mucus layer. Intriguingly, in IL10-genetically deficient mice *A. muciniphila* contributes to colitis (Seregin et al., 2017). However, these degrading microorganisms may stimulate mucin accumulation, renewal, and thickening of the mucosal layer. In addition, the complexity of the mucin *O*-glycans makes these structures protective and inaccessible to most bacteria, except those that have developed consumption mechanisms such as *A. muciniphila* and *Ba. thetaiotaomicron*.

The genome analysis of *A. muciniphila* ATCC BAA-835, isolated from the human gastrointestinal tract, predicted the presence of a large number of mucinases (van Passel et al., 2011). This is a general term referring to GHs or lyases with combined activity against mucin. It was also reported that 26% of its proteome contains a signal peptide site, indicating a preference for extracellular degradation of macromolecules. Consequently, the putative strategy employed by this microorganism is to secrete an extensive array of extracellular proteins that hydrolyze *N*-glycans and *O*-glycans in simple sugars, some of which are later internalized (Shin et al., 2019). Regarding its enzymatic machinery, *A. muciniphila* is predicted

to contain α/β-D-galactosidase, α-L-fucosidase, α/β-*N*-acetylgalactosaminidase, two α-*N*-Acetyl-glucosaminidases, neuraminidase, and a sulfatase. Few studies have confirmed the activities of some of these enzymes (Wang et al., 2018; Shin et al., 2019; Meng et al., 2020), including sulfatases and proteases targeting MUC2. Major end-metabolites produced by this bacterium are propionate, acetate, and sulfate (Ottman et al., 2017).

UTILIZATION OF GMP-DERIVED GLYCANS BY GUT MICROBES

Glycomacropeptide is a highly sialylated glycopeptide resulting from the cheesemaking process. It is an abundant source of *O*-glycans. However, not much attention has been paid to its potential applications for promoting a healthy gut microbiota. Compared to mucin, most studies have focused on the utilization of GMP by probiotic microorganisms *in vitro*, sometimes with contradictory results (Córdova-Dávalos et al., 2019). Early work from Idota et al. (1994) showed that certain *Bifidobacterium*, including *Bi. bifidum*, *Bi. breve*, *Bi. longum*, and *Bifidobacterium adolescentis* could grow on GMP with OD_{600nm} values ranging from 0.7 to 2.70. Although purified GMP contains small amounts of lactose, which could contribute to the above results, the latter two microorganisms do not have the sialidases required to hydrolyze the GMP *O*-glycans (O'Callaghan and van Sinderen, 2016).

Bifidobacterium longum subsp. *infantis* ATCC 15697 (*Bifidobacterium infantis*) is a dominant bacterium in the gut microbiota of newborns. It has been well characterized by its ability to fully utilize several HMO as a carbon source, with protective effects on the infant (Thompson et al., 2018; Henrick et al., 2019). The utilization strategy for this microorganism relies on ABC for internalization of intact glycans, subsequently degraded by intracellular GHs (**Figure 4B**; Zúñiga et al., 2018). *Bifidobacterium infantis* shows a preference for host glycans containing within its genome 16 GH, including several α-fucosidases, β-galactosidases, β-hexosaminidases, and α-sialidases (Thompson et al., 2018). This microorganism has also evolved determinants and mechanisms for the vigorous utilization of milk-derived *N*-glycans (Garrido et al., 2012b; Karav et al., 2016).

Considering the structural similarity between HMO, *N*-glycans, and *O*-glycans, it could be expected that this microorganism also targets mucins as a growth substrate. Interestingly, *B. infantis* is not able to access the *O*-glycans of these glycoproteins (Kim et al., 2013; Turroni et al., 2018). Most glycosidases in *B. infantis* are intracellular, and it lacks homologs to α-*N*-Acetylgalactosaminidases found in *B. bifidum* and *B. longum*.

Strikingly, *B. infantis* has been shown to grow vigorously using GMP (O'Riordan et al., 2018). A 20.6 ± 3.6% increase in OD_{600nm} was observed in the mid-exponential phase of *B. longum* ssp. *infantis* cultures supplemented with GMP, indicating a growth-promoting effect. *In vitro*, it has been shown that *B. infantis* growth is proportional to GMP concentration in the culture media. GMP periodate treatment (GMP-P) inactivates the glycan portion of the peptide chain, and growth was compared with native GMP. GMP-P resulted in a substantially lower (5.5%) increase in growth compared with full GMP in the mid-exponential phase. Under these conditions, *B. infantis* was not able to grow using GMP-P, indicating that the GMP *O*-glycan portion is essential in *B. infantis* growth.

Transcriptomic analysis of the GMP utilization revealed the induction of two intracellular GH25 enzymes, a family related to bacterial lysozymes, and a solute binding protein from an ABC transporter (O'Riordan et al., 2018). These results provide new insights regarding the adaptations of this probiotic microorganism for a milk-derived substrate. However, how this infant-gut associated bacterium can use GMP *O*-glycans as the sole carbon source is not clear, especially considering it cannot access mucin *O*-glycans. It is possible that GMP, a smaller and structurally and physicochemical simpler molecule, is easier to access. Another possibility is the participation of proteases, which could facilitate the import of GMP-derived *O*-glycans into the bacterial cell for intracellular processing.

CONCLUSION

O-linked glycans are complex oligosaccharides decorating host glycoproteins. They are produced endogenously in mucins or could be found in secretions such as milk. These glycans are accessed, released, and consumed by individual members of the gut microbiota, especially beneficial gut microbes such as *Bifidobacterium*, *Bacteroides*, *Akkermansia*, and stimulate the growth of next-generation probiotics such as *Roseburia* and other butyrate-producing bacteria.

O-glycans serve as signaling molecules for cell secretion and provide greater resistance to proteolysis (Boutrou et al., 2008). Under conditions of dietary fiber depletion, endogenous mucin degradation occurs associated with microbial activity, promoting inflammation, and intestinal disease.

O-glycans derived from milk glycopeptides such as in GMP are an attractive opportunity to use them in foods as emerging prebiotics, promoting a healthy gut microbiota. Unfortunately, there are still several limitations to this goal, and no human studies have evaluated its prebiotic effect. While, we have advanced in understanding some of the molecular mechanisms involved in *O*-linked glycan utilization in single microorganisms, the impact of this utilization in metabolic interactions and networks has been evaluated only in a small number of studies.

These gaps have been hindered in part because we lack enzymes or other tools to recover full *O*-glycans from dairy streams or mucin sources. In contrast, certain enzymes have been described for the recovery of *N*-glycans from dairy byproducts. This offers an opportunity for identifying novel enzymes from gut microbes or improving the activity of current endo-α-*N*-acetylgalactosaminidases.

Moreover, the consequences for the host of the microbial utilization of *O*-glycans, or glycoproteins containing *O*-glycans, have not been evaluated and remains a critical question. Whether gut microbes accessing the mucus layer and releasing *O*-glycans is beneficial for the host is still unclear. Furthermore, the study of *O*-glycans utilization by the gut microbiota, and how these microbes shape mucin glycosylation patterns, should be further studied in the context of intestinal inflammation and IBDs, as well as cancer progression.

AUTHOR CONTRIBUTIONS

KG-M, MV-S, and DG conceived and wrote the manuscript. KG-M prepared the Figures. DG critically reviewed the manuscript. All authors approved the final version of the manuscript.

FUNDING

Funding for this manuscript was provided by Fondecyt grant 1190074.

ACKNOWLEDGMENTS

We thank the support of Fondecyt grant 1190074, ANID Doctorate Scholarships 2019, and Interdisciplinary Seed Fund PUC 2018.

REFERENCES

Anderson, K. L., and Salyers, A. A. (1989). Biochemical evidence that starch breakdown by *Bacteroides thetaiotaomicron* involves outer membrane starch-binding sites and periplasmic starch-degrading enzymes. *J. Bacteriol.* 171, 3192–3198. doi: 10.1128/JB.171.6.3192-3198.1989

Arike, L., and Hansson, G. C. (2016). The densely *O*-glycosylated MUC2 mucin protects the intestine and provides food for the commensal bacteria. *J. Mol. Biol.* 428, 3221–3229. doi: 10.1016/j.jmb.2016.02.010

Arike, L., Holmén-Larsson, J., and Hansson, G. C. (2017). Intestinal Muc2 mucin *O*-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases. *Glycobiology* 27, 318–328. doi: 10.1093/glycob/cww134

Atuma, C., Strugala, V., Allen, A., and Holm, L. (2001). The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280, G922–G929. doi: 10.1152/ajpgi.2001.280.5.G922

Bansil, R., and Turner, B. S. (2018). The biology of mucus: composition, synthesis and organization. *Adv. Drug Deliv. Rev.* 124, 3–15. doi: 10.1016/j.addr.2017.09.023

Baum, L. G., and Cobb, B. A. (2016). The direct and indirect effects of glycans on immune function. *Glycobiology* 27, 619–624. doi: 10.1093/glycob/cwx036

Bell, A., Brunt, J., Crost, E., Vaux, L., Nepravishta, R., Owen, C. D., et al. (2019). Elucidation of a sialic acid metabolism pathway in mucus-foraging *Ruminococcus gnavus* unravels mechanisms of bacterial adaptation to the gut. *Nat. Microbiol.* 4, 2393–2404. doi: 10.1038/s41564-019-0590-7

Belzer, C., Chia, L. W., Aalvink, S., Chamlagain, B., Piironen, V., Knol, J., et al. (2017). Microbial metabolic networks at the mucus layer lead to diet-independent butyrate and vitamin B12 production by intestinal symbionts. *mBio* 8, e00770–e00717. doi: 10.1128/mBio.00770-17

BeMiller, J. N. (2019). "Oligosaccharides" in *Carbohydrate chemistry for food scientists*. ed. J. N. BeMiller (PA: Woodhead Publishing and AACC International Press (Elsevier)), 49–74.

Brockhausen, I., and Stanley, P. (2017). "O-GalNAc glycans" in *Essentials of glycobiology*. eds. A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart and M. Aebiet al. (NY: Cold Spring Harbor Laboratory Press).

Bergstrom, K., Fu, J., Johansson, M. E. V., Liu, X., Gao, N., Wu, Q., et al. (2017). Core 1– and 3–derived *O*-glycans collectively maintain the colonic mucus barrier and protect against spontaneous colitis in mice. *Mucosal Immunol.* 10, 91–103. doi: 10.1038/mi.2016.45

Bergstrom, K., and Xia, L. (2013). Mucin-type *O*-glycans and their roles in intestinal homeostasis. *Glycobiology* 23, 1026–1037. doi: 10.1093/glycob/cwt045

Bernard, L., Bonnet, M., Delavaud, C., Delosière, M., Ferlay, A., and Fougère, H. (2018). Milk fat globule in ruminant: major and minor compounds, nutritional regulation and differences among species. *Eur. J. Lipid Sci. Technol.* 120:1700039. doi: 10.1002/ejlt.201700039

Boutrou, R., Jardin, J., Blais, A., Tomé, D., and Léonil, J. (2008). Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J. Agric. Food Chem.* 56, 8166–8173. doi: 10.1021/jf801140d

Brockhausen, I., and Stanley, P. (2017). "O-GalNAc glycans" in *Essentials of glycobiology*. eds. A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart and M. Aebi et al. (NY: Cold Spring Harbor Laboratory Press).

Brosseau, C., Selle, A., Palmer, D. J., Prescott, S. L., Barbarot, S., and Bodinier, M. (2019). Prebiotics: mechanisms and preventive effects in allergy. *Nutrients* 11:1841. doi: 10.3390/nu11081841

Bunesova, V., Lacroix, C., and Schwab, C. (2018). Mucin cross-feeding of infant bifidobacteria and *Eubacterium hallii*. *Microb. Ecol.* 75, 228–238. doi: 10.1007/s00248-017-1037-4

Cani, P. D., and de Vos, W. M. (2017). Next-generation beneficial microbes: the case of *Akkermansia muciniphila*. *Front. Microbiol.* 8:1765. doi: 10.3389/fmicb.2017.01765

Chen, G., Ran, X., Li, B., Li, Y., He, D., Huang, B., et al. (2018). Sodium butyrate inhibits inflammation and maintains epithelium barrier integrity in a TNBS-induced inflammatory bowel disease mice model. *EBioMedicine* 30, 317–325. doi: 10.1016/j.ebiom.2018.03.030

Chung, L., Thiele Orberg, E., Geis, A. L., Chan, J. L., Fu, K., DeStefano Shields, C. E., et al. (2018). *Bacteroides fragilis* toxin coordinates a pro-carcinogenic inflammatory cascade via targeting of colonic epithelial cells. *Cell Host Microbe* 23, 203.e5–214.e5. doi: 10.1016/j.chom.2018.01.007

Cockburn, D. W., and Koropatkin, N. M. (2016). Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease. *J. Mol. Biol.* 428, 3230–3252. doi: 10.1016/j.jmb.2016.06.021

Collins, J. W., Keeney, K. M., Crepin, V. F., Rathinam, V. A. K., Fitzgerald, K. A., Finlay, B. B., et al. (2014). *Citrobacter rodentium*: infection, inflammation and the microbiota. *Nat. Rev. Microbiol.* 12, 612–623. doi: 10.1038/nrmicro3315

Córdova-Dávalos, L., Jiménez, M., and Salinas, E. (2019). Glycomacropeptide bioactivity and health: a review highlighting action mechanisms and signaling pathways. *Nutrients* 11:598. doi: 10.3390/nu11030598

Corfield, A. P. (2000). Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut* 47, 589–594. doi: 10.1136/gut.47.4.589

Corfield, A. P. (2015). Mucins: a biologically relevant glycan barrier in mucosal protection. *Biochim. Biophys. Acta* 1850, 236–252. doi: 10.1016/j.bbagen.2014.05.003

Crost, E. H., Tailford, L. E., Monestier, M., Swarbreck, D., Henrissat, B., Crossman, L. C., et al. (2016). The mucin-degradation strategy of *Ruminococcus gnavus*: the importance of intramolecular trans-sialidases. *Gut Microbes* 7, 302–312. doi: 10.1080/19490976.2016.1186334

Crouch, L. I., Liberato, M. V., Urbanowicz, P. A., Baslé, A., Lamb, C. A., Stewart, C. J., et al. (2020). Prominent members of the human gut microbiota express endo-acting *O*-glycanases to initiate mucin breakdown. *Nat. Commun.* 11:4017. doi: 10.1038/s41467-020-17847-5

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., et al. (2016). *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* 65, 426–436. doi: 10.1136/gutjnl-2014-308778

Davis, J. C. C., Totten, S. M., Huang, J. O., Nagshbandi, S., Kirmiz, N., Garrido, D. A., et al. (2016). Identification of oligosaccharides in feces of

breast-fed infants and their correlation with the gut microbial community. *Mol. Cell. Proteomics* 15, 2987–3002. doi: 10.1074/mcp.M116.060665

Demouveaux, B., Gouyer, V., Gottrand, F., Narita, T., and Desseyn, J. L. (2018). Gel-forming mucin interactome drives mucus viscoelasticity. *Adv. Colloid Interface Sci.* 252, 69–82. doi: 10.1016/j.cis.2017.12.005

Depommier, C., Everard, A., Druart, C., Plovier, H., Van Hul, M., Vieira-Silva, S., et al. (2019). Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat. Med.* 25, 1096–1103. doi: 10.1038/s41591-019-0495-2

Desai, M. S., Seekatz, A. M., Koropatkin, N. M., Kamada, N., Hickey, C. A., Wolter, M., et al. (2016). A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell* 167, 1339.e21–1353.e21. doi: 10.1016/j.cell.2016.10.043

De Weirdt, R., and Van de Wiele, T. (2015). Micromanagement in the gut: microenvironmental factors govern colon mucosal biofilm structure and functionality. *NPJ Biofilms Microbiomes* 1:15026. doi: 10.1038/npjbiofilms.2015.26

Dhanisha, S. S., Guruvayoorappan, C., Drishya, S., and Abeesh, P. (2018). Mucins: structural diversity, biosynthesis, its role in pathogenesis and as possible therapeutic targets. *Crit. Rev. Oncol. Hematol.* 122, 98–122. doi: 10.1016/j.critrevonc.2017.12.006

Donovan, S. M. (2019). "Human milk proteins: composition and physiological significance" in *Human milk: composition, clinical benefits and future opportunities*. eds. S. M. Donovan, J. B. German, B. Lönnerdal and A. Lucas (Switzerland: Nestlé Nutrition Institute/Basel: S. Karger AG), 93–101.

Earley, H., Lennon, G., Balfe, Á., Coffey, J. C., Winter, D. C., and O'Connell, P. R. (2019). The abundance of *Akkermansia muciniphila* and its relationship with sulphated colonic mucins in health and ulcerative colitis. *Sci. Rep.* 9:15683. doi: 10.1038/s41598-019-51878-3

Egan, M., O'Connell Motherway, M., Kilcoyne, M., Kane, M., Joshi, L., Ventura, M., et al. (2014). Cross-feeding by *Bifidobacterium breve* UCC2003 during co-cultivation with *Bifidobacterium bifidum* PRL2010 in a mucin-based medium. *BMC Microbiol.* 14:282. doi: 10.1186/s12866-014-0282-7

Everard, A., Lazarevic, V., Gaïa, N., Johansson, M., Ståhlman, M., Backhed, F., et al. (2014). Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. *ISME J.* 8, 2116–2130. doi: 10.1038/ismej.2014.45

Fiat, A. M., and Jollès, P. (1989). Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. *Mol. Cell. Biochem.* 87, 5–30. doi: 10.1007/BF00421079

Fujita, K., Fusako, O., Nagamine, N., Katayama, T., Hiratake, J., Sakata, K., et al. (2005). Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type *O*-glycan-specific endo-α-*N*-acetylgalactosaminidase from *Bifidobacterium longum*. *J. Biol. Chem.* 280, 37415–37422. doi: 10.1074/jbc.M506874200

Garrido, D., Barile, D., and Mills, D. A. (2012a). A molecular basis for bifidobacterial enrichment in the infant gastrointestinal tract. *Adv. Nutr.* 3, 415S–421S. doi: 10.3945/an.111.001586

Garrido, D., Nwosu, C., Ruiz-Moyano, S., Aldredge, D., German, J. B., Lebrilla, C. B., et al. (2012b). Endo-β-*N*-acetylglucosaminidases from infant gut-associated bifidobacteria release complex *N*-glycans from human milk glycoproteins. *Mol. Cell. Proteomics* 11, 775–785. doi: 10.1074/mcp.M112.018119

Garrido, D., Ruiz-Moyano, S., Lemay, D. G., Sela, D. A., German, J. B., and Mills, D. A. (2015). Comparative transcriptomics reveals key differences in the response to milk oligosaccharides of infant gut-associated bifidobacteria. *Sci. Rep.* 5:13517. doi: 10.1038/srep13517

Gibson, G. R., McCartney, A. L., and Rastall, R. A. (2005). Prebiotics and resistance to gastrointestinal infections. *Br. J. Nutr.* 93, S31–S34. doi: 10.1079/BJN20041343

Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., and Roberfroid, M. B. (2017). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 14, 259–275. doi: 10.1079/NRR200479

Graziani, F., Pujol, A., Nicoletti, C., Dou, S., Maresca, M., Giardina, T., et al. (2016). *Ruminococcus gnavus* E1 modulates mucin expression and intestinal glycosylation. *J. Appl. Microbiol.* 120, 1403–1417. doi: 10.1111/jam.13095

Groux-Degroote, S., Cavdarli, S., Uchimura, K., Allain, F., and Delannoy, P. (2020). Glycosylation changes in inflammatory diseases. *Adv. Protein Chem. Struct. Biol.* 119, 111–156. doi: 10.1016/bs.apcsb.2019.08.008

Guan, Q., and Zhang, J. (2017). Recent advances: the imbalance of cytokines in the pathogenesis of inflammatory bowel disease. *Mediators Inflamm.* 2017:4810258. doi: 10.1155/2017/4810258

Hall, A. B., Yassour, M., Sauk, J., Garner, A., Jiang, X., Arthur, T., et al. (2017). A novel *Ruminococcus gnavus* clade enriched in inflammatory bowel disease patients. *Genome Med.* 9:103. doi: 10.1186/s13073-017-0490-5

Henrick, B. M., Chew, S., Casaburi, G., Brown, H. K., Frese, S. A., Zhou, Y., et al. (2019). Colonization by *B. infantis* EVC001 modulates enteric inflammation in exclusively breastfed infants. *Pediatr. Res.* 86, 749–757. doi: 10.1038/s41390-019-0533-2

Idota, T., Kawakami, H., and Nakajima, I. (1994). Growth-promoting effects of *N*-acetylneuraminic acid-containing substances on bifidobacteria. *Biosci. Biotechnol. Biochem.* 58, 1720–1722. doi: 10.1271/bbb.58.1720

Itano, N. (2019). Implications of altered *O*-glycosylation in tumour immune evasion. *J. Biochem.* 165, 387–390. doi: 10.1093/jb/mvz003

Itzkowitz, S. H., Yuan, M., Montgomery, C. K., Kjeldsen, T., Takahashi, H. K., Bigbee, W. L., et al. (1989). Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. *Cancer Res.* 49, 197–204.

Jin, C., Kenny, D. T., Skoog, E. C., Padra, M., Adamczyk, B., Vitizeva, V., et al. (2017). Structural diversity of human gastric mucin glycans. *Mol. Cell. Proteomics* 16, 743–758. doi: 10.1074/mcp.M117.067983

Johansson, M. E. V., Jakobsson, H. E., Holmén-Larsson, J., Schütte, A., Ermund, A., Rodríguez-Piñeiro, A. M., et al. (2015). Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host Microbe* 18, 582–592. doi: 10.1016/j.chom.2015.10.007

Karav, S., Le Parc, A., Nobrega de Moura Bell, J. M. L., Frese, S. A., Kirmiz, N., Block, D. E., et al. (2016). Oligosaccharides released from milk glycoproteins are selective growth substrates for infant-associated bifidobacteria. *Appl. Environ. Microbiol.* 82, 3622–3630. doi: 10.1128/AEM.00547-16

Katoh, T., Maeshibu, T., Kikkawa, K., Gotoh, A., Tomabechi, Y., Nakamura, M., et al. (2017). Identification and characterization of a sulfoglycosidase from *Bifidobacterium bifidum* implicated in mucin glycan utilization. *Biosci. Biotechnol. Biochem.* 81, 2018–2027. doi: 10.1080/09168451.2017.1361810

Kesimer, M., and Sheehan, J. K. (2012). Mass spectrometric analysis of mucin core proteins. *Methods Mol. Biol.* 842, 67–79. doi: 10.1007/978-1-61779-513-8_4

Kim, J. -H., An, H. J., Garrido, D., German, J. B., Lebrilla, C. B., and Mills, D. A. (2013). Proteomic analysis of *Bifidobacterium longum* subsp. infantis reveals the metabolic insight on consumption of prebiotics and host glycans. *PLoS One* 8:e57535. doi: 10.1371/journal.pone.0057535

Kirmiz, N., Robinson, R. C., Shah, I. M., Barile, D., and Mills, D. A. (2018). Milk glycans and their interaction with the infant-gut microbiota. *Annu. Rev. Food Sci. Technol.* 9, 429–450. doi: 10.1146/annurev-food-030216-030207

Kiyohara, M., Nakatomi, T., Kurihara, S., Fushinobu, S., Suzuki, H., Tanaka, T., et al. (2012). α-*N*-acetylgalactosaminidase from infant-associated bifidobacteria belonging to novel glycoside hydrolase family 129 is implicated in alternative mucin degradation pathway. *J. Biol. Chem.* 287, 693–700. doi: 10.1074/jbc.M111.277384

Koutsioulis, D., Landry, D., and Guthrie, E. P. (2008). Novel endo-α-*N*-acetylgalactosaminidases with broader substrate specificity. *Glycobiology* 18, 799–805. doi: 10.1093/glycob/cwn069

Lamacchia, C., Musaico, D., Henderson, M. E., Bergillos-meca, T., Roul, M., Landriscina, L., et al. (2018). Temperature-treated gluten proteins in gluten-friendly™ bread increase mucus production and gut-barrier function in human intestinal goblet cells. *J. Funct. Foods* 48, 507–514. doi: 10.1016/j.jff.2018.07.047

Lee, S. M., Donaldson, G. P., Mikulski, Z., Boyajian, S., Ley, K., and Mazmanian, S. K. (2013). Bacterial colonization factors control specificity and stability of the gut microbiota. *Nature* 501, 426–429. doi: 10.1038/nature12447

Le Parc, A., Karav, S., Maria, J., Nobrega, L., Bell, D. M., Frese, S. A., et al. (2015). A novel endo-β-*N*-acetylglucosaminidase releases specific *N*-glycans depending on different reaction conditions. *Biotechnol. Prog.* 31, 1323–1330. doi: 10.1002/btpr.2133

Li, Z., and Chai, W. (2019). Mucin *O*-glycan microarrays. *Curr. Opin. Struct. Biol.* 56, 187–197. doi: 10.1016/j.sbi.2019.03.032

Lillehoj, E. P., Kato, K., Lu, W., and Kim, K. C. (2013). Cellular and molecular biology of airway mucins. *Int. Rev. Cell Mol. Biol.* 303, 139–202. doi: 10.1016/B978-0-12-407697-6.00004-0

Lopez-Siles, M., Enrich-Capó, N., Aldeguer, X., Sabat-Mir, M., Duncan, S. H., Garcia-Gil, L. J., et al. (2018). Alterations in the abundance and co-occurrence of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* in the colonic

mucosa of inflammatory bowel disease subjects. *Front. Cell. Infect. Microbiol.* 8:281. doi: 10.3389/fcimb.2018.00281

Lordan, C., Thapa, D., Ross, R. P., and Cotter, P. D. (2019). Potential for enriching next-generation health-promoting gut bacteria through prebiotics and other dietary components. *Gut Microbes* 11, 1–20. doi: 10.1080/19490976.2019.1613124

Luis, A. S., Briggs, J., Zhang, X., Farnell, B., Ndeh, D., Labourel, A., et al. (2018). Dietary pectic glycans are degraded by coordinated enzyme pathways in human colonic *Bacteroides*. *Nat. Microbiol.* 3, 210–219. doi: 10.1038/s41564-017-0079-1

Magalhães, A., Rossez, Y., Robbe-Masselot, C., Maes, E., Gomes, J., Shevtsova, A., et al. (2016). MUC5AC gastric mucin glycosylation is shaped by FUT2 activity and functionally impacts *Helicobacter pylori* binding. *Sci. Rep.* 6:25575. doi: 10.1038/srep25575

Magnelli, P. E., Bielik, A. M., and Guthrie, E. P. (2011). Identification and characterization of protein glycosylation using specific endo- and exoglycosidases. *J. Vis. Exp.* 58:e3749. doi: 10.3791/3749

Manso, M. A., and López-Fandiño, R. (2004). κ-casein macropeptides from cheese whey: physicochemical, biological, nutritional, and technological features for possible uses. *Food Rev. Int.* 20, 329–355. doi: 10.1081/FRI-200033456

Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., et al. (2011). Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host Microbe* 10, 507–514. doi: 10.1016/j.chom.2011.10.007

Martens, E. C., Chiang, H. C., and Gordon, J. I. (2008). Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* 4, 447–457. doi: 10.1016/j.chom.2008.09.007

Meng, X., Wu, H., Wang, W., Lan, T., Yang, W., Yu, D., et al. (2020). A purified aspartic protease from *Akkermansia muciniphila* plays an important role in degrading Muc2. *Int. J. Mol. Sci.* 21:72. doi: 10.3390/ijms21010072

Milani, C., Turroni, F., Duranti, S., Lugli, G. A., Mancabelli, L., Ferrario, C., et al. (2016). Genomics of the genus *Bifidobacterium* reveals species-specific adaptation to the glycan-rich gut environment. *Appl. Environ. Microbiol.* 82, 980–991. doi: 10.1128/AEM.03500-15

Morio, A., Xu, J., Masuda, A., Kinoshita, Y., Hino, M., Morokuma, D., et al. (2019). Expression, purification, and characterization of highly active endo-α-*N*-acetylgalactosaminidases expressed by silkworm-baculovirus expression system. *J. Asia Pac. Entomol.* 22, 404–408. doi: 10.1016/j.aspen.2019.01.009

Neelima, Sharma, R., Rajput, Y. S., and Mann, B. (2013). Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: a review. *Dairy Sci. Technol.* 93, 21–43. doi: 10.1007/s13594-012-0095-0

Nishiyama, K., Yamamoto, Y., Sugiyama, M., Takaki, T., Urashima, T., Fukiya, S., et al. (2017). *Bifidobacterium bifidum* extracellular sialidase enhances adhesion to the mucosal surface and supports carbohydrate assimilation. *mBio* 8, e00928–e01117. doi: 10.1128/mBio.00928-17

O'Callaghan, A., and van Sinderen, D. (2016). Bifidobacteria and their role as members of the human gut microbiota. *Front. Microbiol.* 7:925. doi: 10.3389/fmicb.2016.00925

O'Riordan, N., Callaghan, J. O., Buttò, L. F., Kilcoyne, M., Joshi, L., and Hickey, R. M. (2018). Bovine glycomacropeptide promotes the growth of *Bifidobacterium longum* ssp. *infantis* and modulates its gene expression. *J. Dairy Sci.* 101, 6730–6741. doi: 10.3168/jds.2018-14499

Ottman, N., Geerlings, S. Y., Aalvink, S., de Vos, W. M., and Belzer, C. (2017). Action and function of *Akkermansia muciniphila* in microbiome ecology, health and disease. *Best Pract. Res. Clin. Gastroenterol.* 31, 637–642. doi: 10.1016/j.bpg.2017.10.001

Ouwerkerk, J. P., van der Ark, K. C. H., Davids, M., Claassens, N. J., Finestra, T. R., de Vos, W. M., et al. (2016). Adaptation of *Akkermansia muciniphila* to the oxic-anoxic interface of the mucus layer. *Appl. Environ. Microbiol.* 82, 6983–6993. doi: 10.1128/AEM.01641-16

Padra, M., Adamczyk, B., Benktander, J., Flahou, B., Skoog, C., Padra, J. T., et al. (2018). *Helicobacter suis* binding to carbohydrates on human and porcine gastric mucins and glycolipids occurs via two modes. *Virulence* 9, 898–918. doi: 10.1080/21505594.2018.1460979

Pinho, S. S., and Reis, C. A. (2015). Glycosylation in cancer: mechanisms and clinical implications. *Nat. Rev. Cancer* 15, 540–555. doi: 10.1038/nrc3982

Pluvinage, B., Massel, P. M., Burak, K., and Boraston, A. B. (2019). Structural and functional analysis of four family 84 glycoside hydrolases from the opportunistic pathogen *Clostridium perfringens*. *Glycobiology* 30, 49–57. doi: 10.1093/glycob/cwz069

Png, C. W., Lindén, S. K., Gilshenan, K. S., Zoetendal, E. G., McSweeney, C. S., Sly, L. I., et al. (2010). Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *Am. J. Gastroenterol.* 105, 2420–2428. doi: 10.1038/ajg.2010.281

Podolsky, D. K. (1985). Oligosaccharide structures of human colonic mucin. *J. Biol. Chem.* 260, 8262–8271.

Praharaj, A. B., Dehury, B., Mahapatra, N., Kar, S. K., and Behera, S. K. (2018). Molecular dynamics insights into the structure, function, and substrate binding mechanism of mucin desulfating sulfatase of gut microbe *Bacteroides fragilis*. *J. Cell. Biochem.* 119, 3618–3631. doi: 10.1002/jcb.26569

Puccio, G., Alliet, P., Cajozzo, C., Janssens, E., Corsello, G., Sprenger, N., et al. (2017). Effects of infant formula with human milk oligosaccharides on growth and morbidity. *J. Pediatr. Gastroenterol. Nutr.* 64, 624–631. doi: 10.1097/MPG.0000000000001520

Pudlo, N. A., Urs, K., Kumar, S. S., German, J. B., Mills, D. A., and Martens, E. C. (2015). Symbiotic human gut bacteria with variable metabolic priorities for host mucosal glycans. *mBio* 6, e01282–e01315. doi: 10.1128/mBio.01282-15

Purcell, R. V., Pearson, J., Aitchison, A., Dixon, L., Frizelle, F. A., and Keenan, J. I. (2017). Colonization with enterotoxigenic *Bacteroides fragilis* is associated with early-stage colorectal neoplasia. *PLoS One* 12:e0171602. doi: 10.1371/journal.pone.0171602

Quintana-Hayashi, M., Padra, M., Padra, J., Benktander, J., and Lindén, S. (2018). Mucus-pathogen interactions in the gastrointestinal tract of farmed animals. *Microorganisms* 6:55. doi: 10.3390/microorganisms6020055

Ravcheev, D. A., and Thiele, I. (2017). Comparative genomic analysis of the human gut microbiome reveals a broad distribution of metabolic pathways for the degradation of host-synthetized mucin glycans and utilization of mucin-derived monosaccharides. *Front. Genet.* 8:111. doi: 10.3389/fgene.2017.00111

Ringot-Destrez, B., Kalach, N., Mihalache, A., Gosset, P., Michalski, J. C., Léonard, R., et al. (2017). How do they stick together? Bacterial adhesins implicated in the binding of bacteria to the human gastrointestinal mucins. *Biochem. Soc. Trans.* 45, 389–399. doi: 10.1042/BST20160167

Rivière, A., Selak, M., Lantin, D., Leroy, F., and De Vuyst, L. (2016). Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. *Front. Microbiol.* 7:979. doi: 10.3389/fmicb.2016.00979

Robbe, C., Capon, C., Maes, E., Rousset, M., Zweibaum, A., Zanetta, J. -P., et al. (2003). Evidence of regio-specific glycosylation in human intestinal mucins. *J. Biol. Chem.* 278, 46337–46348. doi: 10.1074/jbc.M302529200

Roberts, G., Tarelli, E., Homer, K. A., Philpott-Howard, J., and Beighton, D. (2000). Production of an endo-β-*N*-acetylglucosaminidase activity mediates growth of *Enterococcus faecalis* on a high-mannose-type glycoprotein. *J. Bacteriol.* 182, 882–890. doi: 10.1128/JB.182.4.882-890.2000

Rojas, E., and Torres, G. (2013). Isolation and recovery of glycomacropeptide from milk whey by means of thermal treatment. *Food Sci. Technol.* 33, 14–20. doi: 10.1590/S0101-20612013005000027

Ross, S. A., Lane, J. A., Kilcoyne, M., Joshi, L., and Hickey, R. M. (2015). "The milk fat globule membrane" in *Biotechnology of bioactive compounds*. eds. V. K. Gupta, M. G. Tuohy, A. O'Donovan and M. Lohani (Chichester, UK: John Wiley & Sons, Ltd.), 631–668.

Rossez, Y., Maes, E., Lefebvre Darroman, T., Gosset, P., Ecobichon, C., Joncquel Chevalier Curt, M., et al. (2012). Almost all human gastric mucin *O*-glycans harbor blood group a, B or H antigens and are potential binding sites for *Helicobacter pylori*. *Glycobiology* 22, 1193–1206. doi: 10.1093/glycob/cws072

Saito, T., and Itoh, T. (1992). Variations and distributions of *O*-glycosidically linked sugar chains in bovine κ-casein. *J. Dairy Sci.* 75, 1768–1774. doi: 10.3168/jds.S0022-0302(92)77936-3

Sawicki, C., Livingston, K., Obin, M., Roberts, S., Chung, M., and McKeown, N. (2017). Dietary fiber and the human gut microbiota: application of evidence mapping methodology. *Nutrients* 9:125. doi: 10.3390/nu9020125

Schroeder, B. O., Birchenough, G. M. H., Ståhlman, M., Arike, L., Johansson, M. E. V., Hansson, G. C., et al. (2018). Bifidobacteria or fiber protects against diet-induced microbiota-mediated colonic mucus deterioration. *Cell Host Microbe* 23, 27.e7–40.e7. doi: 10.1016/j.chom.2017.11.004

Schulz, B. L., Sloane, A. J., Robinson, L. J., Prasad, S. S., Lindner, R. A., Robinson, M., et al. (2007). Glycosylation of sputum mucins is altered in cystic fibrosis patients. *Glycobiology* 17, 698–712. doi: 10.1093/glycob/cwm036

Seregin, S. S., Golovchenko, N., Schaf, B., Chen, J., Pudlo, N. A., Mitchell, J., et al. (2017). NLRP6 protects Il10−/− mice from colitis by limiting colonization of *Akkermansia muciniphila*. *Cell Rep.* 19, 733–745. doi: 10.1016/j.celrep.2017.03.080

Sheridan, P., Martin, J. C., Lawley, T. D., Browne, H. P., Harris, H. M. B., Bernalier-Donadille, A., et al. (2016). Polysaccharide utilization loci and nutritional specialization in a dominant group of butyrate-producing human colonic *Firmicutes*. *Microb. Genom.* 2:e000043. doi: 10.1099/mgen.0.000043

Shimada, Y., Watanabe, Y., Wakinaka, T., Funeno, Y., Kubota, M., Chaiwangsri, T., et al. (2015). α-*N*-acetylglucosaminidase from *Bifidobacterium bifidum* specifically hydrolyzes α-linked *N*-acetylglucosamine at nonreducing terminus of *O*-glycan on gastric mucin. *Appl. Microbiol. Biotechnol.* 99, 3941–3948. doi: 10.1007/s00253-014-6201-x

Shin, J., Noh, J. R., Chang, D. H., Kim, Y. H., Kim, M. H., Lee, E. S., et al. (2019). Elucidation of *Akkermansia muciniphila* probiotic traits driven by mucin depletion. *Front. Microbiol.* 10:1137. doi: 10.3389/fmicb.2019.01137

Shokryazdan, P., Faseleh Jahromi, M., Navidshad, B., and Liang, J. B. (2017). Effects of prebiotics on immune system and cytokine expression. *Med. Microbiol. Immunol.* 206, 1–9. doi: 10.1007/s00430-016-0481-y

Sicard, J. -F., Le Bihan, G., Vogeleer, P., Jacques, M., and Harel, J. (2017). Interactions of intestinal bacteria with components of the intestinal mucus. *Front. Cell. Infect. Microbiol.* 7:387. doi: 10.3389/fcimb.2017.00387

Stanley, P., Taniguchi, N., and Aebi, M. (2017). "N-glycans" in *Essentials of glycobiology*. eds. A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart and M. Aebi et al. (NY: Cold Spring Harbor Laboratory Press).

Sunds, A. V., Poulsen, N. A., and Larsen, L. B. (2019). Short communication: application of proteomics for characterization of caseinomacropeptide isoforms before and after desialidation. *J. Dairy Sci.* 102, 8696–8703. doi: 10.3168/jds.2019-16617

Tailford, L. E., Crost, E. H., Kavanaugh, D., and Juge, N. (2015). Mucin glycan foraging in the human gut microbiome. *Front. Genet.* 6:81. doi: 10.3389/fgene.2015.00081

Thomä-Worringer, C., Sørensen, J., and López-Fandiño, R. (2006). Health effects and technological features of caseinomacropeptide. *Int. Dairy J.* 16, 1324–1333. doi: 10.1016/j.idairyj.2006.06.012

Thompson, P., Medina, D. A., and Garrido, D. (2018). Human milk oligosaccharides and infant gut bifidobacteria: molecular strategies for their utilization. *Food Microbiol.* 75, 37–46. doi: 10.1016/j.fm.2017.09.001

Turroni, F., Bottacini, F., Foroni, E., Mulder, I., Jae-Han, K., Zomer, A., et al. (2010). Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc. Natl. Acad. Sci. U. S. A.* 107, 19514–19519. doi: 10.1073/pnas.1011100107

Turroni, F., Milani, C., Duranti, S., Mahony, J., van Sinderen, D., and Ventura, M. (2018). Glycan utilization and cross-feeding activities by bifidobacteria. *Trends Microbiol.* 26, 339–350. doi: 10.1016/j.tim.2017.10.001

Vandeputte, D., Kathagen, G., D'hoe, K., Vieira-Silva, S., Valles-Colomer, M., Sabino, J., et al. (2017). Quantitative microbiome profiling links gut community variation to microbial load. *Nature* 551, 507–511. doi: 10.1038/nature24460

van der Ark, K. C. H., Aalvink, S., Suarez-Diez, M., Schaap, P. J., de Vos, W. M., and Belzer, C. (2018). Model-driven design of a minimal medium for *Akkermansia muciniphila* confirms mucus adaptation. *Microb. Biotechnol.* 11, 476–485. doi: 10.1111/1751-7915.13033

van Passel, M. W. J., Kant, R., Zoetendal, E. G., Plugge, C. M., Derrien, M., Malfatti, S. A., et al. (2011). The genome of *Akkermansia muciniphila*, a dedicated intestinal mucin degrader, and its use in exploring intestinal metagenomes. *PLoS One* 6:e16876. doi: 10.1371/journal.pone.0016876

van Tassell, M. L., and Miller, M. J. (2011). *Lactobacillus* adhesion to mucus. *Nutrients* 3, 613–636. doi: 10.3390/nu3050613

Varki, A., and Lowe, J. B. (2009). "Biological roles of glycans" in *Essentials of glycobiology*. eds. A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart and J. Marth (NY: Cold Spring Harbor Laboratory Press).

Vliegenthart, J. F. (2017). The complexity of glycoprotein-derived glycans. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 93, 64–86. doi: 10.2183/pjab.93.005

Wang, M., Zhang, X. Y., Guo, R. R., Cai, Z. P., Hu, X. C., Chen, H., et al. (2018). Cloning, purification and biochemical characterization of two β-*N*-acetylhexosaminidases from the mucin-degrading gut bacterium *Akkermansia muciniphila*. *Carbohydr. Res.* 457, 1–7. doi: 10.1016/j.carres.2017.12.007

Wexler, A. G., and Goodman, A. L. (2017). An insider's perspective: bacteroides as a window into the microbiome. *Nat. Microbiol.* 2:17026. doi: 10.1038/nmicrobiol.2017.26

Wilson, B., and Whelan, K. (2017). Prebiotic inulin-type fructans and galacto-oligosaccharides: definition, specificity, function, and application in gastrointestinal disorders. *J. Gastroenterol. Hepatol.* 32, 64–68. doi: 10.1111/jgh.13700

Wrzosek, L., Miquel, S., Noordine, M. L., Bouet, S., Chevalier-Curt, M. J., Robert, V., et al. (2013). *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. *BMC Biol.* 11:61. doi: 10.1186/1741-7007-11-61

Yamada, T., Hino, S., Iijima, H., Genda, T., Aoki, R., Nagata, R., et al. (2019). Mucin *O*-glycans facilitate symbiosynthesis to maintain gut immune homeostasis. *EBioMedicine* 48, 513–525. doi: 10.1016/j.ebiom.2019.09.008

Yamamoto, T., Ugai, H., Nakayama-Imaohji, H., Tada, A., Elahi, M., Houchi, H., et al. (2018). Characterization of a recombinant *Bacteroides fragilis* sialidase expressed in *Escherichia coli*. *Anaerobe* 50, 69–75. doi: 10.1016/j.anaerobe.2018.02.003

Yassour, M., Jason, E., Hogstrom, L. J., Arthur, T. D., Tripathi, S., Siljander, H., et al. (2018). Strain-level analysis of mother-to-child bacterial transmission during the first few months of life. *Cell Host Microbe* 24, 146.e4–154.e4. doi: 10.1016/j.chom.2018.06.007

Zhang, T., Yang, Y., Liang, Y., Jiao, X., and Zhao, C. (2018). Beneficial effect of intestinal fermentation of natural polysaccharides. *Nutrients* 10:1055. doi: 10.3390/nu10081055

Zúñiga, M., Monedero, V., and Yebra, M. J. (2018). Utilization of host-derived glycans by intestinal *Lactobacillus* and *Bifidobacterium* species. *Front. Microbiol.* 9:1917. doi: 10.3389/fmicb.2018.01917

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 November 2020
doi: 10.3389/fmicb.2020.544097

Dietary Supplementation of ε-Polylysine Beneficially Affects Ileal Microbiota Structure and Function in Ningxiang Pigs

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Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 May 2020*
Accepted: *28 October 2020*
Published: *16 November 2020*

Citation:
Zhang X, Hou Z, Xu B, Xie C, Wang Z, Yu X, Wu D, Yan X and Dai Q (2020) Dietary Supplementation of ε-Polylysine Beneficially Affects Ileal Microbiota Structure and Function in Ningxiang Pigs. Front. Microbiol. 11:544097. doi: 10.3389/fmicb.2020.544097

Intestinal microbiota plays an important role in the health of animals. However, little is known about the gut microbiota in Ningxiang pigs. Thus, we investigated how dietary supplementation with different ε-polylysine concentrations (0, 20, 40, 80, and 160 ppm) affected the ileal microbiota in Ningxiang pigs using a replicated 5 × 5 Latin square method. Each experimental period included 10 days for diet adaptation, 3 days for feces collection and 2 days for digesta collection. The ileal contents were collected and used for sequencing of the V3–V4 hypervariable region of the 16S rRNA gene. The results revealed that ε-polylysine significantly decreased the digestibility of crude protein and crude fiber, as well as the utilization of metabolizable energy ($P < 0.05$). The relative abundances of 19 bacterial genera significantly increased, while those of 26 genera significantly decreased ($P < 0.05$). In addition, ε-polylysine increased the abundance of some bacteria (e.g., *Faecalibacterium*, *Bifidobacterium*, and lactic acid bacteria) and inhibited some other bacteria (e.g., *Micrococcaceae*, *Acinetobacter*, *Anaerococcus*, *Peptoniphilus*, *Dehalobacterium*, *Finegoldia*, *Treponema*, and *Brevundimonas*). Furthermore, based on the 16S rRNA gene data and data from the precalculated GreenGenes database, bacterial communities in the ileal contents exhibited enhanced functional maturation, including changes in the metabolism of carbohydrates, amino acids (e.g., alanine, lysine, tryptophan, cysteine, and methionine), cofactors, and vitamins (e.g., biotin, thiamine, and folate), as well as in the activity of the insulin signaling pathway. This study suggests that ε-polylysine may influence the utilization of feed nutrients by Ningxiang pigs, including proteins, lipids, metabolizable energy, and fiber, by regulating the gut microbiota.

Keywords: ε-polylysine, Ningxiang pig, ileum, microbiota, nutrient digestibility, metabolism

INTRODUCTION

Intestinal microbiota is a key to many aspects of nutrition and health, including the immune system (Zhang et al., 2015; Hu et al., 2018), neurobehavioral traits (Chu et al., 2019), digestion, and metabolism (Sonnenburg and Sonnenburg, 2014; Rothschild et al., 2018), as microorganisms enable dietary fiber fermentation and affect energy metabolism (Valdes et al., 2018). An interactive

relationship exists between intestinal microbiota and nutrition, wherein intestinal microbiota affects the digestion/absorption of nutrients and diet/nutrition affects the diversity of the gut microbiota (Flint et al., 2012; Oriach et al., 2016; Valdes et al., 2018).

The nutritional food additive ε-polylysine achieves its antibacterial effects by increasing the permeability of the cell membrane. ε-Polylysine exhibits a broad spectrum of bacteriostatic properties and affects gram-positive bacteria, fungi, and some viruses. Studies in the field of food and nutrition have demonstrated that ε-polylysine exerts antimicrobial activity against *Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella* Typhimurium (Geornaras and Sofos, 2005; Geornaras et al., 2007; Chang et al., 2010; Zhou et al., 2011). Antibacterial tests indicated that the membrane fraction between 2 and 5 kDa exhibited the highest antibacterial activity compared with that of other fractions against test strains of *Staphylococcus aureus, Micrococcus luteus*, *Bacillus subtilis*, *E. coli*, and *Shigella* spp. (Jia et al., 2010). Furthermore, ε-polylysine is easily adsorbed by DNA owing to its negative charge, which makes ε-polylysine suitable for the treatment of liver diseases. Polymerized protein-carrying biological macromolecules of ε-polylysine can effectively treat hepatitis virus infection (Sospedra et al., 1999a,b). One study reported that when antimicrobial ε-polylysine was incorporated into food, it transiently altered the gut microbial communities, as well as their predicted functions, in mice, which indicates a dynamic, yet resilient microbiome that adapts to microbial-active dietary components (You et al., 2017). ε-Polylysine can decrease triacylglycerols by inhibiting pancreatic lipase activity (Kido et al., 2003; Tsujita et al., 2006). Furthermore, Hosomi et al. (2015) reported that hepatic acetyl-coenzyme A carboxylase and glucose-6-phosphate dehydrogenase, two key enzymes of fatty acid biosynthesis, were enhanced in rats that were fed a diet with ε-polylysine.

The present study was performed on the Ningxiang pig (also known as the Caochong or Liusha River pig), a famous native pig breeds in the Hunan province in China, which possesses unique hereditary properties, including a high reproduction rate and good adaptability (Xing et al., 2009). At present, research on ε-polylysine is mainly concentrated in the fields of food and medicine, and ε-polylysine is considered to play an important role as a nutritional antiseptic and antibacterial agent. However, its application in poultry and livestock nutrition has not yet been reported; in particular, its effects on animal gut microbiota remain to be explored. Thus, we aimed to assess whether dietary supplementation with ε-polylysine could regulate the digestion of nutrients and beneficially affect the microbiota structure and function in the ileum of Ningxiang pigs.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols were carried out with the approval of the Institute of Bast Fiber Crops of the Chinese Academy of Agricultural Sciences, Hunan province, China.

TABLE 1 | 5 × 5 Latin square design.

Pigs Periods	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5
Section 1	0 ppm	20 ppm	40 ppm	80 ppm	160 ppm
Section 2	20 ppm	40 ppm	80 ppm	160 ppm	0 ppm
Section 3	40 ppm	80 ppm	160 ppm	0 ppm	20 ppm
Section 4	80 ppm	160 ppm	0 ppm	20 ppm	40 ppm
Section 5	160 ppm	0 ppm	20 ppm	40 ppm	80 ppm

Animals and Sample Collection

Five adult Ningxiang pigs were surgically fitted, between the ileum and cecum, with a T-shaped cranial cannula of approximately 15 cm in length, made of polyvinylchloride plastisol, according to the procedures suggested by Sauer et al. (1977). After a 1-month convalescence period, the pigs were tested in separate stainless-steel metabolic cages. The intubation did not appear to affect the growth of the animals, as indicated by the weight gain and feeding efficiency. The trial was conducted as a replicated 5 × 5 Latin square design with pigs and feeding periods as blocking factors, which is shown in **Table 1**. Each experimental period lasted 15 days, including 10 days of diet adaptation and intake adjustment, 3 days of feces collection and 2 days for the collection of digesta from the ileum (12 h/day). All the pigs were fed a basal diet supplemented with 0, 20, 40, 80, or 160 ppm ε-polylysine. The composition of the basal diet is shown in **Table 2**. Feces were collected and weighed every day. Sulfuric acid (H_2SO_4) added was 10% of the weight of fresh feces to protect samples from decaying. Feces were collected and mixed well in a plastic bag, and 10% of the mixed feces was kept for analysis. The collected feces and feed samples were dried in a forced-air drying oven at 65°C and then ground to pass through a 40-mesh sieve for analysis. The ileal content samples were collected from the ileal fistula of the pigs. The pH of the ileal contents was measured using a pH meter (model S210 SevenCompact™; Mettler Toledo Instruments Co., Ltd., Shanghai, China) immediately after sampling. Afterward, all ileal content samples were stored at −80°C until further use.

Nutrient Digestibility Measurements

Samples of feeds and feces were analyzed for crude protein (CP; AOAC official method 990.03), crude fiber (CF; AOAC official method 978.10), and metabolizable energy (ME). CP was determined by the Kjeldahl method using an auto Kjeldahl system (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Höganäs, Sweden) after acid digestion. ME was determined using a calorimeter (5E-C5508; Kaiyuan, Changsha, China).

DNA Extraction

DNA was extracted from the samples using the E.Z.N.A.® stool DNA kit (D4015; Omega, Inc., United States) according to the manufacturer's instructions. DNA was quantified using an ND-2000C spectrophotometer (NanoDrop Technologies, United States), and its purity was confirmed by agarose gel electrophoresis. The DNA samples were stored at −80°C for polymerase chain reaction (PCR).

TABLE 2 | Compositions and nutrient levels of the experimental diets (air-dry basis%).

Items	Content (Basic diet)
Ingredient (%)	
Corn	39.50
Wheat bran	20.00
Soybean meal (43%)	19.50
Corn starch	8.50
Rice husk powder	5.00
Soybean oil	2.50
Stone powder	1.80
Premix[1]	3.20
Total	100.00
Nutrient levels (%)	
DE/(kcal/kg)[2]	3108.80
CP[3]	14.53
CF[3]	6.32
EE[3]	4.16
Ca[3]	0.73
Total P[3]	0.46
Non-phytate P[3]	0.17
Lys[3]	0.79
Met[3]	0.24
Thr[3]	0.56

[1]*The premix provided the following per kg of the diet: Fe 66 mg, Cu 6 mg, Zn 54 mg, Mn 15 mg, I 0.24 mg, Se 0.18 mg, VA 18 000 IU, VD 35 000 IU, VE 35 IU, VK 5 mg, VB_1 5 mg, VB_2 10 mg, VB_{12} 35 μg, nicotinic acid 40 mg, pantothenic acid 20 mg, folic acid 1.5 mg.*
[2]*Calculated values.*
[3]*Analyzed values.*

PCR Amplification and 16S rDNA Sequencing

The V3–V4 region of the bacterial 16S rRNA gene was amplified with slightly modified versions of the 338F forward (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R reverse (5′-GGACTACHVGGGTWTCTAAT-3′) primers (Fadrosh et al., 2014). The 5′ ends of the primers were tagged with specific barcode per sample and sequencing universal primers. The reaction mixture (25 μL) for PCR amplification contained 25 ng of template DNA, 12.5 μL of PCR premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume (Caporaso et al., 2011). The PCR cycling conditions were as follows: initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 54°C/52°C for 30 s, and elongation at 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR products were confirmed by 2% agarose gel electrophoresis. Ultrapure water was used as a negative control to exclude the possibility of false-positive results. The PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, United States) and quantified using Qubit (Invitrogen, United States). Amplicon pools were used for sequencing, and the size and number of amplicon libraries were evaluated using an Agilent 2100 bioanalyzer (Agilent, United States) and an Illumina library quantification kit (Kapa Biosciences, Woburn, MA, United States). PhiX control libraries (v3) (Illumina) were merged with amplicon libraries (expected at 30%).

Sequence Processing and Bioinformatics Analysis

Samples were sequenced on an Illumina MiSeq platform. Based on their unique barcodes, truncated paired-end reads were assigned to samples and merged using FLASH (Magoč and Salzberg, 2011). Quality filtering of the raw tags was performed to obtain high-quality clean tags using fqtrim (v0.94). The procedure used for filtering sequence reads was as follows: (1) barcodes and joint sequence were removed from reads; (2) paired-end reads were combined into a longer tag; (3) a window quality scan was performed on reads, with a default scan window of 100 bp; when the average quality value in the window was lower than 20, the part of the read from the beginning of the window to the 3′ end was cut off; (4) sequences less than 100 bp in length after truncation were removed; (5) sequences with more than 5% of Ns after truncation were removed; (6)Chimera sequences were filtered using the Vsearch (Rognes et al., 2016) software (v2.3.4). Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs) using Vsearch (v2.3.4). Subsequently, representative sequences for each OTU were chosen, and taxonomic data were assigned to each representative sequence using the Ribosomal Database Project classifier. To analyze the dominant species in different groups and to study phylogenetic relationships of different OTUs, multiple sequence alignment was conducted using the MAFFT software (v7.310). The abundance of each OTU was normalized relative to the sample with the fewest sequences. Alpha diversity was used to analyze the complexity of species in a sample by applying the Chao1, Shannon, and Simpson indices, which were calculated using QIIME (version 1.8.0). Beta diversity was used to evaluate the complexity of species among the samples and was calculated using principal coordinates analysis (PCoA) and cluster analysis in the QIIME software (version 1.8.0).

Functional Profile Analysis of Bacterial Communities Using PICRUSt

Based on the 16S rRNA gene data and the precalculated GreenGenes (v13.5) database (Langille et al., 2013), PICRUSt (v1.1.0) was used to predict the abundances of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs and KEGG pathways in the bacterial communities. Functional differences among the samples were compared using the STAMP software (Parks et al., 2014).

Statistical Analysis

The nutrient digestibility data were analyzed using the general linear model procedure in SAS (version 9.2; SAS Institute, Inc., Cary, NC, United States). Tukey's contrasts were used for *post hoc* comparisons of the means. $P < 0.01$ was considered highly significant, and $P < 0.05$ was considered significant. Statistical analyses of the 16S rRNA gene data were carried out using the GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, United States), R (v3.0.3), and STAMP software.

Statistical comparisons of weighted UniFrac distances among the groups were performed by analysis of similarities. One-way analysis of variance was used for the comparison of alpha diversity between groups. After statistical comparison of the taxa, we used the Benjamini–Hochberg correction to control the false discovery rate using the package "p.adjust" in R. The STAMP software with false discovery rate correction was applied to detect the differentially abundant KEGG pathways between groups. *P* (corrected) <0.05 was considered to indicate statistical significance. Statistical analysis of the taxonomy of the ileal contents among the five groups was conducted using wilcox.test in R. Differences were considered significant at $P < 0.05$. Heatmap diagrams and other plots were created in the R environment (v3.1.2). The relationship between intestinal microorganisms and the utilization of nutrients was calculated using the Pearson product correlation. Statistical and correlation analyses were performed using GraphPad Prism 8.0 (GraphPad Software).

RESULTS

Apparent Nutrient Digestibility and pH of the Ileal Contents in Ningxiang Pigs

The effects of dietary supplementation with ε-polylysine on nutrient digestibility are presented in **Table 3**. The average daily feed intake (ADFI) was not influenced by ε-polylysine ($P > 0.05$). However, significant differences were observed in nutrient digestibility. The CP digestibility and ME utilization were significantly higher in the control group than in the 40, 80, and 160 ppm ε-polylysine experimental groups ($P = 0.036$ and $P = 0.028$, respectively). The CF digestibility was significantly higher in the control group than in the 40 and 80 ppm ε-polylysine groups ($P = 0.040$). However, the pH values of the ileal contents exhibited no significant differences ($P > 0.05$).

Characterization of Sequencing Data and Ileal Microbiota in Different Groups

For the five pig groups, a total of 38,170, 36,354, 31,464, 41,236, and 37,242 raw sequences were obtained, of which 76.7, 79.5, 86.2, 83.4, and 78.6% valid sequences, respectively, remained after filtering out chimeras, removing low-quality sequences, and splitting each file in four. Among the high-quality sequences, approximately 58.9% were between 300 and 400 bp, 39.4% were between 400 and 500 bp, and the rest were shorter than 300 bp (**Supplementary Figure S1**). The Good's coverage for all samples was >99.5%, which indicated that the sequencing data were reliable (**Supplementary Figure S2**). Based on the 97% sequence similarity, the sequences of the V3–V4 region were assigned to a total of 3790 bacterial OTUs. The taxonomic analysis revealed a total of 17 bacterial phyla, 32 classes, 54 orders, 116 families, 344 genera, and 672 species.

Figure 1 shows specific bacterial indices (OTUs, Chao1, Shannon, and Simpson) and *P*-values for each group. Significantly higher OTU levels were observed in the control group ($P < 0.01$) and 20 and 80 ppm groups ($P < 0.05$) compared with 160 ppm group (**Figure 1A**). ε-Polylysine influenced the Chao1 index, which was higher ($P < 0.05$) in the control group than in the treatment groups (**Figure 1B**); however, no effects were observed on the Shannon and Simpson diversity indices ($P > 0.05$) (**Figures 1C,D**). The Venn diagrams displayed the unique and shared OTUs in the Ningxiang pigs. Among a total of 3970 OTUs, 37.5% (1490 core OTUs) were shared among the five groups. The numbers of unique OTUs in each group were 49, 25, 17, 12, and 14 (**Figure 1E**).

The microbial community structure of all samples was analyzed using the phylogeny-based Bray–Curtis method and visualized using PCoA (**Figure 2**). The first two factors (PC1 and PC2) accounted for 39.01 and 14.15% of the sample variation, respectively. These results demonstrated that the microbial communities from different groups were distinguishable from one another. The PCoA based on the bacterial OTUs showed that samples clustered together and indicated a shift in the gut bacterial community after ε-polylysine supplementation (**Figure 2A**). The distance between the experimental groups and control group was 0.33 ± 0.02 ($P > 0.05$) (**Figure 2B**).

Effects of ε-Polylysine on the Taxonomic Composition of Gut Bacteria

The ileal microbiotas in the experimental and control groups were examined using the non-parametric Wilcoxon rank-sum test to compare the mean relative abundances of predominant bacteria. Across the samples, 16 different phyla were identified, of which only five phyla had a relative abundance of >1%, namely, Firmicutes (82.35%), Bacteroidetes (7.20%), Proteobacteria (4.23%), Actinobacteria (3.06%), and Fusobacteria (2.00%). The data indicated that the microbial community structure of the ileal contents was similar among the groups at the phylum level. The abundance of Firmicutes significantly increased ($P < 0.05$) with an increase in dietary ε-polylysine. However, the relative abundances of two other phyla (Candidatus Saccharibacteria and Spirochetes) significantly decreased ($P < 0.05$) with an increase in ε-polylysine levels (**Supplementary Data 1**). The five most abundant phyla accounted for >98.84% of the total sequences in the samples, regardless of the amount of the ε-polylysine supplement. Furthermore, the phyla Proteobacteria, Actinobacteria, and Bacteroidetes exhibited no significant differences ($P > 0.05$). However, certain genera and species within these phyla exhibited significant differences among the groups ($P < 0.05$) (**Figure 3**).

The taxonomic compositions of the gut bacterial communities were further investigated in the Ningxiang pigs, and a total of 344 genera were identified. Abundant genera were defined as those containing >1% of the total group sequences. The following 23 abundant genera were identified: *Lactobacillus* (9.93%), *Roseburia* (8.19%), *Romboutsia* (6.34%), *Turicibacter* (4.84%), *Clostridium* _XVIII (4.69%), *Lachnospiraceae*_unclassified (4.44%), *Ruminococcaceae*_unclassified (2.89%), *Blautia* (2.69%), *Clostridium_sensu_stricto* (2.56%), *Terrisporobacter* (2.45%), *Clostridiaceae*_1_unclassified (2.29%), *Eubacterium* (2.00%), *Streptococcus* (2.15%), *Fusobacterium* (1.96%), *Coprococcus* (1.81%), *Oscillibacter* (1.81%), *Clostridium*_XIVa

TABLE 3 | Effects of dietary ε-polylysine supplemental level on nutrition digestibility (%) of Ningxiang pigs.

Items	Treatment group					Statistics	
	Control	20 ppm	40 ppm	80 ppm	160 ppm	SEM	*P*-value
ADFI/kg	2.58	2.41	2.46	2.45	2.49	0.052	0.062
CP digestibility/%	89.93^{a}	89.23^{ab}	87.55^{b}	85.08^{b}	87.79^{b}	0.541	0.036
CF digestibility/%	56.66^{a}	47.03^{ab}	37.51^{b}	43.24^{b}	47.29^{ab}	2.020	0.040
ME utilization/%	86.72^{a}	84.82^{ab}	81.41^{b}	80.68^{b}	82.09^{b}	0.739	0.028
pH of ileum contents	7.52	7.66	7.40	7.49	7.18	0.072	0.359

Data are expressed as MEAN with STDEV; Means with different letters within a column differ ($P < 0.05$); n = 5 per treatment. Treatment Control group, 0 ppm ε-polylysine; Experiment groups: 20 ppm, 40 ppm, 80 ppm, 160 ppm ε-polylysine.
ADFI, average daily feed intake; CP, crude protein; CF, crude fiber; ME, metabolizable energy.

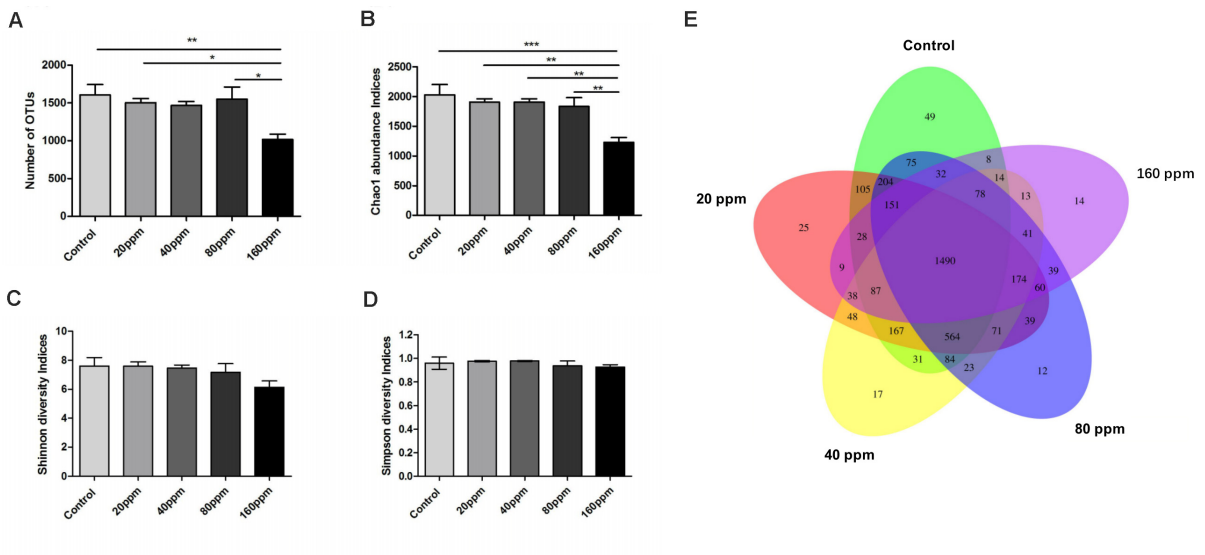

FIGURE 1 | Variations in alpha diversity of the ileum chyme bacteria in Ningxiang pigs. The OTUs level among groups **(A)**. **(B)** Bacterial alpha diversity determined by Chao1 index. **(C)** Bacterial alpha diversity determined by Shannon index. **(D)** Bacterial alpha diversity determined by Simpson index. Each group is represented in a different color. Venn diagrams **(E)** show the numbers of unique and shared OTUs among all groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (with FDR adjust).

(1.75%), *Bacteroides* (1.56%), *Veillonellaceae*_unclassified (1.27%), *Psychrobacter* (1.01%), *Anaerorhabdus* (1.07%), *Prevotella* (1.08%), and *Phascolarctobacterium* (1.01%). The genus *Lactobacillus* (Firmicutes) was the most abundant in the gut bacterial communities (**Supplementary Data 1**). Many genera from Firmicutes exhibited an increasing trend ($P < 0.05$), including *Vagococcus*, *Roseburia*, *Enterococcus*, *Facklamia*, *Lactobacillus*, *Faecalibacterium*, unclassified *Anaerostipes*, *Lachnobacterium*, *Coprococcus*, *Anaerovibrio*, *Clostridium_sensu_stricto*, *Faecalicoccus*, and *Staphylococcus*, while many genera others exhibited a decreasing trend ($P < 0.05$), including *Sporosarcina*, *Oscillibacter*, *Peptoniphilus*, *Anaerobium*, unclassified *Sporobacter*, *Amphibacillus*, *Faecalicatena*, and *Butyrivibrio* (**Figure 3** and **Supplementary Data 2**). Among Proteobacteria, the abundances of the genera *Psychrobacter* and *Proteus* significantly increased ($P < 0.05$), and those of *Acinetobacter*, *Pseudochrobactrum*, and *Enterobacteriaceae*_unclassified significantly decreased ($P < 0.05$). Among Actinobacteria, *Atopobium* exhibited an increasing trend ($P < 0.05$), whereas *Leucobacter* and *Corynebacterium* exhibited a decreasing trend ($P < 0.05$). Furthermore, ε-polylysine significantly increased ($P < 0.05$) *Empedobacter* (Bacteroidetes), *Saccharibacteria_genera_incertae_sedis* (Candidatus_Saccharibacteria), and *Treponema* (Spirochetes), whereas *Bacteroides*, *Butyricimonas*, *Petrimonas*, and *Alistipes* significantly decreased ($P < 0.05$).

We further examined the taxonomic compositions of the ileal microbiotas in the Ningxiang pigs at the species level. In total, 672 significant species were identified. The 20 most abundant species included *Lactobacillus amylovorus*, *Romboutsia*, *Turicibacter* sp., *Clostridium*_XVIII, *Lachnospiraceae*, *Ruminococcaceae*, *Roseburia* sp., *Clostridium_sensu_stricto*, *Terrisporobacter*, *Clostridiaceae*_1, *Roseburia*, *Roseburia* sp. 831b, *Eubacterium* sp., *Clostridium*_XlVa, *Coprococcus* sp., *Oscillibacter*, *Fusobacterium* sp., *Blautia*_sp., *Veillonellaceae*, and *Anaerorhabdus* sp. Dietary ε-polylysine significantly increased or decreased ($P < 0.05$) the abundances of several species (**Figure 3** and

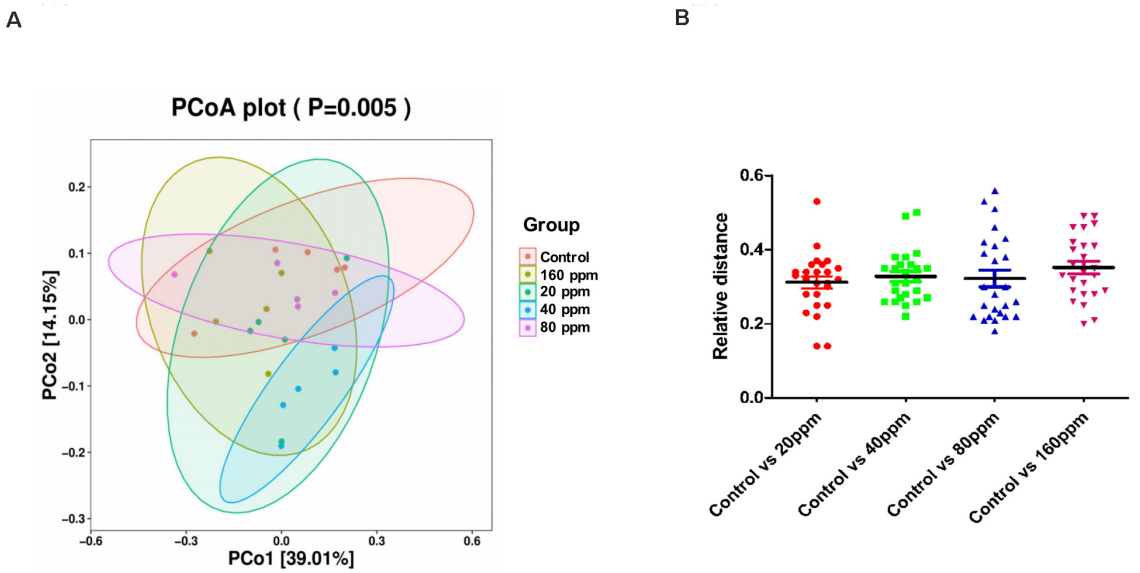

FIGURE 2 | Beta diversity of the ileum chyme bacteria in Ningxiang pigs. PCoA was performed at the operational taxonomic unit (OTU) level based on Bray–Curtis metrics for all samples **(A)** and relative distance of four experimental groups, respectively, compared to the control group **(B)**.

Supplementary Data 3, 4). The relative abundances (>0.05%) of 16 species, including *Roseburia* sp. 831b, *Roseburia faecis*, *Facklamia* sp. 2320B-03, and *Coprococcus* sp. increased, whereas those of 11 species, including *Acinetobacter* sp. WX-19, *Bacteroides salyersiae*, and *Bacteroides caccae* decreased. Most of these species belong to the phylum Firmicutes (**Supplementary Data 4**).

Prediction of Ileal Microbiota Function in Ningxiang Pigs

The PICRUSt analysis, which was used to investigate the functional profiles of the microbiota, suggested distinct nutrient source utilization patterns in the ileum, depending on the bacterial composition (**Figure 4**). Dietary ε-polylysine supplementation resulted in a significant increase in metabolic activity ($P < 0.05$), as the abundances of genes involved in carbohydrate, amino acid, and fatty acid metabolism increased. The bacterial community exhibited significant increases ($P < 0.05$) in the relative abundances of genes for carbohydrate digestion and absorption, carbon fixation pathways in prokaryotes, tricarboxylic acid cycle, pentose and glucuronate interconversions, and starch, sucrose, and pyruvate metabolism. Furthermore, the relative abundances of genes involved in the metabolism of alanine, aspartate, glutamate, tryptophan, tyrosine, cysteine, methionine, D-glutamine, and D-glutamate, as well as in the degradation of lysine, valine, leucine, and isoleucine and in the biosynthesis of phenylalanine, tyrosine, and tryptophan, were also predicted to increase. The relative abundances of genes involved in the biosynthesis and metabolism of glycan varied. Dietary ε-polylysine significantly increased ($P < 0.05$) the proportions of genes for polyketide sugar unit biosynthesis, glycosphingolipid biosynthesis–globo series, and other glycan degradation significantly decreased ($P < 0.05$) the proportions of genes for *N*-glycan and lipopolysaccharide biosynthesis. Furthermore, fatty acid biosynthesis and metabolism were affected by the increased proportions of genes for glycerophospholipid metabolism and primary and secondary bile acid biosynthesis, as well as by the decreased proportions of genes for alpha-linolenic acid metabolism. Additionally, the bacterial community exhibited significantly increased relative abundances of genes for biotin, thiamine, and retinol metabolism, folate biosynthesis, and the folate-dependent one-carbon pool (**Figure 4**), which are all important for maintaining normal physiological functions in animals. The results also showed increases in the proportions of genes involved in molecular processes essential for cell functions and maintenance, such as DNA repair and recombination proteins, DNA replication, DNA replication proteins, ribosomes, translation factors, protein export, protein folding, and associated processing, whereas basal transcription factors were decreased. Finally, the bacterial community exhibited increased proportions of genes for the insulin signaling pathway (**Supplementary Data 5**).

Correlation Between Intestinal Microorganisms and Utilization of Nutrients

At the phylum level, the abundances of Firmicutes and Candidatus Saccharibacteria were positively correlated with CP digestibility ($P < 0.05$), while that of Proteobacteria was negatively correlated with CP digestibility ($P < 0.05$). The abundance of Candidatus Saccharibacteria was positively

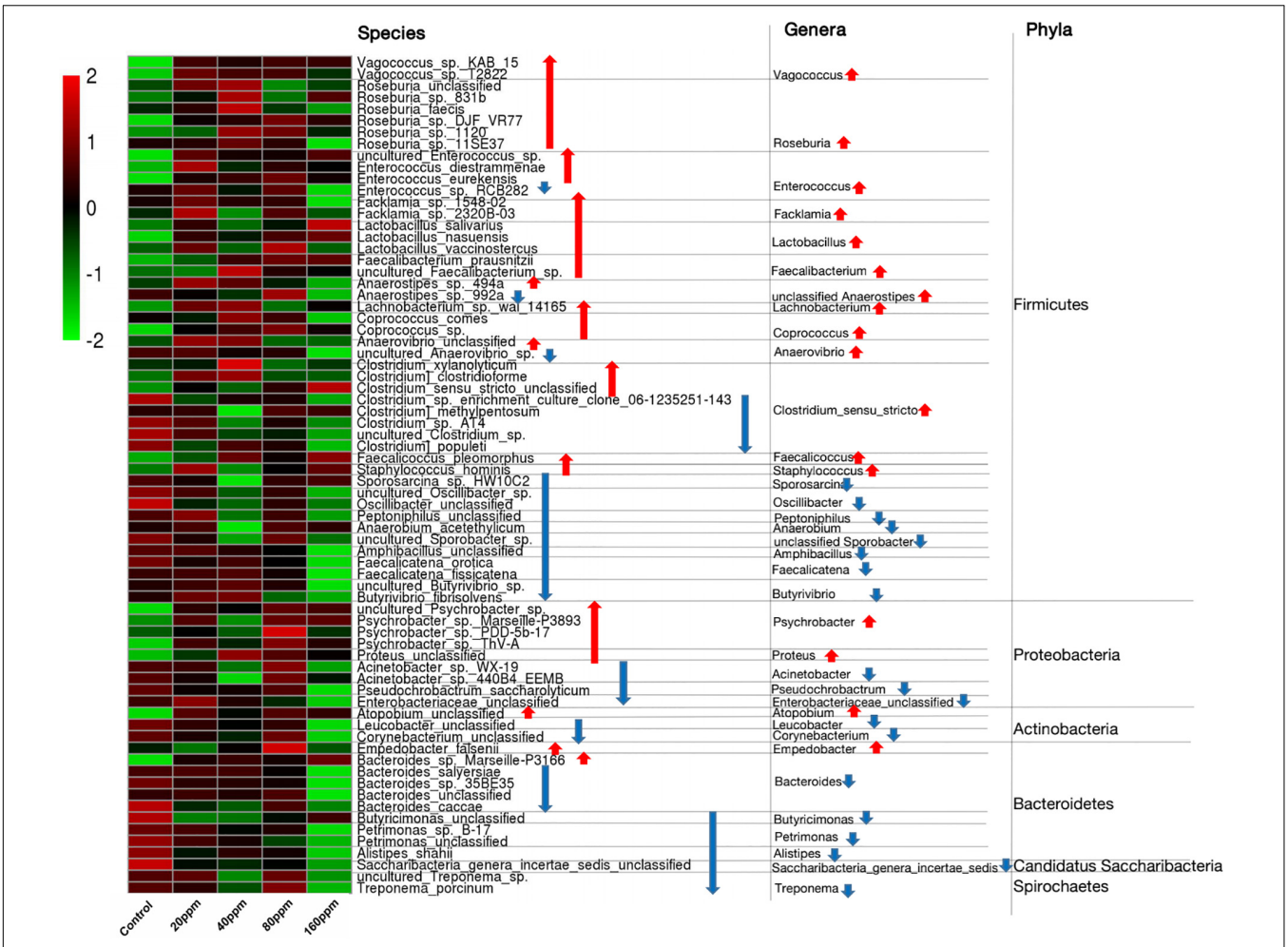

FIGURE 3 | Shifts in the ileum bacterial compositions. Heat map and hierarchical clustering of differentially abundant gut bacterial in piglets with different ε-polylysine supplement level (0, 20, 40, 80, 160 ppm). The values of color in the heat map represent the normalized relative abundances of species (Log 10). Detailed data for heat map were shown in the **Supplementary Data 4**. Statistics were conducted by ANOVA with Tukey-Kramer test and Benjamini-Hochberg correction among five groups (n = 5), and the P-value lower than 0.05. Detailed data for the heat map were shown in the **Supplementary Data 4**.

correlated with the efficiency of ME utilization and CF digestibility ($P < 0.01$). At the genus level, the abundances of *Guggenheimella* and *Saccharibacteria_genera_incertae_sedis* were positively correlated with CP digestibility ($P < 0.05$). However, the abundances of *Phascolarctobacterium*, *Coprococcus*, *Faecalicoccus*, *Pseudoflavonifractor*, *Fretibacterium*, *Ignatzschineria*, and *Bittarella* were negatively correlated with CP digestibility ($P < 0.05$). The abundances of *Guggenheimella*, *Saccharibacteria_incertae_sedis*, *Leucobacter*, *Petrimonas*, *Jeotgalicoccus*, and *Halomonas* were positively correlated with the efficiency of ME utilization and CF digestibility ($P < 0.05$), but those of *Phascolarctobacterium*, *Coprococcus*, *Faecalicoccus*, *Pseudoflavonifractor*, *Fretibacterium*, and *Faecalibacterium* were negatively correlated with the efficiency of ME utilization and CF digestibility ($P < 0.05$). In addition, the abundances of *Blautia* and *Ignatzschineria* were negatively correlated with the efficiency of ME utilization ($P < 0.05$) (**Table 4**).

DISCUSSION

To date, no study has investigated the effects of ε-polylysine on digestibility in pigs. However, previous studies investigated the effects of dietary lysine on digestibly and found that lysine deficiency or excess could influence nutrient digestibility in pigs (Kim et al., 2011; Zeng et al., 2013; Elsbernd et al., 2017), which can affect nitrogen retention and whole-body protein turnover (Roy et al., 2000; Yin et al., 2018). In the current study, ε-polylysine showed some effects on CP and CF digestibility and ME utilization in Ningxiang pigs. A previous study showed that changes in the abundances of bacterial genera were correlated with apparent CF digestibility and the abundance of *Clostridium* was associated with dietary fiber metabolism (Niu et al., 2015). Our findings were also similar to those of previous studies, wherein dietary supplementation with amino acids was reported to mediate the gut microbiota composition and diversity, which may further affect the host metabolism and

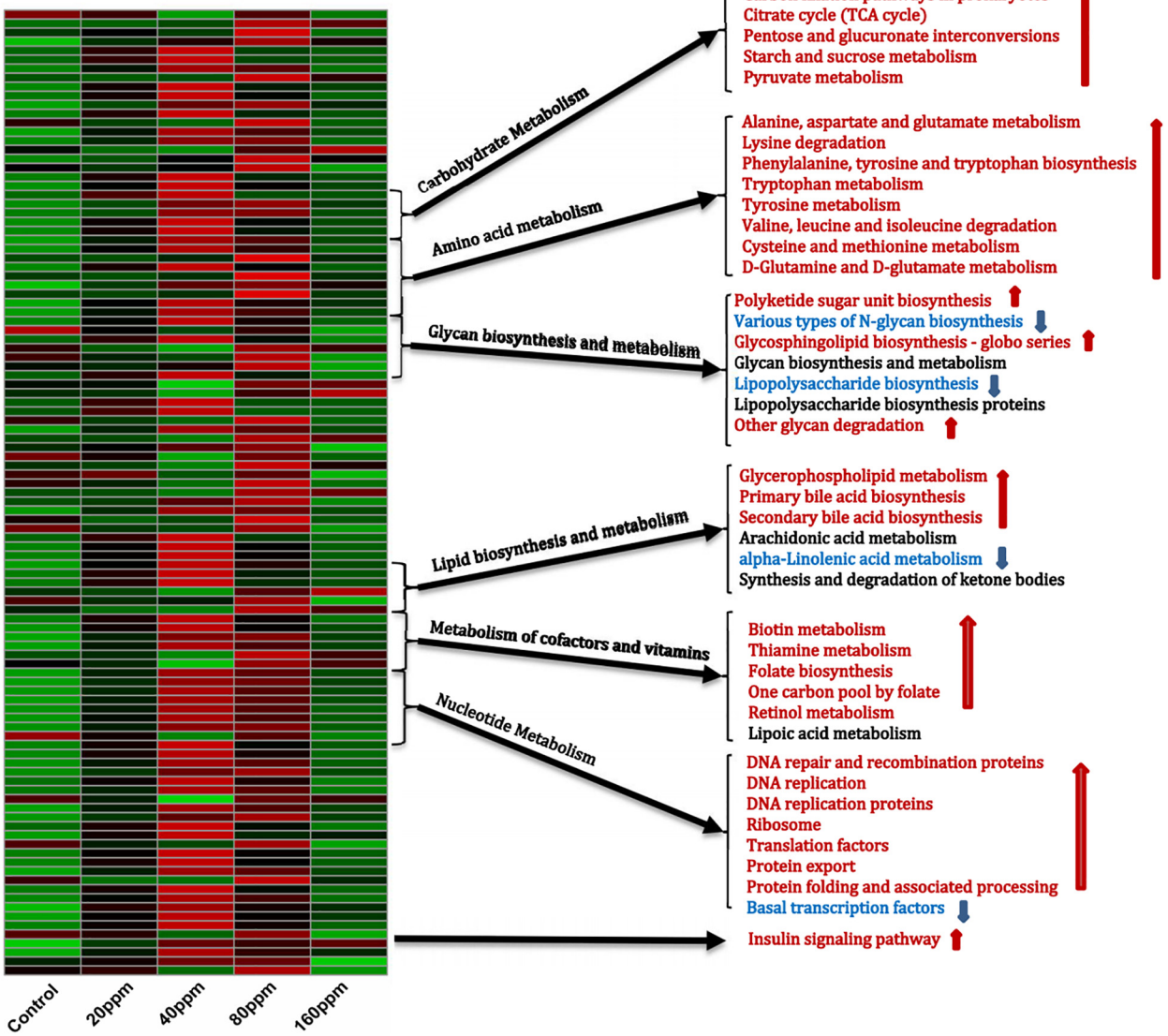

FIGURE 4 | Shifts in ileum bacterial functional profiles as ε-polylysine supplement level. Heat map and hierarchical clustering of different abundant KEGG pathways identified at different ε-polylysine supplement level (0, 20, 40, 80, 160 ppm). The values of color in the heat map represent the normalized relative abundance of KEGG pathways (Log 10). Detailed data for the heat map were shown in the **Supplementary Data 5**.

health (Ren et al., 2014; Bin et al., 2017; Ji et al., 2018). Jumpertz et al. (2011) showed that nutrients could change the gut (fecal) bacterial community structure over a short period of time, and the observed associations between gut microbes and nutrient absorption indicated a possible role of the human gut microbiota in the regulation of the nutrient balance.

Therefore, we investigated the alterations in the ileal microbiota of Ningxiang pigs that were fed a diet supplemented with different concentrations of ε-polylysine. Our results demonstrated alterations in the gut microbiota composition at the phylum, genus, and species levels. Consistent with the data of previous studies (Chen et al., 2018; Wang et al., 2019), Firmicutes, Bacteroidetes, and Proteobacteria were the three most dominant phyla in the gut microbiota of pigs, of which the most abundant phylum was Firmicutes, followed by Bacteroidetes and Proteobacteria. Our findings were also similar to those of Lee et al. (2018), who reported that the abundance of Spirochetes in pigs was decreased by the supplementation of plant extracts. Wang et al. (2018) demonstrated that diabetic cognitive dysfunction in mice was associated with increased production of bile acids in the liver and activation of bile acid signaling in the intestine. Moreover, the bacterial community composition was altered in the cecum of these mice and was characterized by a marked increase in the population of Candidatus Saccharibacteria. Because the abundance of Candidatus Saccharibacteria was decreased by ε-polylysine in

TABLE 4 | Intestinal microorganisms correlated to the utilization of nutrients and Pearson's correlation between gut microbiota and utilization of nutrients.

Items	CP digestibility Pearson's correlation	ME utilization Pearson's correlation	CF digestibility Pearson's correlation
Phyla			
Firmicutes	0.4262*	0.1842	0.1644
Candidatus Saccharibacteria	0.4682*	0.5200**	0.5086**
Proteobacteria	−0.5106**	−0.1535	−0.0079
Genera			
Guggenheimella	0.3980**	0.5172*	0.5702**
Saccharibacteria_ genera_ incertae_ sedis	0.4682*	0.5200**	0.5086**
Leucobacter	0.2566	0.4848*	0.5342**
Petrimonas	0.3872	0.4870*	0.5156**
Jeotgalicoccus	0.1413	0.4939*	0.5454**
Halomonas	0.2341	0.4048*	0.5140**
Phascolarctobacterium	−0.7221**	−0.6130**	0.5143**
Coprococcus	−0.4568*	−0.5385**	−0.4939*
Faecalicoccus	−0.4316*	−0.4588*	−0.4660*
Pseudoflavonifractor	−0.5376**	−0.4557*	−0.4585*
Fretibacterium	−0.4468*	−0.5038*	−0.4687*
Faecalibacterium	−0.3221	−0.4007*	−0.4867*
Ignatzschineria	−0.7358**	−0.5266**	−0.3427
Bittarella	−0.5637**	−0.4677*	−0.3113

**The correlation is significant at a level of 0.05; **the correlation is significant at a level of 0.01.*

the present study, its action may be associated with the suppression of bile secretion and primary and secondary bile acid biosynthesis. However, this association should be elucidated in further studies.

At the genus level, *Lactobacillus*, including *L. salivarius*, *L. nasuensis*, and *L. vaccinostercus*, has been demonstrated to be essential for improving the intestinal microbial balance (Zhang et al., 2019). In addition, *L. salivarius* has probiotic properties; it activates a broad range of cytokines and chemokines and elicits immunomodulatory activity by enhancing innate and acquired immune responses (Perez-Cano et al., 2010; Zhang et al., 2011; Larsen et al., 2013). *Roseburia*, which is a core genus in representative populations of the world, along with *Faecalibacterium*, *Eubacterium*, *Clostridium*, *Blautia*, and *Ruminococcus* (Dehingia et al., 2015), was the second most predominant genus in this study. Five species of *Roseburia*, including *R. faecis*, *Roseburia* sp. 831b, *Roseburia* sp. DJF VR77, *Roseburia* sp. 1120, and *Roseburia* sp. 11SE37, were increased by ε-polylysine. Gut *Roseburia* spp. metabolized dietary components, which stimulated their proliferation and metabolic activities (**Supplementary Data 3, 4**). Tamanai-Shacoori et al. (2017) reported that the genus *Roseburia* included commensal bacteria that produce short-chain fatty acids (SCFAs), especially butyrate, which affects colonic motility, immunity maintenance, and anti-inflammatory properties. In this study, the increase in the *Roseburia* abundance by ε-polylysine may have induced SCFA production and improved gut immunity. Furthermore, PICRUSt analysis suggested that ε-polylysine exerted strong effects on carbohydrate metabolism, glycan biosynthesis, and other metabolic functions, which may be related to the presence of bacteria of the genera *Vagococcus*, *Enterococcus*, *Facklamia*, *Rothia*, *Lachnobacterium*, *Proteus*, *Coprococcus*, *Atopobium*, *Anaerovibrio*, *Anaerorhabdus*, and *Staphylococcus*. Butyrate, a fermentation product of these bacteria can decompose glycan and carbohydrates, and also affects the biosynthesis and metabolism of fatty acids (Leonel and Alvarez-Leite, 2012). However, ε-polylysine could also exhibit inhibitory effects on the biosynthesis and metabolism of carbohydrates, glycan, and fatty acids by inducing changes in the abundances of some other genera (*Butyricimonas*, *Leucobacter*, *Atopostipes*, *Amphibacillus*, *Alloiococcus*, *Alistipes*, and *Butyrivibrio*). Furthermore, ε-polylysine increased the relative abundances of *Faecalibacterium* and *Clostridium_sensu_stricto*, which are beneficial bacteria that can inhibit pathogens. *Faecalibacterium* spp. can promote the development and proliferation of probiotics, such as *Bifidobacterium* spp. and lactic acid bacteria (Duncan et al., 2002; Yu et al., 2019). In addition, ε-polylysine inhibited some pathogenic bacteria (*Micrococcaceae* unclassified, *Acinetobacter*, *Anaerococcus*, *Peptoniphilus*, *Dehalobacterium*, *Finegoldia*, *Treponema*, and *Brevundimonas*). Moreover, Krizova et al. (2002) investigated enzymes of the butyrate pathway and fermentation patterns and reported that *Coprococcus* sp. from the human gut, which produces high levels of butyric acid *in vitro* and is a net producer of acetate, had detectable butyrate kinase, acetate kinase, and butyryl-CoA:acetate-CoA transferase activities. Butyric acid is an important SCFA, and Oh et al. (2019) demonstrated that SCFAs can activate the AMPK/PPAR pathway directly or by activating adipose tissue and can ultimately regulate fatty acid oxidation. Thus, *Coprococcus* sp. could be associated with fatty acid biosynthesis and metabolism in the present study.

Consistent with the data of a previous study on gut microbiome in pigs (Yang and Liao, 2019), the results of our study suggested an increase in the metabolism of carbohydrates, amino acids, cofactors, and vitamins. Furthermore, significant increases in almost all KEGG pathways were associated with nucleotide metabolism, except for basal transcription factors. A remarkable increase in the activity of the insulin signaling pathway was also predicted. Regarding fatty acid biosynthesis and metabolism, the predicted changes included an increase in the glycerophospholipid metabolism and biosynthesis of primary and secondary bile acids, as well as a decrease in the alpha-linolenic acid metabolism. Primary bile acids are produced by the liver to dissolve dietary lipids and fat-soluble vitamins in the small intestine. The primary bile acid pool mainly circulates back to the liver; however, a small portion of the bile acid pool (approximately 5%) enters the large intestine and is further metabolized into secondary bile acids by intestinal microorganisms (Chiang, 2009). The two main bile acid receptors that regulate the host metabolism are G-protein-coupled bile acid receptor 1 and the farnesol X receptor, which, together with bile acids and the intestinal microbiome, regulate the synthesis, metabolism, and distribution of bile acids *in vivo*

(Molinaro et al., 2018). Jia et al. (2018) reported that bile acids played an important role in the lipid balance, carbohydrate metabolism, insulin sensitivity, and innate immunity. In addition, ε-polylysine exerted complex effects on glycan biosynthesis and metabolism, thereby regulating the body energy balance via changes in the gut microbiota in Ningxiang pigs.

This study revealed changes in nutrient digestibility caused by different ε-polylysine levels and the relationships between the abundance and diversity of the gut microbiota in Ningxiang pigs. Jumpertz et al. (2011) indicated a possible role of the gut microbiota in the regulation of nutrient absorption in humans. In a previous study (Ashida et al., 2012), the gut microbiota was shown to degrade dietary fiber, and the abundances of bacteria belonging to the genera *Guggenheimella*, *Saccharibacteria incertae sedis*, *Leucobacter*, *Petrimonas*, *Jeotgalicoccus*, and *Halomonas* were positively correlated with CF digestibility. Jha and Berrocoso (2015) reported that dietary fiber negatively affected energy and nutrient digestibility, which may indicate a balance between CP and CF digestibility. The relative abundances of nine genera, namely, *Guggenheimella*, *Saccharibacteria incertae sedis*, *Phascolarctobacterium*, *Coprococcus*, *Faecalicoccus*, *Pseudoflavonifractor*, *Fretibacterium*, *Ignatzschineria*, and *Bittarella*, were correlated with CP digestibility. The gut microbiota plays a key role in controlling the energy balance via energy expenditure and storage (Ridaura et al., 2013). Foditsch et al. (2014) suggested that *Faecalibacterium prausnitzii* was related to the energy-harvesting capacity of the intestinal microbiota, which is consistent with our results. Therefore, the relationship between nutrient (CP, ME, and CF) digestibility and the gut microbiota should be the focus of further study.

In conclusion, considering that there were no significant differences in ADFI, the reduced nutrient digestibility led to an increased excretion of nutrients and increased metabolism of carbohydrates, amino acids, fatty acids, and glycan, which may signify a reduced nutritional requirement for Ningxiang pigs. We determined the structure and function of the microbiota in the ileal contents of Ningxiang pigs, which were fed dietary ε-polylysine, and predicted that ε-polylysine could enhance the level of nutrients, including carbohydrates, vitamins, and glycan, thus providing health benefits to pigs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

XZ, DW, QD, and XYan designed the research. XZ and XYu conducted the research. XZ, BX, CX, and ZW analyzed the data. XZ and DW wrote the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by the Agricultural Science and Technology Innovation project of Chinese Academy of Agricultural Sciences (ASTIP-IBFC).

ACKNOWLEDGMENTS

We thank all present and past members of Wu laboratory and Yan laboratory who have contributed comments and ideas.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.544097/full#supplementary-material

Supplementary Figure 1 | The length distribution of sequences.

Supplementary Figure 2 | Bacterial rarefaction curves based on good_coverage was used to assess the depth of coverage for each sample. Each sample was distinguished by different colors of lines.

REFERENCES

Ashida, H., Ogawa, M., Kim, M., Mimuro, H., and Sasakawa, C. (2012). Bacteria and host interactions in the gut epithelial barrier. *Nat. Chem. Biol.* 8, 36–45. doi: 10.1038/nchembio.741

Bin, P., Liu, S., Chen, S., Zeng, Z., Huang, R., Yin, Y., et al. (2017). The effect of aspartate supplementation on the microbial composition and innate immunity on mice. *Amino Acids* 49, 2045–2051. doi: 10.1007/s00726-017-2467-5

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108(Suppl. 1), 4516–4522. doi: 10.1073/pnas.1000080107

Chang, S. S., Lu, W. Y. W., Park, S. H., and Kang, D. H. (2010). Control of foodborne pathogens on ready-to-eat roast beef slurry by ε-polylysine. *Int. J. Food Microbiol.* 141, 236–241. doi: 10.1016/j.ijfoodmicro.2010.05.021

Chen, J., Kang, B., Jiang, Q., Han, M., Zhao, Y., Long, L., et al. (2018). Alpha-ketoglutarate in low-protein diets for growing pigs: effects on cecal microbial communities and parameters of microbial metabolism. *Front. Microbiol.* 9:1057. doi: 10.3389/fmicb.2018.01057

Chiang, J. Y. (2009). Bile acids: regulation of synthesis. *J. Lipid Res.* 50, 1955–1966. doi: 10.1194/jlr.R900010-JLR200

Chu, C., Murdock, M. H., Jing, D., Won, T. H., Chung, H., Kressel, A. M., et al. (2019). The microbiota regulate neuronal function and fear extinction learning. *Nature* 574, 543–548. doi: 10.1038/s41586-019-1644-y

Dehingia, M., Talukdar, N. C., Talukdar, R., Reddy, N., Mande, S. S., Deka, M., et al. (2015). Gut bacterial diversity of the tribes of India and comparison with the worldwide data. *Sci. Rep.* 5:18563. doi: 10.1038/srep18563

Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., and Flint, H. J. (2002). Acetate utilization and butyryl coenzyme A (CoA): acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl. Environ. Microbiol.* 68, 5186–5190. doi: 10.1128/AEM.68.10.5186-5190.2002

Elsbernd, A. J., De Lange, C. F. M., Stalder, K. J., Karriker, L. A., and Patience, J. F. (2017). SID lysine requirement of immunologically and physically castrated male pigs during the grower, early and late finisher periods. *J. Anim. Sci.* 95, 1253–1263. doi: 10.2527/jas2016.0544

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., et al. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2:6. doi: 10.1186/2049-2618-2-6

Flint, H. J., Scott, K. P., Louis, P., and Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nat. Rev. Gastroenterol. Hepatol.* 9, 577–589. doi: 10.1038/nrgastro.2012.156

Foditsch, C., Santos, T. M., Teixeira, A. G., Pereira, R. V., Dias, J. M., Gaeta, N., et al. (2014). Isolation and characterization of *Faecalibacterium prausnitzii* from calves and piglets. *PLoS One* 9:e116465. doi: 10.1371/journal.pone.0116465

Geornaras, I., and Sofos, J. N. (2005). Activity of ε–polylysine against *Escherichia coli* O157: H7, *Salmonella* Typhimurium, and *Listeria monocytogenes*. *J. Food Sci.* 70, M404–M408. doi: 10.1111/j.1365-2621.2005.tb08325.x

Geornaras, I., Yoon, Y., Belk, K. E., Smith, G. C., and Sofos, J. N. (2007). Antimicrobial activity of ϵ-polylysine against *Escherichia coli* O157: H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in various food extracts. *J. Food Sci.* 72, M330–M334. doi: 10.1111/j.1750-3841.2007.00510.x

Horwitz, W. (2005). "AOAC official method 978.10. fiber (crude) in animal feed," *Official Methods of Analysis of AOAC International. 18th ed. AOAC International*. Gaithersburg: MD.

Horwitz, W., Latimer, G. W. (2005). "AOAC official method 990.03. protein (crude) in animal feed, combustion method," *Official Methods of Analysis of AOAC International. 18th ed. AOAC International* (Gaithersburg: MD), 30–31.

Hosomi, R., Yamamoto, D., Otsuka, R., Nishiyama, T., Yoshida, M., and Fukunaga, K. (2015). Dietary ϵ-Polylysine decreased serum and liver lipid contents by enhancing fecal lipid excretion irrespective of increased hepatic fatty acid biosynthesis-related enzymes activities in rats. *Prevent. Nutr. Food Sci.* 20:43. doi: 10.3746/pnf.2015.20.1.43

Hu, J., Ma, L., Nie, Y., Chen, J., Zheng, W., Wang, X., et al. (2018). A microbiota-derived bacteriocin targets the host to confer diarrhea resistance in early-weaned piglets. *Cell Host Microbe* 24, 817–832. doi: 10.1016/j.chom.2018.11.006

Jha, R., and Berrocoso, J. D. (2015). Dietary fiber utilization and its effects on physiological functions and gut health of swine. *Animal* 9, 1441–1452. doi: 10.1017/S1751731115000919

Ji, Y., Guo, Q., Yin, Y., Blachier, F., and Kong, X. (2018). Dietary proline supplementation alters colonic luminal microbiota and bacterial metabolite composition between days 45 and 70 of pregnancy in Huanjiang mini-pigs. *J. Anim. Sci. Biotechnol.* 9:18. doi: 10.1186/s40104-018-0233-5

Jia, S., Fan, B., Dai, Y., Wang, G., Peng, P., and Jia, Y. (2010). Fractionation and characterization of ϵ-poly-l-lysine from *Streptomyces albulus* CGMCC 1986. *Food Sci. Biotechnol.* 19, 361–366. doi: 10.1007/s10068-010-0051-9

Jia, W., Xie, G., and Jia, W. (2018). Bile acid–microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. *Nat. Rev. Gastroenterol. Hepatol.* 15, 111–128. doi: 10.1038/nrgastro.2017.119

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., et al. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *Am. J. Clin. Nutr.* 94, 58–65. doi: 10.3945/ajcn.110.010132

Kido, Y., Hiramoto, S., Murao, M., Horio, Y., Miyazaki, T., Kodama, T., et al. (2003). ε-Polylysine inhibits pancreatic lipase activity and suppresses postprandial hypertriacylglyceridemia in rats. *J. Nutr.* 133, 1887–1891. doi: 10.1093/jn/133.6.1887

Kim, Y. W., Ingale, S. L., Kim, J. S., Kim, K. H., and Chae, B. J. (2011). Effects of dietary lysine and energy levels on growth performance and apparent total tract digestibility of nutrients in weanling pigs. *Asian Austral. J. Anim. Sci.* 24, 1256–1267. doi: 10.5713/ajas.2011.11134

Krizova, L., Maixnerova, M., Sedo, O., and Nemec, A. (2002). International journal of systematic and evolutionary microbiology. *Soc. Gen. Microbiol.* 52, 2141–2146. doi: 10.1099/ijs.0.02241-0

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Larsen, N., Vogensen, F. K., Gøbel, R. J., Michaelsen, K. F., Forssten, S. D., Lahtinen, S. J., et al. (2013). Effect of *Lactobacillus salivarius* Ls-33 on fecal microbiota in obese adolescents. *Clin. Nutr.* 32, 935–940. doi: 10.1016/j.clnu.2013.02.007

Lee, S., Guevarra, R., Lee, J., Marimuthu, V., Kim, D., Kim, S., et al. (2018). PSX-35 Effects of dietary plant extracts on growth performance and intestinal microbiota composition in weaned piglets. *J. Anim. Sci.* 96(Suppl. 3), 489. doi: 10.1093/jas/sky404.1068

Leonel, A. J., and Alvarez-Leite, J. I. (2012). Butyrate: implications for intestinal function. *Curr. Opin. Clin. Nutr. Metab. Care* 15, 474–479. doi: 10.1097/MCO.0b013e32835665fa

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Molinaro, A., Wahlström, A., and Marschall, H. U. (2018). Role of bile acids in metabolic control. *Trends Endocrinol. Metab.* 29, 31–41. doi: 10.1016/j.tem.2017.11.002

Niu, Q., Li, P., Hao, S., Zhang, Y., Kim, S. W., Li, H., et al. (2015). Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Sci. Rep.* 5:9938. doi: 10.1038/srep09938

Oh, H. Y. P., Visvalingam, V., and Wahli, W. (2019). The PPAR–microbiota–metabolic organ trilogy to fine-tune physiology. *FASEB J.* 33, 9706–9730. doi: 10.1096/fj.201802681RR

Oriach, C. S., Robertson, R. C., Stanton, C., Cryan, J. F., and Dinan, T. G. (2016). Food for thought: the role of nutrition in the microbiota-gut–brain axis. *Clin. Nutr. Exp.* 6, 25–38. doi: 10.1016/j.yclnex.2016.01.003

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi: 10.1093/bioinformatics/btu494

Perez-Cano, F. J., Dong, H., and Yaqoob, P. (2010). *In vitro* immunomodulatory activity of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: two probiotic strains isolated from human breast milk. *Immunobiology* 215, 996–1004. doi: 10.1016/j.imbio.2010.01.004

Ren, W., Chen, S., Yin, J., Duan, J., Li, T., Liu, G., et al. (2014). Dietary arginine supplementation of mice alters the microbial population and activates intestinal innate immunity. *J. Nutr.* 144, 988–995. doi: 10.3945/jn.114.192120

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341:1241214. doi: 10.1126/science.1241214

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., et al. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555, 210–215. doi: 10.1038/nature25973

Roy, N., Lapierre, H., and Bernier, J. F. (2000). Whole-body protein metabolism and plasma profiles of amino acids and hormones in growing barrows fed diets adequate or deficient in lysine. *Can. J. Anim. Sci.* 80, 585–595. doi: 10.4141/A98-057

Sauer, W. C., Stothers, S. C., and Phillips, G. D. (1977). Apparent availabilities of amino acids in corn, wheat and barley for growing pigs. *Can. J. Anim. Sci.* 57, 585–597. doi: 10.4141/cjas77-075

Sonnenburg, E. D., and Sonnenburg, J. L. (2014). Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. *Cell Metab.* 20, 779–786. doi: 10.1016/j.cmet.2014.07.003

Sospedra, P., Haro, I., Alsina, M. A., Reig, F., and Mestres, C. (1999a). Physicochemical interaction of a lipophilic derivative of HAV antigen VP3 (110–121) with lipid monolayers. *Mater. Sci. Eng. C* 8, 543–549. doi: 10.1016/S0928-4931(99)00055-7

Sospedra, P., Nagy, I. B., Haro, I., Mestres, C., Hudecz, F., and Reig, F. (1999b). Interaction of polylysine-[HAV-VP3 peptide] constructs with DPPC mono

and bilayers. *Biomed. Chromatogr.* 13, 159–160. doi: 10.1002/(SICI)1099-0801(199904)13:2<159::AID-BMC846>3.0.CO;2-H

Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S. B., et al. (2017). *Roseburia* spp.: a marker of health? *Future Microbiol.* 12, 157–170. doi: 10.2217/fmb-2016-0130

Tsujita, T., Takaichi, H., Takaku, T., Aoyama, S., and Hiraki, J. (2006). Antiobesity action of ε-polylysine, a potent inhibitor of pancreatic lipase. *J. Lipid Res.* 47, 1852–1858. doi: 10.1194/jlr.M600168-JLR200

Valdes, A. M., Walter, J., Segal, E., and Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ* 361:k2179. doi: 10.1136/bmj.k2179

Wang, J., Han, Y., Zhao, J. Z., Zhou, Z. J., and Fan, H. (2019). Pyrosequencing-based analysis of the complex microbiota located in the gastrointestinal tracts of growing-finishing pigs. *Anim. Product. Sci.* 59, 870–878. doi: 10.1071/AN16799

Wang, X., Wang, F., Zhang, Y., Xiong, H., Zhang, Y., Zhuang, P., et al. (2018). Diabetic cognitive dysfunction is associated with increased bile acids in liver and activation of bile acid signaling in intestine. *Toxicol. Lett.* 287, 10–22. doi: 10.1016/j.toxlet.2018.01.006

Xing, X. W., Hawthorne, W. J., Yi, S., Simond, D. M., Dong, Q., Ye, B., et al. (2009). Investigation of porcine endogenous retrovirus in the conservation population of Ningxiang pig. *Transpl. Proc.* 41, 4389–4393. doi: 10.1016/j.transproceed.2009.09.051

Yang, Z., and Liao, S. F. (2019). Physiological effects of dietary amino acids on gut health and functions of swine. *Front. Vet. Sci.* 6:169. doi: 10.3389/fvets.2019.00169

Yin, J., Li, Y., Han, H., Liu, Z., Zeng, X., Li, T., et al. (2018). Long-term effects of lysine concentration on growth performance, intestinal microbiome, and metabolic profiles in a pig model. *Food Funct.* 9, 4153–4163. doi: 10.1039/C8FO00973B

You, X., Einson, J. E., Lopez-Pena, C. L., Song, M., Xiao, H., McClements, D. J., et al. (2017). Food-grade cationic antimicrobial ε-polylysine transiently alters the gut microbial community and predicted metagenome function in CD-1 mice. *NPJ Sci. Food* 1, 1–10. doi: 10.1038/s41538-017-0006-0

Yu, D., Zhu, X., Feng, J., Kang, C., Zhang, M., Zhu, W., et al. (2019). Effect of low-protein diet on hormones associated with growth and on gut microbiota in weaned piglets. *Acta Microbiol. Sin.* 58, 1695–1704. doi: 10.13343/j.cnki.wsxb.20190107

Zeng, P. L., Yan, H. C., Wang, X. Q., Zhang, C. M., Zhu, C., Shu, G., et al. (2013). Effects of dietary lysine levels on apparent nutrient digestibility and serum amino acid absorption mode in growing pigs. *Asian Austral. J. Anim. Sci.* 26:1003. doi: 10.5713/ajas.2012.12555

Zhang, D., Liu, H., Wang, S., Zhang, W., Wang, J., Tian, H., et al. (2019). Fecal microbiota and its correlation with fatty acids and free amino acids metabolism in piglets after a *Lactobacillus* strain oral administration. *Front. Microbiol.* 10:785. doi: 10.3389/fmicb.2019.00785

Zhang, H., Sparks, J. B., Karyala, S. V., Settlage, R., and Luo, X. M. (2015). Host adaptive immunity alters gut microbiota. *ISME J.* 9, 770–781. doi: 10.1038/ismej.2014.165

Zhang, J., Deng, J., Wang, Z., Che, C., Li, Y. F., and Yang, Q. (2011). Modulatory effects of *Lactobacillus salivarius* on intestinal mucosal immunity of piglets. *Curr. Microbiol.* 62, 1623–1631. doi: 10.1007/s00284-011-9906-4

Zhou, C., Li, P., Qi, X., Sharif, A. R. M., Poon, Y. F., Cao, Y., et al. (2011). A photopolymerized antimicrobial hydrogel coating derived from epsilon-poly-L-lysine. *Biomaterials* 32, 2704–2712. doi: 10.1016/j.biomaterials.2010.12.040

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer XK declared a shared affiliation with several of the authors, XZ, XYu, ZH, DW, and QD, to the handling editor at the time of review.

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METHODS
published: 16 November 2020
doi: 10.3389/fmicb.2020.581571

Identification of Genetic Variants *via* Bacterial Respiration Gas Analysis

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Indole is a signal molecule derived from the conversion of tryptophan, and it is present in bacterial respiratory gas. Besides influencing bacterial growth, indole exhibits effects on human health, including a positive effect on inflammation and protection against pathogens. However, a high fecal indole concentration (FIC) can suggest an unbalanced gut flora or the presence of certain pathogens. To analyze the indole produced by bacteria, its collection and detection is required. Traditional methods usually require centrifugation of liquid bacterial culture medium and subsequent extraction of indole from the medium or partial purification of indole from fecal samples (e.g., by distillation or extraction). In this study, we demonstrate the possibility of identifying gas contents directly from bacteria, and we distinguish the difference in species and their genetics without the need to centrifuge or extract. Using an absorbent sheet placed above a liquid culture, we were able to collect gas content directly from bacteria. Gas chromatography-mass spectrometry (GC-MS) was used for the analysis. The GC-MS results showed a clear peak attributed to indole for wild-type *Escherichia coli* cells (MG1655 and MC4100 strains), whereas the indole peak was absent in the chromatograms of cells where proteins, part of the indole production pathway from tryptophan (TnaA and TnaB), were not expressed (by using *tnaAB*-deleted cells). The indole observed was measured to be present in a low nmol-range. This method can distinguish whether the bacterial genome contains the *tnaAB* gene or not and can be used to collect gas compounds from bacterial cultures quickly and easily. This method is useful for other goals and future research, such as for measurements in restrooms, for food-handling facilities, and for various applications in medical settings.

Keywords: bacteria, gas, indole, gas chromatography-mass spectrometry, *tnaAB*, *Escherichia coli*

OPEN ACCESS

Edited by:
Sonia G. Sáyago-Ayerdi, Instituto Tecnológico de Tepic, Mexico

Reviewed by:
Aaron F. Gonzalez Cordova, Consejo Nacional de Ciencia y Tecnología (CONACYT), Mexico
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Specialty section:
This article was submitted to Microbiotechnology, a section of the journal Frontiers in Microbiology

Received: *13 July 2020*
Accepted: *27 October 2020*
Published: *16 November 2020*

Citation:
Koga N, Hosomi T, Zwama M, Jirayupat C, Yanagida T, Nishino K and Yamasaki S (2020) Identification of Genetic Variants via Bacterial Respiration Gas Analysis. Front. Microbiol. 11:581571. doi: 10.3389/fmicb.2020.581571

INTRODUCTION

Human gastrointestinal tract bacteria can survive in oxygen-deprived (anaerobic) conditions. These microbes produce signal substances, which not only regulate bacterial growth, but also influence human health by affecting biological functions (Berstad et al., 2015). Indole is the main metabolite produced by enteric bacteria from tryptophan, a quorum-sensing compound (Kim and Park, 2013) and exhibits a major influence on host metabolism (Chimerel et al., 2014). We previously reported the contribution of multidrug efflux pumps expressed in bacteria to the removal of indole-derivative compounds under anaerobic conditions (Hirakawa et al., 2004; Zhang et al., 2011). Indole compounds are released into the natural environment of

anaerobic bacteria in the human gut, as a component of their respiratory gas. Indole regulates various bacterial functions, including drug resistance, virulence, and biofilm formation (Lee et al., 2009; Lee and Lee, 2010; Nikaido et al., 2012). As indole is believed to exhibit a significant influence on host metabolism, it directly impacts human health. Sonowal et al. (2017) studied the effects of indole in geriatric worms, flies, and mice, in which indole extended health spans, suggesting that indole helps to retain a "young gene expression profile" in animals. Additionally, indole produced by intestinal bacteria was found to relieve inflammation of the liver in mice (Beaumont, 2018).

Some of the health effects of indole and indole derivatives were linked to the activation of the aryl hydrocarbon receptor by acting as agonists (Hubbard et al., 2015; Puccetti et al., 2018), an action which is also directly linked to protection against pathogenic bacteria (Romani et al., 2014). The effect of microorganisms on human health has been of increased interest to researchers (Cani, 2019). However, as well as its beneficial properties, indole also was suggested to be potentially harmful to the human body. A high concentration of the indole derivative, indoxyl sulfate, was related to chronic kidney disease and vascular disease in humans (Zhang and Davies, 2016).

As indole is therefore involved in human health, developments in its collection and detection methods are important. Examples of procedures used to measure indole produced by bacteria include steam distillation (Happold and Hoyle, 1934) and gas chromatography-mass spectrometry (GC-MS; Jensen and Jensen, 1994). These methods usually require the following steps: centrifugation of the liquid culture medium with cultured bacteria, and extraction of indole from the medium using an organic solvent, such as hexane or chloroform (more details regarding different techniques are mentioned in the Discussion section). These steps are very time-consuming, and, in particular, the extraction step often requires high volumes of reagent and is easily influenced by the experimenter. In addition, the identification of bacteria is usually based on biochemical reactions, such as in the analytical profile index (API), or genetic screening, which checks RNA- or DNA-specific sequences. Currently, no practical methods that obtain bacterial profiles directly from samples and measure them easily and quickly exist. Therefore, in the present study, we aimed to develop a single-step method to identify genetic features based on bacterial respiratory gas. We demonstrate that indole gas, gathered directly from samples, can be collected easily, without centrifuging the samples or extracting indole from the medium. We were able to clearly distinguish the genomic traits of the different bacterial strains used. If we can distinguish the species of bacteria and their genetics based on the compounds that the bacteria produce, the method can be applied to various settings, including the medical field and food hygiene management.

MATERIALS AND METHODS

Strains and Conditions

The *Escherichia coli* strains used in this study were MG1655 and MC4100 (reference NKE104 and NKE259, respectively; **Table 1**). They were prepared from stocks of MG1655 (Blattner et al., 1997) or MC4100 (Casadaban, 1976). The gene-deletion strains used in this study are NKE256 (MG1655Δ*tnaAB*) and NKE258 (MC4100Δ*tnaAB*; Hirakawa et al., 2004). MG1655Δ*tnaAB* was created by the method described by Link et al. (1997) with primers *tnaAB*-No, *tnaAB*-Ni, *tnaAB*-Ci, and *tnaAB*-Co (**Table 2**). With 10% skim milk for long-term storage, the bacterial strains were stored at −80°C. The strains were cultured in Luria-Bertani (LB) broth (BD Difco) at 37°C overnight. The primers used to create the *tnaAB*-deletion strains and their sequences are provided in **Table 2**.

Collection of Gas Ingredients From Live Microbes

The procedure overview can be seen in **Figure 1**. Each strain was cultured in 4 ml LB broth at 37°C overnight. The following day, stainless steel cups (Eco-Cup SF, Frontier Laboratories Ltd., Japan) were attached to a wire (Eco-Stick DF, Frontier Laboratories Ltd., Japan) equipped with a thermal desorption adsorbent (MonoTrap RGPS TD, GL Sciences B.V., The Netherlands). The wire was pierced through the covers of the bottles (septa, blue PTFE/white silicone, Supelco). The amber glass vials (screw-top vial, Supelco) contained 1 cm of bacterial liquid culture (3 ml volume). The adsorbent was placed 2 cm above the culture, and it was immediately cultured at 37°C for 1 h, without shaking. The vapor pressure of indole is high even at room temperature (0.016 hPa at 25°C); therefore, the effect of temperature disturbance is considered to be quite small. After collecting the gas ingredients, the adsorbents were put into 1.5 ml anti-static tubes and stored at 4°C until further use. As a negative control, the same protocol was performed in LB media without bacteria. Each experiment was repeated at least three times.

TABLE 1 | Strains and plasmids used in this study.

Strains	Description	Source or reference
***Escherichia coli* strains**		
NKE104	MG1655	Blattner et al., 1997
NKE259	MC4100	Casadaban, 1976
NKE256	MG1655Δ*tnaAB*	Hirakawa, unpublished
NKE258	MC4100Δ*tnaAB*	Hirakawa et al., 2004

*Wild-type E. coli strains MG1655 and MC4100 were used to create the tnaAB knock-out mutants (explained in Methods and **Table 2**).*

TABLE 2 | Primers and sequences used in this study.

Primers	Sequence (5'–3')
tnaAB-No	cgcggatcctttctccagcttctgtattgg
tnaAB-Ni	cacgcaataaccttcacactccaaatttataaccatttatttttaattacagtgatccctg
tnaAB-Ci	gttataaatttggagtgtgaaggttattgcgtgtaaatccttcaagaagccagccattcg
tnaAB-Co	cgcgtcgacgacagcactttagcccgacg

Primers used for the gene-deletion of tnaAB in MG1655 and MC4100 E. coli cells. Primer names in accordance with the method description by Link et al. (1997).

GC-MS Analysis of Bacterial Respiratory Gas

Before GC-MS analyses, the gas-adsorbed adsorbents prepared in the section of "Collection of gas ingredients from live microbes" were transferred to a liner (diameter: 4.5 mm and length: 80 mm) with tweezers. The liner was immediately set to an inlet port of a gas chromatography-mass spectrometer (GCMS-QP2020A, Shimazu Corporation, Japan). The inlet port temperature was controlled by an inlet-temperature control system (OPTIC-4 inlet, GL Sciences B.V., The Netherlands). First, the inlet temperature was kept at 35°C. For the gas-desorption from the adsorbent, the injection port was heated to 250°C with a rate of 5°C/s, and kept at this temperature until the end of the measurements. The desorbed gas was flowed into a capillary column (SLB-IL60 capillary GC column, Supelco) and detected by the MS detector (**Figure 1**). The column oven temperature was first kept at 40°C for 6 min, followed by heating to 280°C with a rate of 5°C/min, and then kept at the temperature for 5 min. The amount of desorbed indole was estimated by comparing the peak area of indole with that of a calibration standard.

RESULTS

To detect and check for indole in gaseous state, we used wild-type *E. coli* strains and strains lacking the *tnaAB* genes (see Materials and Methods), which encode tryptophan-specific transporter TnaB and tryptophanase TnaA (which converts tryptophan into indole and pyruvate). In addition, considering the difference in pedigrees, we performed the experiment for two *E. coli* K12 strains, MC4100, and MG1655.

Figure 2 shows the GC-MS chromatogram results for the biogas collected from each of the four strains: MC4100, MG1655, and their *tnaAB*-deletion counterparts. Two sharp peaks at early retention times were observed in the all chromatograms, and they were attributed to ethanol (EtOH; from the vaporized EtOH used for disinfection) and trimethylsilanol (TMS-OH; from the adsorbent). For both wild-type strains, a clear, sharp peak appeared at 37.6 min retention time (**Figures 2A,B**). Based on the mass fragment pattern using mass spectrometry, this peak was attributed to indole. In the chromatograms of both MC4100 and MG1655, the indole peak showed a significantly higher intensity compared to any other bacterial volatile compound. The indole peak was completely absent in the chromatograms for the *tnaAB*-deletion strains (**Figures 2C,D**). This sharp contrast can be directly explained by the lack of indole transporter and indole production enzymes in the *tnaAB*-deleted strains. Repeated experiments proved that this difference was statically significant (**Supplementary Figure S1**). A box plot of the different measurements is shown in **Figure 3**. The amount of indole could also determine quantitatively by using a standard curve, and was 1.5 and 1.4 nmol for MC4100 and MG1655 respectively, while 0 nmol for the knock-out cells (**Supplementary Table S1**).

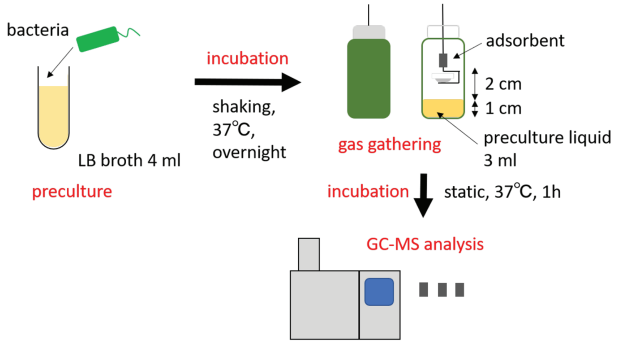

FIGURE 1 | Overview of the indole analysis method applied in this study. Bacteria cultured overnight were transferred to a vial, where the bacterial gas was absorbed above the culture. The absorbent was then analyzed by gas chromatography-mass spectrometry (GC-MS).

DISCUSSION

During the past century, various methods were employed to measure the indole produced by bacteria (Bergman, 1917). For example, Happold and Hoyle (1934) extracted indole from bacterial culture media using multiple distillation steps. Jensen and Jensen (1994) measured indole present in bacterial culture media by GC-MS. These methods are still frequently used today. However, in order to prepare the measurement samples, these methods require an additional step to extract indole from the bacterial media. Other methods were also developed. Darkoh et al. (2015) describe a method to quantify the amount of indole in biological samples using the hydroxylamine-based indole assay, which can detect indole specifically (but not indole analogs). While this assay is rapid and precise in quantitation, it lacks the ability to assess indole from bacterial gas and also requires extraction from the samples.

In this study, we gathered indole gas directly from live microbes, from the atmosphere above a liquid bacterial medium and then analyzed it using GC-MS. **Figure 2** shows that the wild-type K12 *E. coli* strains (MC4100 and MG1655) produced a sharp, clear indole peak, while the *tnaAB*-deleted strains did not produce this peak. The results illustrated in **Figure 3** show that the indole produced by the wild-type strains and caught by the absorbent was in ample abundance for detection, and it produced a significantly higher total amount compared to the *tnaAB*-deleted strains. The expectation exists that a *tnaAB*-deletion strain does not produce indole (Kobayashi et al., 2006), and these strains were therefore used as a negative control to verify the indole peak present in the wild-type strains' chromatograms. Based on these results, we demonstrated that, with this quick and easy method, detecting indole and obtaining a clear and sharp peak in the chromatograms of samples from indole-producing bacteria is possible. The MonoTrap system is in essence miniaturized solid-phase microextraction (SPME), which we chose for its relatively fast analytical properties and compared to other SPME methods, and does not require solvents which may pollute the samples, and can if needed be used

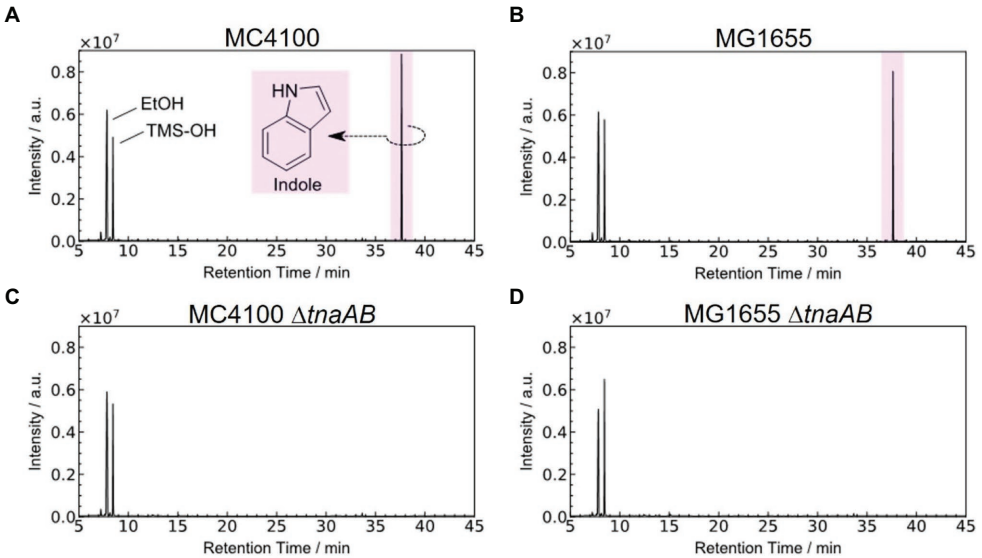

FIGURE 2 | Typical GC-MS chromatograms of the gas released from different *Escherichia coli* strains. Chromatograms of the gas present in the absorbent for all four strains, **(A)** MC4100, **(B)** MG1655, **(C)** MC4100Δ*tnaAB*, and **(D)** MG1655Δ*tnaAB*. The detected organic species were identified from their mass/charge ratio. Trimethylsilanol is considered to be derived from the adsorbent material. Each experiment was repeated at least three times. EtOH, ethanol; TMS-OH, trimethylsilanol. Data shown is one of the results. Repeats gave similar results (**Supplementary Figure S1**). Indole was obtained and measured in a low nmol-range (**Supplementary Table S1**).

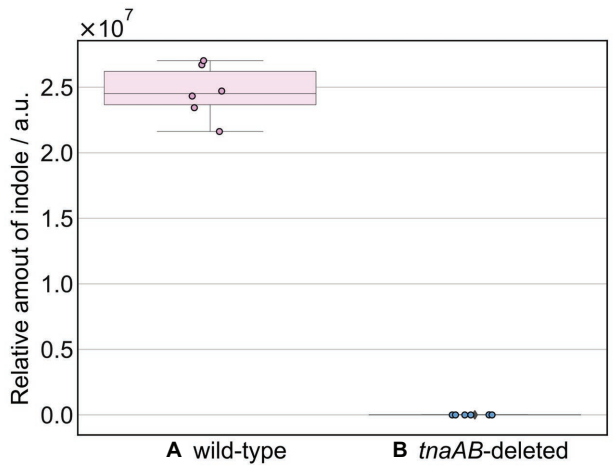

FIGURE 3 | Box plot of the relative amounts of indole. The amount of indole produced by **(A)** wild-type strains (MC4100 and MG1655) and **(B)** *tnaAB*-deleted strains (MC1655Δ*tnaAB* and MG1655Δ*tnaAB*). The amount of indole was calculated from the peak area at 37.6 min retention time in the GC-MS spectrum. The error bars represent the 95% confidence boundaries of the measured values. GC-MS analysis of each strain was repeated at least three times.

with both chemical and thermal desorption. In addition, the MonoTrap allowed for a small sample size to be analyzed, due to its high surface area (Dugheri et al., 2020).

As the collection of indole in this method is easy and fast, it can be used to analyze a large number of samples and obtain time-series data. The method could also potentially be automated. As mentioned earlier, indole is a metabolite produced by enteric bacteria. If indications of fecal indole concentrations (FICs) can be rapidly determined from gaseous states, they could be used as markers to detect pathogenic microorganisms or an unhealthy microflora. Zhu et al. (2010) measured the volatile organic components (VOCs) that different species of bacteria produced by second electrospray ionization mass spectrometry (SESI-MS), and they identified bacteria by looking for distinct patterns in VOC detection. Additionally, recently, researchers suggested that FICs can act as biomarkers to indicate bacterial infections (Chappell et al., 2016). A previous study (Darkoh et al., 2015) suggested a specific range of FICs that are valid for the majority of unhealthy individuals. Indole detection and quantification devices could be used as sensors in restrooms, in food-handling facilities, or in medical settings, where rapid identification of bacterial species is a major advantage. Future research is needed to verify the quantification of indole present in bacterial respiratory gas and its correlation to individuals' health and gut flora. We believe our quick indole collection method could be used in the future development of detection devices and therefore contribute to the improvement of human health.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

NK, KN, and SY performed the molecular biological and biochemical experiments. TH, CJ, and TY performed GC-MS analysis. NK, KN, MZ, and SY wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Takeda Science Foundation, the Center of Innovation Program (COI) from the Japan Science and Technology Agency (JST), Grants-in-Aid, Network Joint Research Center for Materials and Devices, and Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), Grant-in-Aid for Scientific Research (B; Kakenhi 17H03983), Challenging Research (Exploratory; 18K19451) and Grand-in-aid for Early-Career Scientists (18K14902, 20K16242) from the Japan Society for the Promotion of Science (JSPS).

ACKNOWLEDGMENTS

We would like to thank Dr. Rustam Aminov for his helpful advice on the manuscript. We also thank the Research Program for CORE lab of Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials in Network Joint Research Center for Materials and Devices.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.581571/full#supplementary-material

REFERENCES

Beaumont, M. (2018). The gut microbiota metabolite indole alleviates liver inflammation in mice. *FASEB J.* 32, 6681–6693. doi: 10.1096/fj.201800544

Bergman, O. (1917). The determination of fecal indole. *J. Biol. Chem.* 32, 17–22.

Berstad, A., Raa, J., and Valeur, J. (2015). Indole-the scent of a healthy 'inner soil'. *Microb. Ecol. Health Dis.* 26:27997. doi: 10.3402/mehd.v26.27997

Blattner, F. R., Plunkett, G. 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., et al. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* 277, 1453–1474. doi: 10.1126/science.277.5331.1453

Cani, P. D. (2019). Microbiota and metabolites in metabolic diseases. *Nat. Rev. Endocrinol.* 15, 69–70. doi: 10.1038/s41574-018-0143-9

Casadaban, M. J. (1976). Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J. Mol. Biol.* 104, 541–555. doi: 10.1016/0022-2836(76)90119-4

Chappell, C. L., Darkoh, C., Shimmin, L., Farhana, N., Kim, D., Okhuysen, P. C., et al. (2016). Fecal indole as a biomarker of susceptibility to cryptosporidium infection. *Infect. Immun.* 84, 2299–2306. doi: 10.1128/IAI.00336-16

Chimerel, C., Emery, E., Summers, D. K., Keyser, U., Gribble, F. M., and Reimann, F. (2014). Bacterial metabolite indole modulates incretin secretion from intestinal enteroendocrine L cells. *Cell Rep.* 9, 1202–1208. doi: 10.1016/j.celrep.2014.10.032

Darkoh, C., Chappell, C., Gonzales, C., and Okhuysen, P. (2015). A rapid and specific method for the detection of indole in complex biological samples. *Appl. Environ. Microbiol.* 81, 8093–8097. doi: 10.1128/AEM.02787-15

Dugheri, S., Mucci, N., Bonari, A., Marrubini, G., Cappelli, G., Ubiali, D., et al. (2020). Solid phase microextraction techniques used for gas chromatography: a review. *Acta Chromatogr.* 32, 1–9. doi: 10.1556/1326.2018.00579

Happold, F. C., and Hoyle, L. (1934). The quantitative determination of indole in bacterial cultures. *Biochem. J.* 28, 1171–1173. doi: 10.1042/bj0281171

Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T., and Yamaguchi, A. (2004). Indole induces the expression of multidrug exporter genes in *Escherichia coli*. *Mol. Microbiol.* 55, 1113–1126. doi: 10.1111/j.1365-2958.2004.04449.x

Hubbard, T. D., Murray, I. A., Bisson, W. H., Lahoti, T. S., Gowda, K., Amin, S. G., et al. (2015). Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles. *Sci. Rep.* 5:12689. doi: 10.1038/srep12689

Jensen, M. T., and Jensen, B. B. (1994). Gas chromatographic determination of indole and 3-methylindole (skatole) in bacterial culture media, intestinal contents and faeces. *J. Chromatogr. B Biomed. Appl.* 655, 275–280. doi: 10.1016/0378-4347(94)00065-4

Kim, J., and Park, W. (2013). Indole inhibits bacterial quorum sensing signal transmission by interfering with quorum sensing regulator folding. *Microbiology* 159, 2616–2625. doi: 10.1099/mic.0.070615-0

Kobayashi, A., Hirakawa, H., Hirata, T., Nishino, K., and Yamaguchi, A. (2006). Growth phase-dependent expression of drug exporters in *Escherichia coli* and its contribution to drug tolerance. *J. Bacteriol.* 188, 5693–5703. doi: 10.1128/JB.00217-06

Lee, J., Attila, C., Cirillo, S. L., Cirillo, J. D., and Wood, T. K. (2009). Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence. *Microb. Biotechnol.* 2, 75–90. doi: 10.1111/j.1751-7915.2008.00061.x

Lee, J., and Lee, J. (2010). Indole as an intercellular signal in microbial communities. *FEMS Microbiol. Rev.* 34, 426–444. doi: 10.1111/j.1574-6976.2009.00204.x

Link, A. J., Phillips, D., and Church, G. M. (1997). Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol.* 179, 6228–6237. doi: 10.1128/jb.179.20.6228-6237.1997

Nikaido, E., Giraud, E., Baucheron, S., Yamasaki, S., Wiedemann, A., Okamoto, K., et al. (2012). Effects of indole on drug resistance and virulence of *Salmonella enterica* serovar typhimurium revealed by genome-wide analyses. *Gut Pathog.* 4, 5–17. doi: 10.1186/1757-4749-4-5

Puccetti, M., Paolicelli, G., Oikonomou, V., De Luca, A., Renga, G., Borghi, M., et al. (2018). Towards targeting the aryl hydrocarbon receptor in cystic fibrosis. *Mediat. Inflamm.* 2018, 1–7. doi: 10.1155/2018/1601486

Romani, L., Zelante, T., De Luca, A., Iannitti, R. G., Moretti, S., Bartoli, A., et al. (2014). Microbiota control of a tryptophan-AhR pathway in disease tolerance to fungi. *Eur. J. Immunol.* 44, 3192–3200. doi: 10.1002/eji.201344406

Sonowal, R., Swimm, A., Sahoo, A., Luo, L., Matsunaga, Y., Wu, Z., et al. (2017). Indoles from commensal bacteria extend healthspan. *Proc. Natl. Acad. Sci. U. S. A.* 114, E7506–E7515. doi: 10.1073/pnas.1706464114

Zhang, L. S., and Davies, S. S. (2016). Microbial metabolism of dietary components to bioactive metabolites: opportunities for new therapeutic interventions. *Genome Med.* 8:46. doi: 10.1186/s13073-016-0296-x

Zhang, Y., Xiao, M., Horiyama, T., Zhang, Y., Li, X., Kunihiko, K., et al. (2011). The multidrug efflux pump MdtEF protects against nitrosative damage during the anaerobic respiration in *Escherichia coli*. *J. Biol. Chem.* 286, 26576–26584. doi: 10.1074/jbc.M111.243261

Zhu, J., Bean, H. D., Kuo, Y., and Hill, J. E. (2010). Fast detection of volatile organic compounds from bacterial cultures by secondary electrospray ionization-mass spectrometry. *J. Clin. Microbiol.* 48, 4426–4431. doi: 10.1128/JCM.00392-10

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 November 2020
doi: 10.3389/fmicb.2020.601233

Exopolysaccharide Producing *Bifidobacterium animalis* subsp. *lactis* Strains Modify the Intestinal Microbiota and the Plasmatic Cytokine Levels of BALB/c Mice According to the Type of Polymer Synthesized

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Bacteria-host interactions are mediated by different microbial associated molecular patterns which are most often surface structures such as, among others, exopolysaccharides (EPSs). In this work, the capability of two isogenic EPS-producing *Bifidobacterium animalis* subsp. *lactis* strains to modulate the gut microbiota of healthy mice, was assessed. Each strain produces a different type of polymer; the ropy strain S89L synthesized a rhamnose-rich, high-molecular weight EPS in highest abundance than the non-ropy DMS10140 one. BALB/c mice were orally fed for 10 days with milk-bifidobacterial suspensions and followed afterward for 7 post-intervention days (wash-out period). The colonic content of mice was collected in several sampling points to perform a metataxonomic analysis. In addition, the influence of specific microbial clades, apparently stimulated by the ropy and non-ropy strains, on mouse plasmatic cytokine levels was investigated through hierarchical association testing. Analysis of 16S rRNA gene sequences showed that the abundance of *Firmicutes* phylum significantly increased 7 days after cessing the treatment with both strains. The relative abundance of *Alloprevotella* genus also rose, but after shorter post-treatment times (3 days for both DMS10140 and S89L strains). Some bacterial clades were specifically modulated by one or another strain. As such, the non-ropy DMS10140 strain exerted a significant influence on *Intestinomonas* genus, which increased after 4 post-administration days. On the other hand, feeding with the ropy strain S89L led to an increase in sequences of *Faecalibaculum* genus at 4 post-treatment days, while the abundance of *Erysipelotrichaceae* and *Lactobacillaceae* families increased for

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Edited by:
Sonia G. Sáyago-Ayerdi, Instituto Tecnológico de Tepic, Mexico

Reviewed by:
Hugo Garcia, Tecnologico Nacional de Mexico/IT Veracruz, Mexico
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *31 August 2020*
Accepted: *05 November 2020*
Published: *26 November 2020*

Citation:
Sabater C, Molinero-García N, Castro-Bravo N, Diez-Echave P, Hidalgo-García L, Delgado S, Sánchez B, Gálvez J, Margolles A and Ruas-Madiedo P (2020) Exopolysaccharide Producing Bifidobacterium animalis subsp. lactis Strains Modify the Intestinal Microbiota and the Plasmatic Cytokine Levels of BALB/c Mice According to the Type of Polymer Synthesized. Front. Microbiol. 11:601233. doi: 10.3389/fmicb.2020.601233

prolonged times. Association testing revealed that several lactobacilli and bifidobacterial significantly stimulated by ropy S89L strain were positively associated with the levels of certain cytokines, including IL-5 and IL-27. These results highlight relevant changes in mice gut microbiota produced after administration of the ropy S89L strain that were associated to a potential immune modulation effect.

Keywords: exopolysaccharide, bifidobacteria, microbiota, mice, cytokine, immune response

INTRODUCTION

Bifidobacterium is part of the human intestinal microbiota being one of the most abundant genera in this ecosystem, mainly in infants. Furthermore, some bifidobacterial strains are considered to have probiotic properties and, thus, they are commonly used in commercial probiotic products. Some of their health properties could be related to the presence of specific surface molecules, such as exopolysaccharides (EPS). EPS are carbohydrate polymers synthesized by some bacteria that can be totally liberated to the extracellular milieu, or can remain loosely attached to the bacterial surface. EPS play an important role in the intestinal ecosystem by mediating bacterial-host interactions, modulating the immune system response, and also acting as a fermentable carbon source by other members of the microbiota (Castro-Bravo et al., 2018). Besides, these polymers are of great importance for the producing bacteria due to their protective role against adverse conditions, thus allowing their persistence in the gut for a longer time (Fanning et al., 2012). EPS structures contain different monomers, mainly D-glucose, D-galactose, and L-rhamnose, although others can be found such as *N*-acetyl glucosamine, D-glucose, D-ribose, or fucose. Some of these bacterial EPS can be used by the human gut microbiota leading to a high production of short chain fatty acids (Salazar et al., 2016; Liu et al., 2019). It has been demonstrated that some EPS-producing bifidobacteria can modulate the intestinal microbiota diversity and function (by modifying the profile of released metabolites), as determined *in vitro* by means of pH-controlled fecal batch fermentations or *in vivo* using animal experimental studies (Salazar et al., 2016; Yan et al., 2019). Microbiota modulation in inflammatory processes can be of great importance. Recent studies describe the evolution of gut microbiome during inflammatory bowel disease and detect relevant relationships between certain taxa and serum levels of antibodies through hierarchical association models (Lloyd-Price et al., 2019). Among the biological activities of EPS described, also the structure-immunity relationships have gained great attention (Hidalgo-Cantabrana et al., 2012; Xu et al., 2019). In fact, two recent studies carried out with an EPS-producing *Bifidobacterium longum* strain in a murine model of DSS-induced colitis showed that the strain was able to alleviate the inflammatory symptoms through the microbiota modulation and the maintenance of the mucosal barrier (Yan et al., 2019, 2020).

Several studies dealing with EPS producing *Bifidobacterium animalis* subsp. *lactis* strains, specifically those having a "ropy" phenotype due to the production of a rhamnose-rich high molecular weight (HMW)-EPS, have been carried out in our research group. It has been demonstrated that strains producing this ropy EPS can *in vitro* elicit different immune responses when co-incubated with PBMC (peripheral blood mononuclear cells) from humans (López et al., 2012) or with GALT (gut associated lymphoid tissue) isolated from rats (Hidalgo-Cantabrana et al., 2014), being also able to ameliorate inflammatory symptoms in a DSS-induced colitis mice model (Hidalgo-Cantabrana et al., 2016). However, so far, no studies demonstrating whether the intestinal microbiota could be differentially modulated by ropy and non-ropy strains in a healthy animal model have been reported. Therefore, the aim of this study was to determine the capability of different EPS-producing *B. animalis* subsp. *lactis* strains to modify the intestinal microbiota in healthy BALB/c mice and to explain the influence of relevant clades stimulated by EPS on the production of serum cytokines in animals with a non-disturbed mucosal barrier. To achieve this, a recently developed bacterial model, based on the wild-type strain *B. animalis* subsp. *lactis* DSM10140 (non-ropy) and its ropy isogenic mutant S89L that produces the HMW-EPS in higher abundance, was selected. Mutant S89L was obtained using gene replacement techniques to substitute the gene *balat_1410* (responsible for the determination of the EPS chain length) of DSM10140 for the mutated one with a single nucleotide change (Castro-Bravo et al., 2017). Therefore, differences between both strains on microbial and immune modulation capabilities could be only attributed to a single gene which determines the chain size of the polymer. Then, the main difference between both strains is that S89L presents in its surface a bigger amount of the HMW-EPS than its parental DSM10114 strain.

MATERIALS AND METHODS

Bacterial Growth Conditions

The DSM101410 and S89L strains were cultivated in MRSc [MRS (Biokar Diagnostics) supplemented with 0.25% L-cysteine-HCl (Sigma-Chemical Co.)] at 37°C, for 24 h, in a jar under anaerobic conditions (Anaerocult A, Merck). Cultures were washed with PBS and resuspended in heat-treated 11% skimmed milk (BD Difco, Thermo Fisher Scientific Inc.). Bifidobacterial suspensions in milk, containing on average 8.9 ± 0.4 Log CFU/ml, of each strain were daily prepared to be administered (dose of 100 μl) to the mice by means of a gastric tube.

Experimental Design

The animal experimental procedure was approved by the Ethical Committee of Laboratory Animals of the University of Granada (Spain) (Ref. No. CEEA-2010-286). Female BALB/c mice (7–9 weeks old, approximately 20 g) were obtained from Janvier Labs (St Berthevin Cedex) and kept under conventional conditions

with a standard pelleted diet and sterilized water for 1 week before beginning the experiments. A total of 114 animals were randomly distributed in three groups: 48 mice receiving the non-ropy strain (DSM10140 group), 48 mice receiving the ropy strain (S89L group) and the 18 remaining mice did not receive any treatment (reference group). The weight at the beginning of the experimental procedure for each animal group was (mean ± SD): 22.28 ± 1.45 g for the control group, 22.82 ± 1.56 g for DSM10140-treated group and 22.14 ± 1.65 for g the S89L-treated group. The statistical analysis performed (ANOVA) showed no statistical differences in the initial weight among the three groups ($p > 0.05$). The experimental design was as follows: 10 days of intervention with daily oral administration of the milk-bifidobacteria suspensions, followed by 7 days of post-intervention without bifidobacteria intake (wash-out period). No variations in behavior or health status were observed in the three groups of mice during the experimentation period. In different sampling points (5 and 10 days of intervention, and 1, 3, 4, and 7 post-intervention days), 8 mice from each bifidobacterial group, and 3 mice from the reference group (thus, 18 control animals in total), were sacrificed in order to collect the colonic content and the blood serum. For that, each mouse was anesthetized with an overdose of halothane and blood was extracted from the heart using heparinized tubes. After that, animals were sacrificed by cervical dislocation and the gut was excised to collect its content. Samples were stored at −80°C after their collection.

Cytokine Analysis

The ProcartaPlex Multiplex Immunoassay for mouse (Thermo Fisher Scientific Inc.) was used to quantify the levels of different cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17a, IL-18, IL-22, IL-23, IL-27, GM-CSF, IFN-γ, and TNF-α) by means of the FACS Canto II flow cytometer (BD Biosciences). The detection limits (pg/mL) for those detected in our samples were: 2.21 ± 0.36 (IL-5), 5.48 ± 0.72 (IL-6), 15.08 ± 0.62 (IL-9), 2.27 ± 0.01 (IL-10), 1.65 ± 0.36 (IL-17a), 12.30 ± 0.80 (IL-23), 2.68 ± 0.01 (IL-27), and 1.12 ± 0.19 (IFN-γ).

16S rRNA and 16S-23S Internal Transcribed Spacer (ITS) Gene Sequencing

Total DNA was isolated from the colonic content of the 114 samples using the QiaEz DNA-extraction protocol previously optimized in our research group (Milani et al., 2013), consisting in a mechanical cell disruption step, followed by enzymatic lysis and combined with an extraction with the QIAamp Stool DNA kit (Qiagen). Using the primers Probio_Uni and Probio_Rev (Milani et al., 2013), the V3 region of the 16S rRNA gene was amplified. The 16S-23S Internal Transcribed Spacers (ITS) were amplified from extracted DNA using the specific primer pair ProbioBif-ITS_Fw and ProbioBif-ITS_Rev, which targets the variable region between the 16S rRNA and 23S rRNA gene sequences (Milani et al., 2014). Sequencing was performed using an Illumina MiSeq machine at GenProbio S.R.L. (Parma, Italy). Sequence reads were filtered and the resulting ones were processed using a personalized script of QIIME software (Caporaso et al., 2010) matched by pair-ends. Quality control filtering was performed, keeping sequences with a mean sequence quality score >20 and a length between 140 and 400 bp.

Data Analysis

Shapiro–Wilk's Normality test ($p < 0.05$) and Levene's test ($p < 0.05$) to determine the homogeneity of variances were calculated for all data generated. Statistically significant differences between samples were calculated through Kruskal–Wallis and Mann–Whitney statistical tests for non-parametric independent samples followed by False Discovery Rate (FDR) *post hoc* using Benjamini–Hochberg method with a value of 0.25. All statistical analyses were computed on R v3.5.0 and Mothur software.

In order to calculate diversity measures, the 16S rRNA reads were clustered in Operational Taxonomic Units (OTUs) defined at ≥99% sequence homology by meanings of UCLUST software (Edgar, 2010). All reads were classified to the lowest possible taxonomic rank using the QIIME and the reference database SILVA (Quast et al., 2013). Similarity of the microbial communities between the samples was calculated by UniFrac method (Lozupone and Knight, 2005). Phyloseq (McMurdie and Holmes, 2013) and Microbiome (Lahti and Shetty, 2017) packages were also used in the analysis of sequencing data. After assignment of reads to phylum, family, genus and species levels, two differential analyses were performed: Metastast algorithm of Mothur software, and DESeq2 differential abundance testing for sequencing data (Love et al., 2014). To determine statistical differences in taxonomic data according to the strain, treatment time or both p_{adj} lower than 0.05 and log2FoldChange greater than 1.5 were considered.

Relevant taxonomic clades were associated to cytokine plasmatic levels through hierarchical all-against-all association testing (HAIIA)[1] (Lloyd-Price et al., 2019) considering q-values and a Bonferroni False Discovery Rate of 0.25. In addition, a graphical correlation network between taxa and cytokines was computed using ccrepe (Schwager et al., 2019) and qgraph (Epskamp et al., 2012) packages.

RESULTS

Influence of Ropy and Non-ropy Strains on Cytokine Levels

The administration of non-ropy DSM10140 and ropy S89L *B. animalis* subsp. *lactis* strains to BALB/c mice exerted a relevant influence on the levels of some serum cytokines. Statistically significant differences in their production according to the strain selected (ropy or non-ropy) as well as the treatment time (5 and 10 days of intervention, and 1, 3, 4, and 7 post-intervention days) were found (**Figure 1**, the statistical

[1] huttenhower.sph.harvard.edu/halla

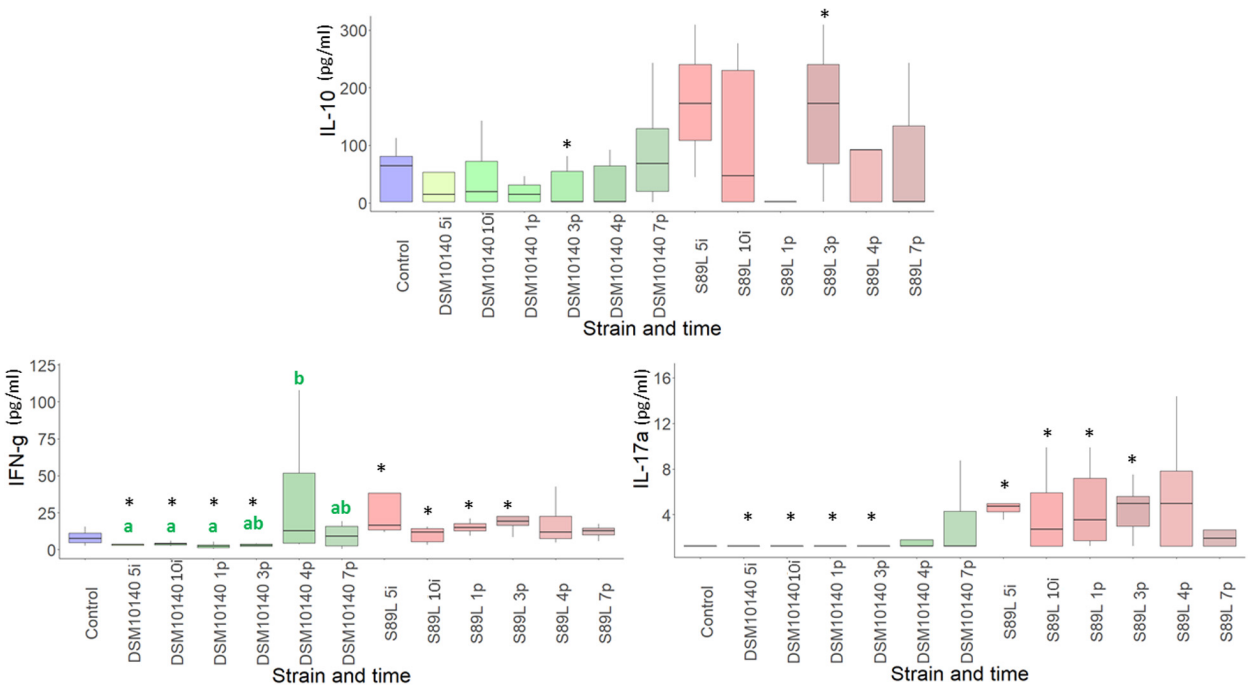

FIGURE 1 | Box plot (median, interquartile range, minimum and maximum values) of the level (pg/ml) of IL-10, IFN-γ, IL-17A quantified in the blood serum of control mice group, as wells as in the groups fed with the non-ropy DSM10140 or with the ropy S89L *Bifidobacterium animalis* subsp. *lactis* strains at different sampling points (5i and 10i days of intervention, and 1p, 3p, 4p, and 7p days post-intervention). Within each strain-treatment group, samples (days) that do not share a common letter are statistically different ($p < 0.05$). Within each treatment day, the statistical differences between the DSM10140 and S89L groups are marked with an asterisk (*$p < 0.05$). For information about other cytokines, see **Supplementary Table 1** in the **Supplementary Material**.

analysis is shown in **Supplementary Table 1**). Regarding the anti-inflammatory IL-10, although no statistical differences were detected (with the exception of sample 3p), levels were higher after S89L administration compared to DMS1040 strain. On the other hand, significantly lower levels of IFN-γ and IL-17a were found in mice treated with DSM10140 than in treated with S89L during the intervention period and until the first 3 post-intervention days. However, no differences were detected between both mice groups when the ratios IL-10/INF-γ or IL-10/IL-17a were calculated, although a tendency to have higher values (i.e., anti-inflammatory profile) for the S89L treated group was detected, probably due to the highest production of IL-10 (**Supplementary Table 1**). The release of other cytokines, such as IL-9, IL-23, and IL-27, was also enhanced in S89L treatments (**Supplementary Table 1**), although after 4 post-treatment days no differences between both mice groups were detected. In both mice groups, similar concentrations of other quantifiable cytokines, such as IL-5 and IL-6, were detected, but the last one tend to be released in lower concentrations in mice fed with the ropy S89L strain. The levels of the others analyzed (IL-1β, IL-2, IL-4, IL-10, IL-12p70, IL-13, IL-18, IL-22, GM-CSF, and TNF-α) were below the limit of detection of the method used (**Supplementary Table 1**).

After a principal component analysis (PCA) of this data, no clear patterns could be inferred in the general cytokine profile obtained, since the PCA explains a low percentage (below 50%) of variance (**Supplementary Figure 1**). These results could be related with the high inter-individual variability found among mice.

Influence of Ropy and Non-ropy Strains on Gut Microbiota

The influence of the ropy and non-ropy EPS-producing strains on mice gut microbiota composition was studied. First, the alpha diversity measuring variability of species within a sample was calculated using different indices (Shannon, Simpson, and Inverse Simpson) which provide complementary information, reflecting different patterns in the core microbiota. These indexes showed a greater dispersion for mice fed with non-ropy DSM10140 and ropy S89L strains in comparison to the control group, which was more accentuated during the post-intervention period, especially in S89L group (**Supplementary Figure 2**). The global Chao1 index, an estimation of the number of species represented by only one individual in the sample, was 90.3, ranging from 89.6 to 93.0 in DSM10140 group and from 82.0 to 91.4 in S89L-treated mice. On the other hand, the beta-diversity (Bray–Curtis distance) measuring differences in composition among samples, revealed that DSM10140 and S89L groups had a more diverse microbiota than the control group (**Figure 2A**), especially at the end of the post-intervention period (**Figure 2B**), achieving a higher number of species at the fourth day of post-intervention for the ropy S89L treatment (**Figure 2C**). These results suggest that the administration of non-ropy DSM10140

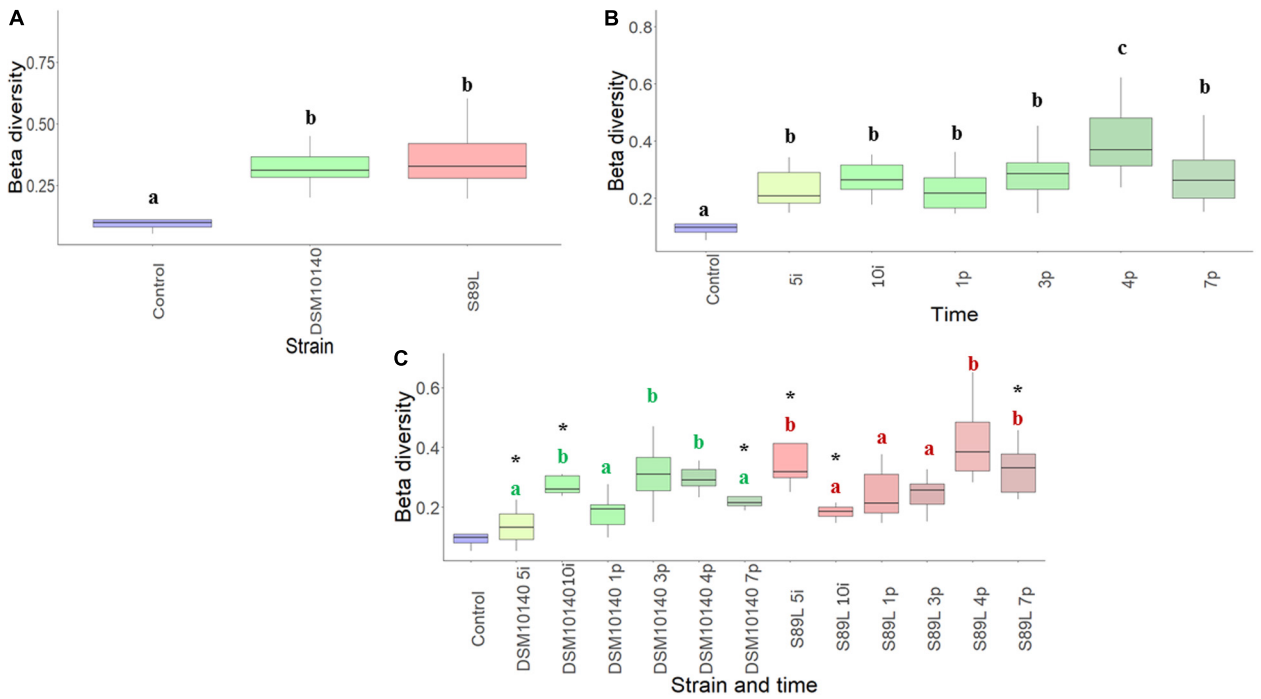

FIGURE 2 | Beta-diversity (Bray–Curtis metrics) of the relative abundance of colonic microbiota samples from mice grouped according to the EPS-strain used for feeding **(A)**, the intervention time **(B)** and a combination of both EPS-strain and sampling (days) points **(C)**. Different letters indicate statistical differences among the different EPS-strain groups **(A)** or sampling days **(B)**; in the combination of both factors **(C)**, within each EPS-strain group samples (days) those that do not share a common letter are statistically different ($p < 0.05$), whereas within each treatment day, the statistical differences between the DSM10140 and S89L groups are marked with an asterisk (*$p < 0.05$).

and ropy S89L strains stimulate a significant number of species that are present in low abundances in the control group. In addition, the ropy EPS seems to exert a stronger effect on gut microbiota that takes place mainly at the final days of the post-treatment period.

The relative abundance of the main (≥1%) bacterial phyla detected in the colon content of the different BALB/c mice groups is represented in **Figure 3** (the complete statistical analysis is provided in **Supplementary Table 2**). In general, the evolution of abundances along the intervention and post-intervention period was similar in both bifidobacterial-treated mice groups. Specifically, percentages remained without changes ($p > 0.05$) between the two sampling points (5i and 10i) of the intervention period and they resemble that of the reference group. However, at 1 and 3 post-intervention days statistical differences ($p < 0.05$) for some phyla were detected with respect to the intervention period and also with respect to longer times of the post-intervention. In fact, it seems that the more time elapsed since the end of intervention (4 and 7 days, post-intervention), the more resembles the profile of the microbiota phyla to the initial state and to the reference (non-treated) group. Thus, the greatest changes were detected after 1 and 3 days of cessation of bifidobacterial intake; an increase in Actinobacteria and Bacteroidetes abundance, to detriment of the Firmicutes phylum, was found. This indicates that the biggest influence of our EPS-producing bifidobacteria on the colonic microbiota occurred during the wash-out period, after finishing the oral administration of both strains, suggesting that this intervention with our EPS-producing bifidobacteria had a delayed effect on microbiota dynamics in agreement with diversity analyses previously described. The other (most abundant) phylum presenting variations along the intervention period was Proteobacteria (**Figure 3** and **Supplementary Table 2**); a reduction in the relative abundance was observed from the beginning of the intervention period to the end of the experimental procedure in both bifidobacterial-treatment groups, but this effect only remained in the group S89L fed with the ropy-EPS bifidobacteria. Indeed after 4 and 7 days post-intervention the group fed with the strain DSM10140 showed higher ($p < 0.05$) relative abundance of this phylum than the S89L-fed group (**Supplementary Table 2**). In both cases, the levels of Proteobacteria were significantly lower than in the control group. To corroborate these statistically significant differences, DESeq2 differential abundance testing was applied to all phyla determined (**Supplementary Figure 3**). It was also found that Tenericutes were significantly higher in mice fed with ropy and non-ropy strains compared to control groups (**Supplementary Figure 3A**). Proteobacteria abundance decreased during post-intervention period when combined data from both treatments (**Supplementary Figure 3B**) in agreement with the results presented in **Figure 3**. Moreover, significant differences were observed for Firmicutes considering both the

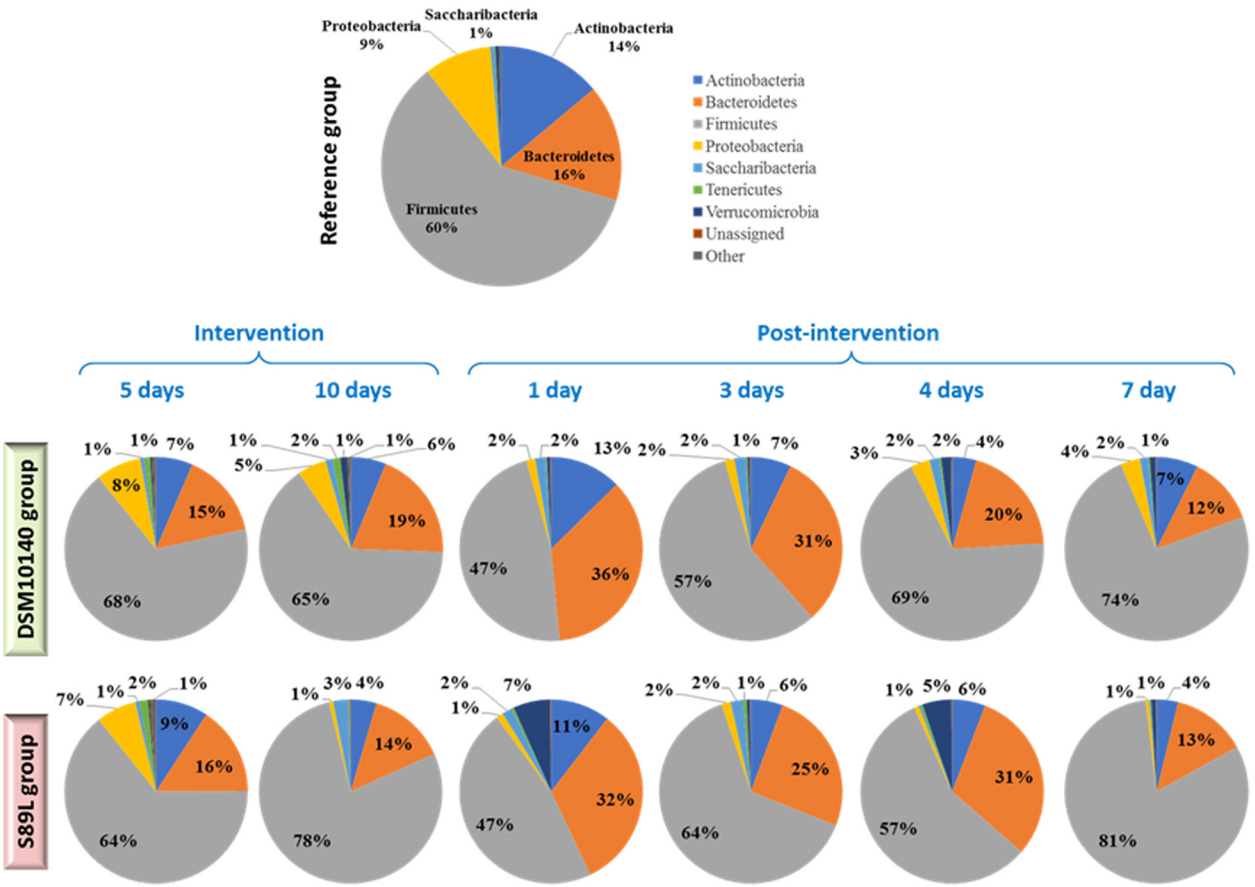

FIGURE 3 | Relative abundance of the main (≥1%) bacterial phyla detected in the colonic content of different BALB/c mice groups ($n \geq 6$ per group and sampling day). The reference group was constituted by non-treated mice of the same litter; treatment groups were fed for 10 days with a daily dose of 8.9 ± 0.4 Log CFU/ml, suspended in skimmed milk, of the EPS-producing strains *B. animalis* subsp. *lactis* DSM10140 (parental, non-ropy strain) or S89L (mutant, ropy strain producing a HMW-EPS). Colonic content samples were obtained from mice sacrificed at two points during the intervention period (5 and 10 days) and at four points post-intervention (without administration of bifidobacteria: 1, 3, 4, and 7 days). For statistical analysis see **Supplementary Table S2** in **Supplementary Material**.

selected strain and intervention time separately (**Supplementary Figure 3C**); higher abundances at the end of the intervention (10i point) in S89L group were observed. However, in both groups of mice, a relevant increase in the relative abundance of this phylum was achieved after 7 post-treatment days in agreement with the non-parametric statistical tests presented in **Figure 3**.

Regarding family level analysis, differences of relative abundances along the experimental points (**Figure 4**), as well as between both treatment mice groups at a given point (**Supplementary Table 3**) were observed. Among the most abundant (>2%) families, the noticeable change at the end of the intervention period (10i) was the significant ($p < 0.05$) increase in *Lactobacillaceae*, which was also corroborated by DESeq2 differential abundance test (**Supplementary Figure 4A**). A decrease in *Bacteroidaceae* and *Ruminococcaceae* (*Clostridia* class) in the S89L group was also denoted (**Figure 4** and **Supplementary Table 3**) then, concomitantly, there was an increase of Firmicutes/Bacteroidetes ratio at this 10i point (**Supplementary Figure 5**). Finally, the S89L treatment group also showed a significant decrease of *Rikenellaceae* (Bacteroidetes phylum) with respect to the DSM10140-fed group (**Supplementary Table 3**). During the post-intervention (wash-out) period, a remarkable increase of *Bacteroidales* S24-7 family was detected at day 1 by both conventional statistical tests (**Figure 4**) and DESeq2 differential abundance testing (**Supplementary Figure 4B**) in both groups of mice, which tended to decline afterward with a concomitant long-term increase of *Lactobacillaceae*. Curiously, in the first post-intervention day the *Bifidobacteriae* family increased its relative abundance in both groups of mice (**Figure 4**), and the percentages fluctuated along wash-out period detecting significant differences between DSM10140 and S89L groups at the 4th post-intervention day (**Supplementary Table 3**). At this day, there were also significant ($p < 0.05$) differences on the relative abundance of *Desulfovibrionaceae* family between treatment groups, being lower the percentage in S89L-treated mice. It should be noted that advanced DESeq2 differential abundance test also detected significant changes in minor families present in mice microbiota (>0.1%). For example, *Lachnospiraceae* increased during post-intervention period as well as *Erysipelotrichaceae*, which achieved

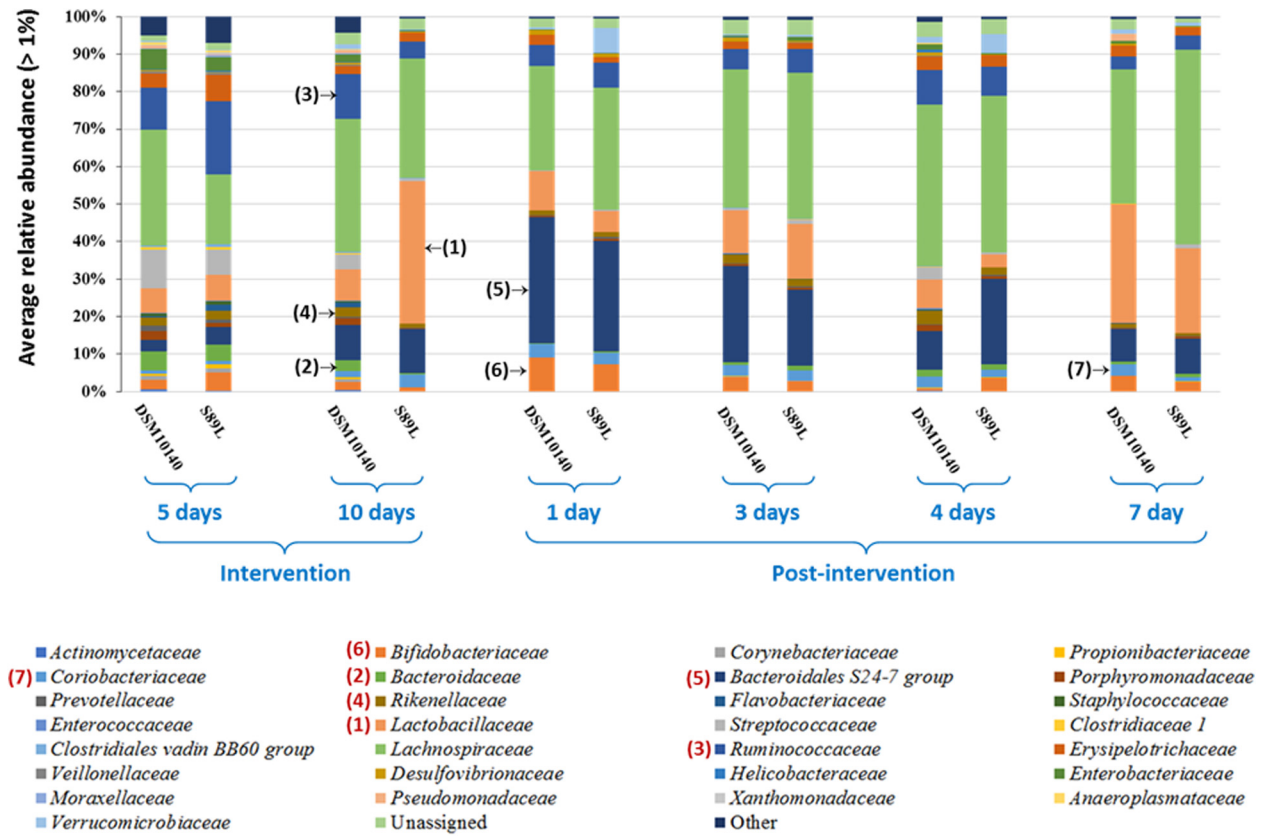

FIGURE 4 | Mean ($n \geq 6$ mice per group and sampling day) of the bacterial families, having a relative abundance percentage higher than 1%, present in the colon content of mice treated with the EPS-producing strains *B. animalis* subsp. *lactis* DSM10140 or S89L. The treatment procedure is described in the legend of **Figure 3**. For statistical analysis see **Supplementary Table S3** in **Supplementary Material**.

higher abundances at the fourth day after ropy S89L treatment. Similarly, *Coriobacteriaceae* increased during the first days of post-intervention with both strains and decreased at the seventh day probably indicating a partial loss of the modulatory activity (**Supplementary Figure 4B**). In general, *Verrucomicrobiaceae* showed higher abundances in mice administered with S89L, regardless time (**Supplementary Figure 4A**).

Taxonomic analysis of genera present in samples (relative abundance > 0.1%) using DESeq2 differential abundance test revealed a general increase in *Ruminococcaceae* UCG-014 genus compared to the control group (**Supplementary Figure 6A**). In addition, a stimulation of *Ruminococcus gnavus* group during post-intervention was observed, which was more accentuated in S89L treatment (**Supplementary Figure 6B**) although no differences were found among the three groups of mice (**Supplementary Figure 6A**). An increase in the abundance of this bacterium has been associated with pro-inflammatory states, such as Crohn disease (Henke et al., 2019); however, as we have indicated in previous sentences, the slight increase in the relative abundance of *R. ganvus* did not correlate with a pro-inflammatory state in or experimental model. In addition, S89L administration led to a high abundance of *Eubacterium fissicatena* group and *Faecalibaculum* after 3 and 4 post-treatment days, although this modulatory activity decreased at longer times. Similarly, *Alloprevotella* and *Intestinimonas* achieved higher abundances after 2–3 post-intervention days with both strains (**Supplementary Figure 6B**). Given that this intervention study was carried out with two strains of *Bifidobacterium,* this genus was analyzed in more detail. **Figure 5** shows the "box and whisker" plot representing the relative abundance of the sequences identified as bifidobacteria. Intriguingly, during the intervention procedure a reduction in the relative abundance of *Bifidobacterium* was observed in both bifidobacterial-treated groups with respect the levels found in the reference (non-intervention) mice group. In fact, this is coincident with the lower proportion of Actinobacteria phylum observed in the two treated mice groups in comparison to the reference one (**Figure 3**). It seems that this was the phylum reducing its relative abundance in higher extent during the intervention period and *Bifidobacterium* genus might account for this behavior. Indeed, the recovering of Actinobacteria phylum and *Bifidobacteriaceae* family, denoted at the first post-intervention day, also match with the increase of this genus in both treatment groups (**Figure 5** and **Supplementary Figure 7**). Besides, the statistical differences (**Supplementary Table 3**) observed in the family at the 10th day of intervention, and the 4th post-intervention day between

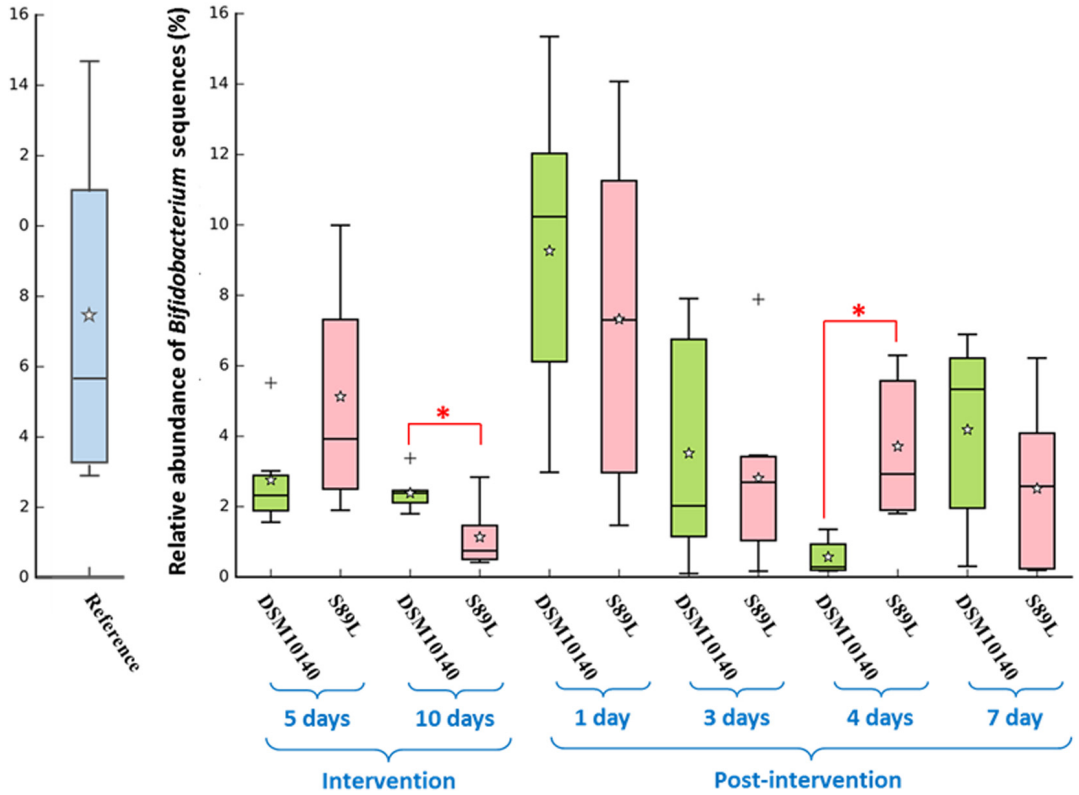

FIGURE 5 | Relative abundance (%) of sequences corresponding to *Bifidobacterium* spp. in the colonic content of mice treated with the EPS-producing strains *B. animalis* subsp. *lactis* DSM10140 or S89L, and non-treated reference group. The treatment procedure is described in the legend of **Figure 3**. For each combination treatment day/strain, the "box and whiskers" plot represents median, interquartile range and minimum and maximum values, calculated from de sequences obtained for at least 6 mice; the average is represented with the white star. Within each treatment day, the non-parametric Mann–Whitney test for 2-independent samples was used to assess differences between both strain (*$p < 0.05$). The IBM SPSS Statistic vs25 package was used for the non-parametric statistical analysis.

the DSM10140 and S89L groups are also coincident with the tendency observed for *Bifidobacterium* spp.

To complete taxonomic characterization, a tentative species-level analysis was also performed. For this purpose, statistically relevant lactobacilli and bifidobacteria (>0.1%) determined by DESeq2 differential abundance test was carried out with the 16S rRNA gene sequences. Abundances of *B. animalis* were increased in both treatments with respect to the control group (**Supplementary Figure 8A**), but no statistical differences were found. Curiously, a great increase in *Lactobacillus reuteri* populations was achieved at the end of post-intervention period with both strains (**Supplementary Figure 8B**), corroborating the delayed effect of the microbiota modulatory activity previously observed for other clades. Further, the ITS regions were sequenced to study the species belonging to *Bifidobacterium* genus (**Figure 6**). Administration of both strains enhanced *B. bifidum* growth compared to the control group, regardless intervention time (**Figure 6A**). On the other hand, the abundances of *B. pseudolongum* subsp. *globosum* and *B. pseudolongum* subsp. *pseudolongum* showed a great abundance during post-intervention in both treatments while *B. pseudolongum* spp. growth significant increased at 4 days of post-treatment in S89L group (**Figure 6B**). It was previously reported that animals fed with EPS-producing bifidobacteria are able to increase the populations of other bifidobacterial species (Salazar et al., 2011), which could be explained by the use of the polymers as fermentable substrates for other microbiota inhabitants, including bifidobacteria (Salazar et al., 2016).

Associations Between Serum Cytokine Levels and Gut Microbiota

To better understand the potential biological effect of the administration of S89L ropy strain in mice, serum cytokine markers and microbial taxonomic data were integrated through correlation networks (**Figure 7**) and hierarchical all-against-all association testing (HAIIA; **Figure 8**). Positive and negative associations between taxonomic data and cytokine profiles were first represented as a correlation network (**Figure 7**). As expected, *Lactobacillaceae* family was positive associated to *Lactobacillus* species while different species of bifidobacteria positively associated to each other. In this sense, positive relationships

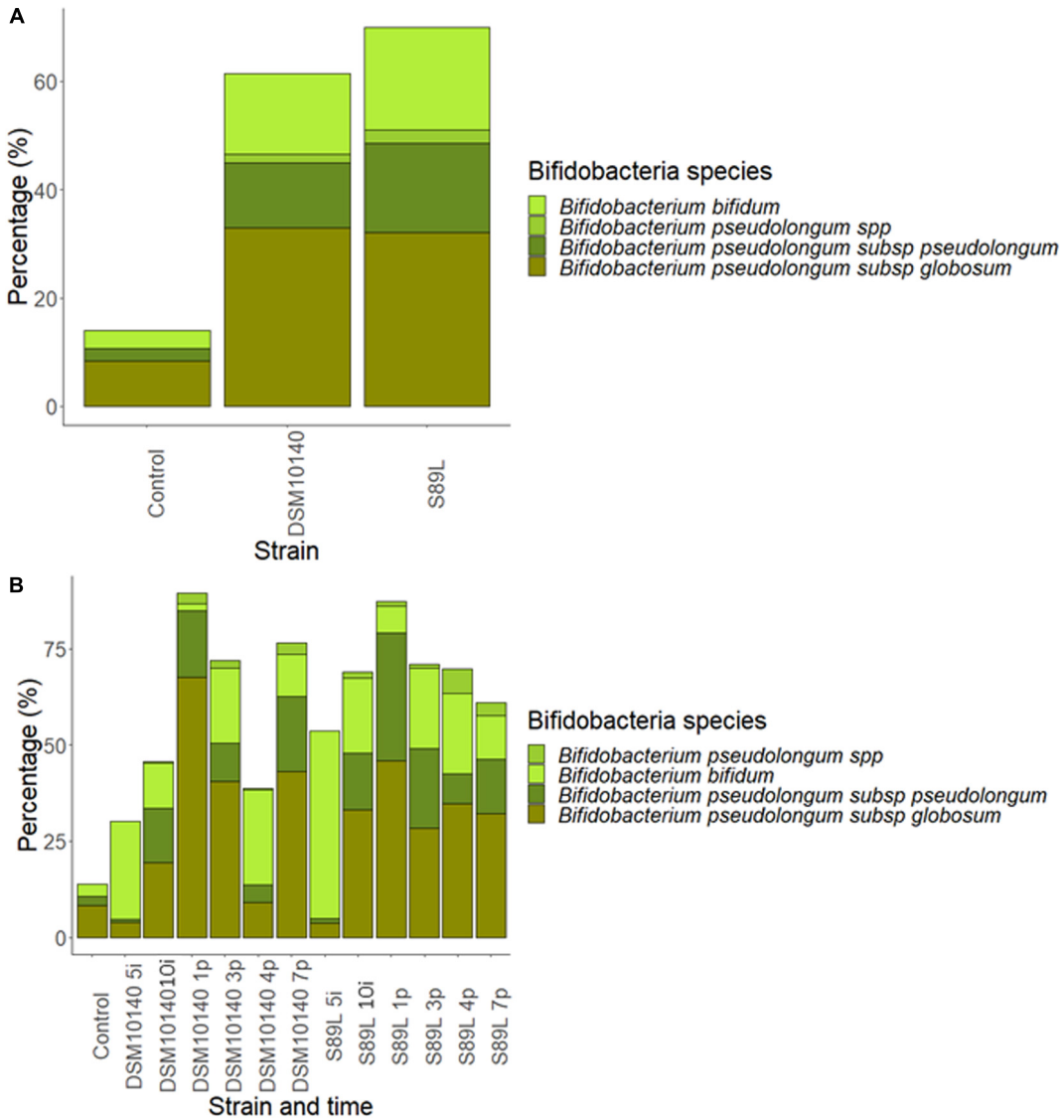

FIGURE 6 | Abundance percentages of statistically significant different *Bifidobacterium* species after Internal Transcribed Spacer (ITS) sequencing according to the EPS-strain selected **(A)** and both EPS-strain and intervention time **(B)**.

between *Coriobacteriaceae* family and *Enterorhabdus* genus are due to the shared class *Coriobacteria*. Positive relationship between *Tenericutes* and *Rumicococcaceae* UCG-014 as well as between *Lachnospiraceae* family and *Intestinimonas* were observed. As expected, abundances of Firmicutes and *Bacteroidales* S-24 group were correlated in a negative way. With regard to cytokine profiles, in general, they showed positive correlations to each other. The HAIIA analysis (**Figure 8**) revealed that *Lactobacillaceae* family and specifically *L. reuteri* were associated to high levels of IL-5. Similarly, populations of *B. pseudolongum* subspecies and *L. reuteri* were associated to higher serum levels of IL-27. It is worth remember that the S89L strain (ropy- EPS) stimulated the growth of these microorganisms, exerting a delayed effect that took place during the post-intervention time. *Verrucomicrobiaceae* family slightly contributed to the release of IL-17a cytokine and the decrease of IL-10/IL-17a ratio. It has also been observed that the IFN-γ was positively associated to *B. bifidum*, and negatively associated to *B. pseudolongum* subsp. *globosum*, indicating a different modulatory pattern. With regard to other clades, Proteobacteria, reduced during post-intervention, is associated to a lower release of interleukins IL-9 and IL-27.

DISCUSSION

A differential modulatory effect of two isogenic EPS-producing *B. animalis* subsp. *lactis* strains on both gut microbiota and cytokine production in a healthy mice model has been found. Potential relationships between the different microbial clades

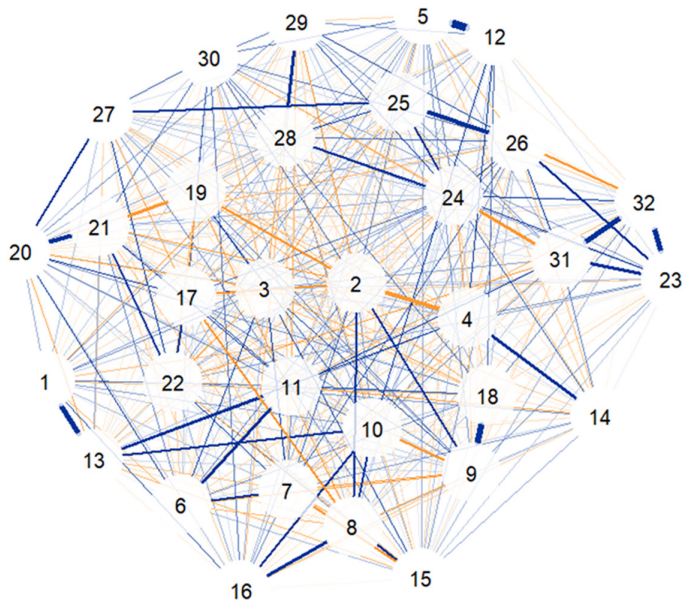

FIGURE 7 | Correlation network illustrating positive and negative associations between taxonomic clades, serum levels of cytokines and inflammatory factors. Blue lines indicate positive associations while saffron lines suggest negative associations. Line thickness is in proportion to magnitude.

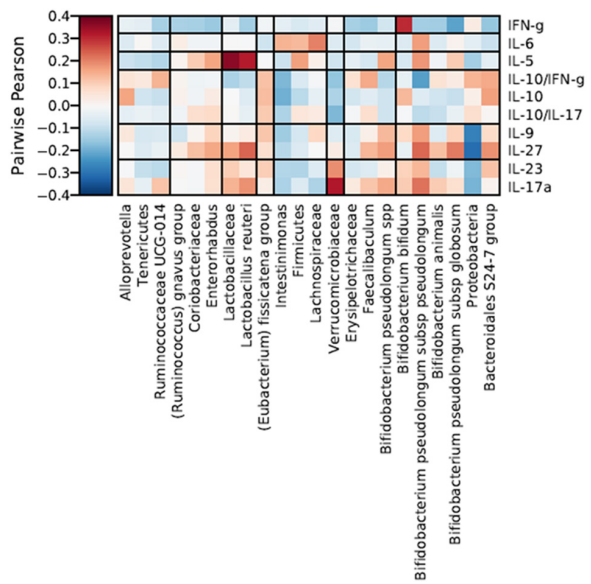

FIGURE 8 | Hierarchical all-against-all association testing (HAllA) describing the effect of specific taxonomic clades on serum levels of cytokines and inflammatory factors, considering Pairwise Pearson correlation coefficients adjusted by False Discovery Rate (FDR < 0.25). Red and blue cells indicate positive and negative correlations, respectively. Color intensity is in proportion to magnitude.

determined were elucidated through correlation networks. As expected, different lactobacilli and bifidobacteria were positively associated with other members of *Lactobacillaceae* and *Bifidobacteriaceae*. Other interestingly relationships suggested include positive correlations between Tenericutes and *Rumicococcaceae* UCG-014 (*Firmicutes*) in agreement with Zhang and Gao (2017) which reported that bacterial genomes in the phyla Tenericutes and Firmicutes are notably positively correlated. *Lachnospiraceae* family and *Intestinimonas* genus were positively correlated as both clades involving butyrate producers (Bui et al., 2016). In contrast, Firmicutes and *Bacteroidales* S-24 group were negatively associated, and antagonic relationships between these two clades have been already reported in humanized mice (Clarke et al., 2012). Indeed, the Firmicutes/Bacteroidetes (F/B) ratio was shown to be an indicator of human gut microbiota status and a variation in this value has been related to different pathological states, such as autoimmune diseases, metabolic syndrome or obesity, among others (Hevia et al., 2014). The tendency is different according to the disease; for example, in obesity the intestinal microbiota dysbiosis was correlated with an increase in the F/B ratio where more abundance of Firmicutes is observed in different types of experiments (Castaner et al., 2018), whereas the contrary was found in systemic lupus erythematous disease (Hevia et al., 2014). In our study, this ratio decreased in both bifidobacterial mice groups in the first stages of post-intervention (**Supplementary Figure 5**); this agrees with a previous study in which *Bifidobacterium pseudocatenulatum* (strain CECT7765) was administered to mice, although in a model of obesity (Moya-Pérez et al., 2015). However, in our case this effect reverted in further post-intervention days tending to reach the initial ratio, or even increase it, at the last day of the experimental follow up. This behavior could be explained by the fact the orally administered bacteria are not able to permanently colonize the

gut ecosystem which will tend to reach the initial microbial homeostasis after cessation the intake of both EPS-producing *B. animalis* subsp. *lactis* strains. Regarding Proteobacteria phylum, which was the decreased in mice at the end of both experimental treatments, is normally present at low levels in a healthy human gut but, under certain conditions, the opportunistic members of the phylum can overgrow, such as in the case of inflammatory diseases or aging. It has been proved a positive correlation between opportunistic enterobacteria and some pro-inflammatory markers in elders (Biagi et al., 2010). Contrarily, treatment with specific *Bifidobacterium* strains can down-regulate the postoperative pro-inflammatory response of patients undergoing colorectal resection (Mizuta et al., 2016). It has been reported that *Rikenellaceae* (Bacteroidetes phylum) family, which decreased in ropy S89L treatment group, seems to be over-represented in genetic and diet-induced obese mice (Kim et al., 2012); thus, it could be interesting to explore the potential application of ropy EPS-producing strains to reduce the levels of *Rikenellaceae* in an obese model. The S24-7 family of the *Bacteroidales* order, which was reduced at prolonged post-intervention times, is a prominent component of the murine gut microbiota, and seems to be present as well within the human intestinal community (Ormerod et al., 2016). This LPS-producing Gram-negative group might be directly involved in the mild-inflammation states related to some physiopathological processes, such as obesity (Kang et al., 2017) or aging (Van Beek et al., 2018). Administration of ropy S89L strain led to a lower accumulation of intestinal sulfate reducing bacteria (SRB), like *Desulfovibrionaceae,* that are directly related to IBD (inflammatory bowel disease) development (Kushkevych et al., 2018). On the other hand, a significant difference at the last sampling point of our experimental procedure (7th post-intervention day) in the family *Coriobacteriaceae* was detected, showing also a reduction in S89L-fed mice group. These bacteria are normal inhabitants of the gut, where they carry out relevant functions such as the conversion of bile salts and steroids as well as the activation of dietary polyphenols. However, their increased occurrence has been associated with a range of pathologies; as an example, this taxon is over-represented in colorectal cancer-associated microbiomes and this is why some of their members could be considered as pathobionts (Tjalsma et al., 2012). The variations detected between the two ropy and non-ropy treatment groups in the relative abundance of several taxa indicate that the presence of different amounts the HMW-EPS between both strains modulated the mice microbiota in a different way. In general, the relative abundance of several bacterial groups prevalent in different immune-related disorders was under-represented in mice fed with the ropy S89L strain. Nevertheless, interventions toward reducing the levels of potential pathobionts with ropy EPS-producing strains should be further explored, being an opportunity for their application as probiotics.

On the other hand, the influence of taxonomic changes induced by these EPS-producing bifidobacteria administration on serum cytokine levels was investigated. Our results agree with those obtained by Yan et al. (2019) that reported a significant decrease in IL-6 levels in mice after treatment with ropy-EPS producing strain from *B. longum*. Immune modulatory activity of EPS from *B. animalis* subsp. *lactis* had been previously reported *in vitro*, regulating the production of IL-6 and TNF-α in a dose-dependent manner in murine macrophage cell line RAW 264.7 (Liu et al., 2017). In fact, a reduction in the IL-6 levels in rats fed with wild-type ropy *B. animalis* subsp. *lactis* strains were previously demonstrated in a rat model (Salazar et al., 2014), in the same way that we have found in the current study the mutant S89L strain. Additionally, administration of EPS from *Lactobacillus fermentum* combined with *B. animalis* subsp. *lactis* led to a relevant decrease in TNF-α and increased IL-10 production in mice (Ale et al., 2019), which was also observed in our study as well as previous ones carried out with closely related ropy *B. animalis* subsp. *lactis* strains (Hidalgo-Cantabrana et al., 2014, 2015, 2016). Moreover, high doses of EPS from *B. animalis* can contribute to maintain IL-2/IL-10 ratio in mice (Xu et al., 2017) and it has been described that EPS modulates IFN-γ and IL-10 (Xu et al., 2019), a behavior also observed in our study. Interestingly, Schiavi et al. (2016) reported that surface-associated EPS from *B. longum* subsp. *longum* modulates IL-17 levels in mice, while hierarchical association testing performed in our work suggested that *B. pseudolongum* subsp. *pseudolongum* was positively associated to a higher release of this cytokine. This test also revealed that IFN-γ was associated with some bifidobacteria, like *B. bifidum*, in agreement with previous studies (Wang et al., 2019). Results presented in the current work reinforce our previous findings about the potential anti-inflammatory effect of ropy EPS from *B. animalis* subsp. *lactis* which could be mediated through the modulation of gut microbiota.

CONCLUSION

In short, the fluctuations of the relative abundance of different taxa in the colonic microbiota of the treated mice observed along this experimental procedure must be directly related to the intake of *B. animalis* subsp. *lactis* strains. The lack of consistency in the evolution of microbial populations could be linked to the high inter-individual differences among mice within each group, mainly taking into account that we have analyzed different animals in each sampling point of the intervention. In spite of this, we have found significant variations and tendencies in certain microbial groups associated with the presence of different EPS in the surface the bifidobacterial strains under study. In general, the ropy S89L strain, covered in higher proportion by rhamnose-rich HMW-EPS, reduced the abundance of microbial groups that could be related with low-degree inflammatory states. In addition, we have found that the influence of specific taxa, stimulated by the ropy and non-ropy strains, on cytokine plasmatic levels investigated through hierarchical association testing also suggests an anti-inflammatory effect of the ropy S89L orally administered in mice. Altogether, results reported here could explain previous observations with ropy EPS-producing strains. Thus, the attenuation of immune response, or the induction of an anti-inflammatory profile, by ropy EPSs could be also related to a differential modulation of the intestinal microbiota with respect to that induced by non-EPS producing

strains. Further studies must be undertaken to correlate the positive and negative relations between ropy EPS-producing strains and specific microbial groups, and to propose their application for restoring the microbial dysbiosis associated with specific inflammatory diseases.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of Laboratory Animals of the University of Granada (Spain) (Ref. No. CEEA-2010-286).

AUTHOR CONTRIBUTIONS

AM and PR-M were in charge of the experimental design of this work. NC-B, PD-E, and LH-G performed the animal experimentation procedure. CS and NM-G performed the data analyses. SD, BS, and JG supervised the work of the Ph.D. students. PR-M wrote the manuscript draft. All authors have revised and approved the final version.

FUNDING

The MicroHealth group acknowledge the projects AGL2015-64901-R (AEI/FEDER, UE) and RTI2018-096339-B-I00 (MCIU/AEI/FEDER, UE), as well as the grant IDI/2018/000236 from the "Plan for Research, Development and Innovation of the Principado de Asturias 2018–2020" co-financed by the European Regional Development Funds (FEDER). NC-B thanks her FPI fellowship to the MINECO (BES2013-063984). CS acknowledges his Postdoctoral research contract funded by the "Instituto de Investigación Sanitaria del Principado de Asturias" (ISPA). The CIBER-EHD was funded by the Instituto de Salud Carlos III. PD-E and LH-G are predoctoral fellows from University of Granada of "Programa de Doctorado: Medicina Clínica y Salud Pública." We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.601233/full#supplementary-material

REFERENCES

Ale, E. C., Bourin, M. J. B., Peralta, G. H., Burns, P. G., Ávila, O. B., Contini, L., et al. (2019). Functional properties of exopolysaccharide (EPS) extract from *Lactobacillus fermentum* Lf2 and its impact when combined with *Bifidobacterium animalis* INL1 in yoghurt. *Int. Dairy J.* 96, 114–125. doi: 10.1016/j.idairyj.2019.04.014

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., et al. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One* 5:e10667. doi: 10.1371/journal.pone.0010667

Bui, T. P. N., Shetty, S. A., Lagkouvardos, I., Ritari, J., Chamlagain, B., Douillard, F. P., et al. (2016). Comparative genomics and physiology of the butyrate-producing bacterium *Intestinimonas butyriciproducens*. *Environ. Microbiol. Rep.* 8, 1024–1037. doi: 10.1111/1758-2229.12483

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Castaner, O., Goday, A., Park, Y. M., Lee, S. H., Magkos, F., Shiow, S. A. T. E., et al. (2018). The gut microbiome profile in obesity: a systematic review. *Int. J. Endocrino.* 2018:4095789. doi: 10.1155/2018/4095789

Castro-Bravo, N., Hidalgo-Cantabrana, C., Rodríguez-Carvajal, M. A., Ruas-Madiedo, P., and Margolles, A. (2017). Gene replacement and fluorescent labeling to study the functional role of exopolysaccharides in *Bifidobacterium animalis* subsp. lactis. *Front. Microbiol.* 8:1405. doi: 10.3389/fmicb.2017.01405

Castro-Bravo, N., Wells, J. M., Margolles, A., and Ruas-Madiedo, P. (2018). Interactions of surface exopolysaccharides from *Bifidobacterium* and *Lactobacillus* within the intestinal environment. *Front. Microbiol.* 9:2426. doi: 10.3389/fmicb.2018.02426

Clarke, S. F., Murphy, E. F., Nilaweera, K., Ross, P. R., Shanahan, F., O'Toole, P. W., et al. (2012). The gut microbiota and its relationship to diet and obesity: new insights. *Gut Microbes* 3, 186–202. doi: 10.4161/gmic.20168

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Epskamp, S., Cramer, A. O., Waldorp, L. J., Schmittmann, V. D., and Borsboom, D. (2012). qgraph: network visualizations of relationships in psychometric data. *J. Stat. Softw.* 48, 1–18. doi: 10.18637/jss.v048.i04

Fanning, S., Hall, L. J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D., et al. (2012). Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2108–2113. doi: 10.1073/pnas.1115621109

Henke, M. T., Kenny, D. J., Cassilly, C. D., Vlamakis, H., Xavier, R. J., and Clardy, J. (2019). Ruminococcus gnavus, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* 116, 12672–12677. doi: 10.1073/pnas.1904099116

Hevia, A., Milani, Ch, López, P., Cuervo, A., Arboleya, S., Duranti, S., et al. (2014). Intestinal dysbiosis associated with systemic lupus erythematosus. *mBio* 5:e01548-14. doi: 10.1128/mBio.01548-14

Hidalgo-Cantabrana, C., Algieri, F., Rodriguez-Nogales, A., Vezza, T., Martínez-Camblor, P., Margolles, A., et al. (2016). Effect of a ropy exopolysaccharide-producing *Bifidobacterium animalis* subsp. lactis strain orally administered on DSS-induced colitis mice model. *Front. Microbiol.* 7:868. doi: 10.3389/fmicb.2016.00868

Hidalgo-Cantabrana, C., López, P., Gueimonde, M., de los Reyes-Gavilán, C. G., Suárez, A., Magolles, A., et al. (2012). Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria. *Probiotics Antimicro. Prot.* 4, 227–237. doi: 10.1007/s12602-012-9110-2

Hidalgo-Cantabrana, C., Nikolic, M., López, P., Suárez, A., Miljkovic, M., Kojic, M., et al. (2014). Exopolysaccharide-producing *Bifidobacterium animalis* subsp. lactis strains and their polymers elicit different responses on immune cells from

blood and gut associated lymphoid tissue. *Anaerobe* 26, 24–30. doi: 10.1016/j.anaerobe.2014.01.003

Hidalgo-Cantabrana, C., Sánchez, B., Álvarez-Martín, P., López, P., Martínez-Álvarez, N., Delley, M., et al. (2015). A single mutation in the gene responsible for the mucoid phenotype of *Bifidobacterium animalis* subsp. lactis confers surface and functional characteristics. *Appl. Environ. Microbiol.* 81, 7960–7968. doi: 10.1128/AEM.02095-15

Kang, C., Wang, B., Kaliannan, K., Wang, X., Lang, H., Hui, S., et al. (2017). Gut microbiota mediates the protective effects of dietary capsaicin against chronic low-grade inflammation and associated obesity induced by high-dat diet. *mBio* 8:e00470-17.

Kim, K. A., Gu, W., Lee, I. A., Joh, E. H., and Kim, D. H. (2012). High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS One* 7:e47713. doi: 10.1371/journal.pone.0047713

Kushkevych, I., Dordević, D., Vítězovɑ, M., and Kollɑr, P. (2018). Cross-correlation analysis of the *Desulfovibrio* growth parameters of intestinal species isolated from people with colitis. *Biologia* 73, 1137–1143. doi: 10.2478/s11756-018-0118-2

Lahti, L., and Shetty, S. (2017). *Tools for Microbiome Analysis in R. Version 2.1.24*. Available online at: http://microbiome.github.com/microbiome (accessed August 8, 2020).

Liu, G., Chen, H., Chen, J., Wang, X., Gu, Q., and Yin, Y. (2019). Effects of bifidobacteria-produced exopolysaccharides on human gut microbiota in vitro. *Appl. Microbiol. Biotechnol.* 103, 1693–1702. doi: 10.1007/s00253-018-9572-6

Liu, L., Li, H., Xu, R. H., and Li, P. L. (2017). Expolysaccharides from *Bifidobacterium animalis* RH activates RAW 264.7 macrophages through toll-like receptor 4. *Food Agr. Immunol.* 28, 149–161. doi: 10.1080/09540105.2016.1230599

Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., et al. (2019). Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 569, 655–662. doi: 10.1038/s41586-019-1237-9

López, P., Monteserín, D. C., Gueimonde, M., de los Reyes-Gavilán, C. G., Margolles, A., Suárez, A., et al. (2012). Exopolysaccharide-producing *Bifidobacterium* strains elicit different in vitro responses upon interaction with human cells. *Food Res. Int.* 46, 99–107. doi: 10.1016/j.foodres.2011.11.020

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Gueimonde, M., et al. (2013). Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PLoS One* 8:e68739. doi: 10.1371/journal.pone.0068739

Milani, C., Lugli, G. A., Turroni, F., Mancabelli, L., Duranti, S., Viappiani, A., et al. (2014). Evaluation of bifidobacterial community composition in the human gut by means of a targeted amplicon sequencing (ITS) protocol. *FEMS Microbiol. Ecol.* 90, 493–503. doi: 10.1111/1574-6941.12410

Mizuta, M., Maeda, H., Shirkawa, K., Abe, F., Endo, I., Udaka, T., et al. (2016). Perioperative supplementation with bifidobacteria improves postoperative nutritional recovery, inflammatory response, and fecal microbiota in patients undergoing colorectal surgery: a prospective, randomized clinical trial. *Biosci. Microb. Food Heal.* 35, 77–87. doi: 10.12938/bmfh.2015-017

Moya-Pérez, A., Neef, A., and Sanz, Y. (2015). *Bifidobacterium pseudocatenulatum* CECT 7765 reduces obesity-associated inflammation by restoring the lymphocyte-macrophage balance and gut microbiota structure in high-fat diet-fed mice. *PLoS One* 10:e0126976. doi: 10.1371/journal.pone.0126976

Ormerod, K. L., Wood, D. L. A., Lachner, N., Gellatly, S. L., Daly, J. N., Parsons, J. D., et al. (2016). Genomic characterization of the uncultured *Bacteroidales* family S24-7 inhabiting the guts of homeothermic animals. *Microbiome* 4:36. doi: 10.1186/s40168-016-0181-2

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Salazar, N., Binetti, A., Gueimonde, M., Alonso, A., Garrido, P., González del Rey, C., et al. (2011). Safety and intestinal microbiota modulation by the exopolysaccharide-producing strains *Bifidobacterium animalis* IPLA R1 and *Bifidobacterium longum* IPLA E44 orally administered to Wistar rats. *Int. J. Food Microbiol.* 144, 342–351. doi: 10.1016/j.ijfoodmicro.2010.10.016

Salazar, N., Gueimonde, M., de los Reyes-Gavilán, C. G., and Ruas-Madiedo, P. (2016). Exopolysaccharides produced by lactic acid bacteria and bifidobacteria as fermentable substrates by the intestinal microbiota. *Crit. Rev. Food Sci. Nutr.* 56, 1440–1453. doi: 10.1080/10408398.2013.770728

Salazar, N., López, P., Garrido, P., Moran, J., Cabello, E., Gueimonde, M., et al. (2014). Immune modulating capability of two exopolysaccharide-producing *Bifidobacterium* strains in a Wistar rat model. *BioMed Res. Int*. 2014:106290. doi: 10.1155/2014/106290

Schiavi, E., Gleinser, M., Molloy, E., Groeger, D., Frei, R., Ferstl, R., et al. (2016). The surface-associated exopolysaccharide of *Bifidobacterium longum* 35624 plays an essential role in dampening host proinflammatory responses and repressing local TH17 responses. *Appl. Environ. Microbiol.* 82, 7185–7196. doi: 10.1128/AEM.02238-16

Schwager, E., Bielski, C., and Weingart, G. (2019). *Ccrepe: Ccrepe_And_Nc.Score. R Package Version 1.22.0*. Available online at: https://bioconductor.org/packages/ccrpe (accessed August 8, 2020).

Tjalsma, H., Boleij, A., Marchesi, J. R., and Dutilh, B. E. (2012). A bacterial driver–passenger model for colorectal cancer: beyond the usual suspects. *Nat. Rev. Microbiol.* 10, 578–582. doi: 10.1038/nrmicro2819

Van Beek, A. A., Fransen, F., Meijer, B., De Vos, P., Knol, E. F., and Savelkoul, H. F. J. (2018). Aged mice display altered numbers and phenotype of basophils, and bone marrow-derived basophil activation, with a limited role for aging-associated microbiota. *Immun. Ageing* 15:32. doi: 10.1186/s12979-018-0135-6

Wang, L., He, Z., Tian, P., and Wang, G. (2019). "Lactic acid bacteria and host immunity," in *Lactic Acid Bacteria*, ed. W. Chen (Singapore: Springer), 261–296. doi: 10.1007/978-981-13-7832-4_9

Xu, R., Shen, Q., Wu, R., and Li, P. (2017). Structural analysis and mucosal immune regulation of exopolysaccharide fraction from *Bifidobacterium animalis* RH. *Food Agric. Immunol.* 28, 1226–1241. doi: 10.1080/09540105.2017.1333578

Xu, Y., Cui, Y., Yue, F., Liu, L., Shan, Y., Liu, B., et al. (2019). Exopolysaccharides produced by lactic acid bacteria and bifidobacteria: structures, physiochemical functions and applications in the food industry. *Food Hydrocol.* 94, 475–499. doi: 10.1016/j.foodhyd.2019.03.032

Yan, S., Yang, B., Ross, R. P., Stanton, C., Zhang, H., Zhao, J., et al. (2020). *Bifidobacterium longum* subsp. longum YS108R fermented milk alleviates DSS induced colitis via anti-inflammation, mucosal barrier maintenance and gut microbiota modulation. *J. Funct. Foods* 73:104153. doi: 10.1016/j.jff.2020.104153

Yan, S., Yang, B., Zhao, J., Zhao, J., Stanton, C., Ross, R. P., et al. (2019). A ropy exopolysaccharide producing strain *Bifidobacterium longum* subsp. longum YS108R alleviates DSS-induced colitis by maintenance of the mucosal barrier and gut microbiota modulation. *Food Funct.* 10, 1595–1608. doi: 10.1039/c9fo00014c

Zhang, G., and Gao, F. (2017). Quantitative analysis of correlation between AT and GC biases among bacterial genomes. *PLoS One* 12:e0171408. doi: 10.1371/journal.pone.0171408

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 December 2020
doi: 10.3389/fmicb.2020.609734

Development of a Dairy-Free Fermented Oat-Based Beverage With Enhanced Probiotic and Bioactive Properties

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Lactobacillus fermentum PC1 with proven probiotic properties was used to ferment oats with added honey to develop a probiotic beverage with enhanced bioactive ingredients. The viable *Lactobacilli* were enumerated during the fermentation and storage at 4°C, as well as after exposure to simulated gastrointestinal tract conditions. Good survival was noted both during storage as well as when exposed to the *in vitro* digestive tract conditions. Comparative analysis of the antioxidant activity, total phenolic content, and phenolic composition indicated fermentation improved the total antioxidant capacity and phenolic acid concentration. An increase of more than 50% of gallic acid, catechin, vanillic acid, caffeic acid, p-coumaric acid, and ferulic acid was observed in the methanol extracts. Moreover, no significant decrease in the β-glucan content was noted during fermentation and storage. In conclusion, this fermented product has a great potential as a functional food with enhanced probiotic survival and increased bioactive ingredients.

Keywords: *Lactobacillus fermentum* PC1, oats, viability, *in vitro* digestion, antioxidant activity, phenolic content

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Edited by:
Sonia G. Sáyago-Ayerdi, Instituto Tecnológico de Tepic, Mexico

Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *24 September 2020*
Accepted: *17 November 2020*
Published: *03 December 2020*

Citation:
Chen L, Wu D, Schlundt J and Conway PL (2020) Development of a Dairy-Free Fermented Oat-Based Beverage With Enhanced Probiotic and Bioactive Properties. Front. Microbiol. 11:609734. doi: 10.3389/fmicb.2020.609734

INTRODUCTION

Probiotics can impart a range of beneficial effects including improving digestion, strengthening the immune system, and modifying the gut microbiome. It is recognized that the probiotic needs to be viable for maximum benefit, and it is generally accepted that the finished product should contain at least 10^6–10^7 viable cells per ml (CFU per ml; Shori, 2015). Consequently, there has been an increasing interest in improving the survival of probiotics in the finished product, during storage and when consumed and exposed to digestive tract conditions. Traditional fermented dairy foods are frequently used for probiotic delivery, because probiotic strains have been shown to survive well under these conditions. However, there is an increasing demand for non-dairy probiotic foods because of the rise in lactose intolerance, milk allergy, and an interest in low cholesterol content products (Gupta and Abu-Ghannam, 2012). Probiotic fermented non-dairy products have the advantage of being lactose free and having a low cholesterol content (Ranadheera et al., 2017). Cereals, fruits, and vegetable-based probiotic fermented products have received increasing attention in recent years because they can be alternatives

to dairy based products and also because such products often contain complex carbohydrates that can be preferentially utilized by the probiotics (Verspreet et al., 2016). Such carbohydrates, referred to as prebiotics, can promote the growth of probiotics and, thereby, enhance the performance of the probiotic, for example, by inhibiting the growth of potentially pathogenic gut microbes (Verspreet et al., 2016).

In recent years, oats consumption has been linked to numerous health benefits, such as anti-inflammatory and antioxidant activity, and shown to have the potential to reduce the risk of cardiovascular diseases (CVD), type 2 diabetes, gastrointestinal disorders, and cancer (Martínez-Villaluenga and Peñas, 2017). Oats contains bioactive compounds, especially natural antioxidant phenolic compounds (Soycan et al., 2019) and β-glucan (Ho et al., 2016). Thus, oats is becoming a popular matrix of choice for innovative functional probiotic containing foods (Angelov et al., 2018). It has been shown that oats can promote the growth of lactic acid bacteria (Herrera-Ponce et al., 2014; Wu et al., 2018). In addition, there are reports of the optimization of the total phenolic content and antioxidant capacity in oats by fermentation using yeast or bacteria (Duru et al., 2019; Bei et al., 2020). Unfortunately, most studies did not achieve improvements in both probiotic and bioactive properties (Duru et al., 2019) or only focused on one aspect (Hole et al., 2012; Călinoiu et al., 2019; Bei et al., 2020).

Fermentation is the breakdown of carbohydrates, such as starch and sugar, by bacteria and yeast. It is an ancient technique for preserving food. Common fermented foods include kimchi, sauerkraut, kefir, tempeh, kombucha, and yogurt. More recently, many health benefits have been proposed for fermented foods and these included reducing heart disease risk, aiding digestion, and enhancing immunity and weight loss (Şanlier et al., 2019). The aim of this work was to develop a non-dairy fermented beverage that delivered novel fermentation products with both improved probiotic and bioactive properties. In preliminary studies, a significant decrease of the β-glucan content was found in oats when no sugar was added in the fermentation, most probably because the β-glucan is a selective substrate of *Lactobacilli* (Jaskari et al., 1998). Thus, we hypothesized that if we wanted to develop an oat-based probiotic food with both improved probiotic viability and bioactive ingredients, we needed to add sugars to promote the growth of the probiotic strain and enhance fermentation. With the target to develop a healthy functional food, honey was used as a sugar source for the probiotic strain, because honey can have prebiotic activity (Conway et al., 2010) and also contains antioxidant and oligosaccharides and, therefore, is a suitable ingredient of functional foods (Das et al. 2015). Moreover, it has been used in old and modern medical practice due to its antimicrobial, anti-inflammatory, and wound-healing properties (Al-Waili et al., 2011).

In our previous work, *Lactobacillus fermentum* PC1 has been shown to have good capacity for attenuating inflammation, working as an oral adjuvant and influencing the gut microbes (Plant and Conway, 2002; Plant et al., 2003; Esvaran and Conway, 2016; Esvaran and Conway, 2019). It has been shown to tolerate well bile salts and low pH and survive passage through the digestive tract when dosed at the high dose, but less well at a low dose (Gibson and Conway, 1994). Furthermore, we have previously shown that the addition of a prebiotic enhanced the survival of probiotics (O'Riordan et al., 2001). Thus, in this study, we aim to evaluate if the viability of *L. fermentum* PC1 during *in vitro* digestion and storage could be improved through the use of oats and honey as a delivery matrix fermented with *L. fermentum* PC1, and if such fermentation could enhance and maintain the bioactive ingredients in the end product. In addition to the viability testing, the sugar consumption and organic acid production were monitored during fermentation and storage. We also evaluated the bioactive ingredients including antioxidant potential, total phenolic acid content, phenolic composition, and β-glucan.

MATERIALS AND METHODS

Materials, Enzymes, and Strain

High performance liquid chromatography (HPLC)-grade formic acid, acetonitrile, and Folin-Ciocalteau reagent as well as phenolic acid standards including gallic acid, chlorogenic acid, Catechin, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, p-coumaric acid, sinapic acid, ferulic acid, and quercetin were all purchased from Sigma-Aldrich (Singapore).

Whole grain oat flour from Bob's Red Mill was purchased from Lazada online shop in Singapore. *L. fermentum* PC1 (FII511400) was obtained from the CRC Food Industry Innovation culture collection. Human α-amylase (A1031), porcine pepsin (P6887), porcine trypsin (T4799), bovine chymotrypsin (C4129), porcine pancreatic lipase (L0382), and fresh bile salts (B8756) were purchased from Sigma-Aldrich (Singapore). DeMann-Rogosa-Sharpe (MRS) broth and agar were obtained from Sigma-Aldrich (Singapore) and prepared according to the manufacturer's instructions.

Fermentation Conditions

Dry oat flour was autoclaved at 121°C for 10 min. Honey from Sardinia (Miele Di Sardegna honey) was suspended in distilled water (3 g honey in 90 ml distilled water) and pasteurized at 80°C for 10 min. The diluted honey was added aseptically to the sterilized oat flour to yield a final concentration of 10% oats (w/v) and 3% honey (w/v). This mixture was heated to 80°C for 10 min with regular stirring in a thermostatically controlled water bath to ensure homogenization. The mixture was cooled to room temperature before inoculation.

Overnight-grown *L. fermentum* PC1 strain in MRS (pH 6.2 ± 0.2) was inoculated into the oat and honey mixture at 1% (v/v) to yield an initial concentration of about 10^7 per ml. The mixture was fermented in screw cap bottles (250 ml) at 37°C, 150 rpm for 72 h, and subsequently stored at 4°C for 14 days. Samples were taken daily during fermentation and then, after 10 and 14 days of storage, analyzed for viable count of *Lactobacilli*, pH values, and bioactive compounds as

well as viable counts after exposure to simulated digestive tract conditions.

Enumeration of Viable *L. fermentum* PC1

Viable *Lactobacilli* in the fermented product were quantified using the standard plate count method (Conway et al., 1987). In brief, 1 ml of fermented product was used to make 10-fold serial dilutions in PBS. Aliquots of 10 µl of appropriate dilutions were plated in triplicate on MRS agar plates using the drop plate method. The plates were incubated at 37°C for 48 h. Colonies were counted and recorded as log CFU (colony forming units) per ml.

Impact of Simulated Digestive Tract Conditions on Survival of *L. fermentum* PC1 in Fermented Oat

Fermented oat products were exposed to conditions which simulated oral, gastric, and small intestinal digestion conditions according to a published method with slight modifications (Minekus et al., 2014). *L. fermentum* PC1 48 h secondary culture grown in MRS was washed and re-suspended in PBS to about 10^7 per ml and used as control. In summary, samples were initially combined with simulated salivary fluid with a final concentration of human α-amylase of 75 U per ml, and the mixture was incubated for 2 min at pH 7, followed by the addition of simulated gastric fluid with final concentration of porcine pepsin of 2000 U per ml and pH 3, and incubated for 2 h. The mixture was then combined with simulated intestinal fluid and incubated for another 2 h after pH adjustment to 7 and with final concentrations of the following enzymes: porcine trypsin (100 U per ml), bovine chymotrypsin (25 U per ml), porcine pancreatic lipase (2000 U per ml), and fresh bile salts (10 mM). All three steps were performed at 37°C. Aliquots (100 µl) of undigested samples, and samples taken after simulated gastric phase and intestinal phase were serially diluted. Aliquots (10 µl) of appropriate dilutions were plated on MRS agar plates using the drop plate method for enumeration of viable *Lactobacilli*.

Glucose, Fructose, Lactic Acid, and Acetic Acid Assay

Samples taken throughout the experiment were centrifuged at 16,000 × *g* for 30 min, and then supernatants diluted three or four times in 5 mM H_2SO_4 prior to filtration using a 0.45 µm membrane (PES, VWR). Glucose, fructose, lactic acid, and acetic acid concentrations in the supernatant were determined using an Ultra-Fast Liquid Chromatography (Shimadzu) equipped with a refractor index detector (RID-10A). An Aminex® HPX-87H column (Bio-Rad, Singapore) was used for the separation with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min according to the manufacturer's instructions. The temperature of the column oven and RID were set at 50°C and 45°C, respectively. Samples (20 µl) were injected in duplicate for each independent experiment. Concentration of glucose, fructose, lactic acid, and acetic acid were calculated according to a standard curve prepared using concentrations ranging from 0.625 to 20 g/L.

Ultrasound-Assisted Extraction of Phenolic Compounds

Samples were extracted using a slightly modified variant of the published ultrasound-assisted extraction method (Călinoiu et al., 2019). Firstly, 40.0 ± 0.1 mg of freeze-dried sample was accurately weighed, and 1.8 ml of hexane was added to remove fats. The mixtures were vortexed for 30 s, sonicated for 10 min, and vortexed for another 30 min. Then, the mixture was centrifuged for 15 min at 8,000 × *g*, the supernatant was discarded, and the wet samples were dried for 30 min at 30°C in a fume hood. These dried samples were extracted by adding 80:20 methanol:water (1.5 ml) and vortexed until fully suspended and held in a sonic bath for 1 h at 40°C. Samples were then vortexed for another 20 min prior to being centrifuged for 15 min at 10,000 × *g*. This extraction was repeated one more time and the supernatants were combined and evaporated to dryness. The dried extracts were reconstituted in 0.2 ml 80% methanol, vortexed for 5 min, and then centrifuged in 10,000 × *g* for 20 min prior to analyses of antioxidant activity, total phenolic content, and HPLC analysis of phenolic compounds.

Determination of Antioxidant Activity and Total Phenolic Content

Total antioxidant activity was analyzed by Total Antioxidant Capacity Assay Kit (Sigma-Aldrich, MAK187) according to the manufacturer's instructions. Briefly, 5 µl of the methanol extracts were mixed with Cu^{2+} reagent and incubated in darkness at room temperature for 90 min, and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Benchmark Plus Microplate Spectrophotometer System). Trolox solutions ranging from 0 to 20 nmol per well were used to prepare a standard curve. The antioxidant activity was expressed as nmol Trolox equivalents per mg sample (nmol TE/mg).

Total phenolic content was analyzed according to the Folin-Ciocalteu method with modification (Călinoiu et al., 2019). Briefly, 20 µl phenolic extract was mixed with 10 µl Folin-Ciocalteu's reagent for 5 min. Then, 30 µl 20% Na_2CO_3 (w/v) and 140 µl of distilled water were added to the solution to reach a final volume of 200 µl. The mixture was incubated in the dark for 60 min at 300 rpm at room temperature. The plate was centrifuged at 200 × *g* and 120 µl samples of supernatant from each well were transferred to a new plate, and the absorbance was read at 760 nm with a microplate reader (Bio-Rad, Benchmark Plus Microplate Spectrophotometer System). A standard curve was prepared using a series of concentrations of gallic acid ranging from 0 to 8.4 µg per well. The results were expressed as mg gallic acid equivalents per g sample (mg GAE/g).

HPLC Analysis of Phenolic Compounds

The HPLC analyses were carried out using an Agilent 1290 Infinity LC system coupled with photodiode array detector. Separation was performed at 25°C on a ZORBAX RRHD SB-C18 column (1.8 µm, 2.1 mm × 150 mm; Agilent Technologies, Singapore). Two solvents were used for the mobile phase: 0.1% formic acid in distilled water (v/v; solvent A) and 0.1% formic acid

in acetonitrile (v/v; solvent B). The following optimized gradient elution (expressed in % B) was used: 0–2 min, 5% B; 2–6 min, 5–14% B; 6–38 min, 14–40% B; 38–40 min; 40–90% B; 40–42 min, 90–5% B; 40–45 min, 5% B. Aliquots (20 µl) of phenolic extracts from each time point were injected into the column. The flow rate was 0.3 ml/min, and detection was performed at 280 nm. Phenolic acids were identified by comparing their retention times and UV visibility with the standards under same analysis conditions. Quantitation was based on linear calibration curves of phenolic acid standards prepared using concentrations ranging from 0.78125 to 100 mg/L. All measurements were performed in triplicate and all the samples were injected in duplicate. The final concentrations of phenolic acids were expressed as µg/g.

Analysis of β-Glucan

The β-glucan content in the fermented oat product was quantified using the Mixed Link (1–3, 1–4) Beta Glucan kit (Megazyme International, Bray, Ireland) with modifications of method B. In brief, 30 mg (±1%) of freeze-dried fermented oat product was weighed to 0.1 mg precision and transferred into a 2 ml plastic screw cap tube. Firstly, the sample was extracted with 1.75 ml of 50% (v/v) aqueous ethanol to remove free sugars and fats. The extraction was repeated two additional times and the supernatant after centrifugation was discarded. Secondly, the pellet was suspended in 1.0 ml of sodium phosphate buffer (20 mM, pH 6.5) and the tube was incubated at 50°C for 5 min. Thirdly, 50 µl of lichenase (2.5 U) was added and the tube was vortexed and incubated for 1 h at 50°C with stirring at 300 rpm. Then, 0.5 ml of sodium acetate buffer (200 mM, pH 4.0) was added and the mixture was vigorously mixed. After that, the tubes were centrifuged for 10 min at 10,000 × *g*. Aliquots (25 µl) were transferred into 2 ml test tubes, and β-glucosidase (25 µl, 0.05 U) in 50 mM sodium acetate buffer (pH 4.0) was added and then the tubes were incubated at 50°C for 10 min. Finally, GOPOD Reagent (0.75 ml) was added to each tube prior to incubation at 50°C for a further 20 min. Glucose concentrations in the samples were measured at 510 nm against a reagent blank using SPECTRONIC 200 (Thermo Scientific, Singapore). Reagent blanks and D-glucose standards of 1 mg/ml were included in duplicate. For every independent assay, the test was carried out in duplicate with a reaction blank. The final β-glucan content was expressed as g/100 g dry weight (DW).

Statistical Analysis

All the experiments were performed at least as three independent experiments, each analyzed in duplicate. The results are expressed as mean ± SD. Statistical analyses were carried out using either the Student's *t*-test or one-way ANOVA in R (version 3.6.3). Values of $p < 0.05$ were considered statistically significant. Correlations between total phenolic content and antioxidant activity were determined using Pearson's correlation. Correlation coefficient $r > 0.5$ is considered to show a strong positive correlation.

RESULTS AND DISCUSSION

L. fermentum PC1 Growth During Fermentation

In this study, whole grain oat flour was used as a delivery vehicle for *L. fermentum* PC1 by fermenting 10% oat flour supplemented with 3% honey in distilled water with no additional ingredients. *L. fermentum* PC1 was inoculated around 10^7 cell per ml. The growth profile shown in **Table 1** demonstrated that there was a significant increase of viable PC1 cells during the first 24 h (7.96 log CFU/ml) as compared to 0 h (7.12 log CFU/ml), with a slight decrease after 48 h and 72 h to 7.28 log CFU/ml and 7.38 log CFU/ml, respectively. The viable count remained relatively stable during storage at 4°C for 10 days (7.40 log CFU/ml) and 14 days (7.32 log CFU/ml). With this increase of cell growth, there was a significant decrease of pH from 6.26 to 4.12 after 24 h, and a further decrease to 3.93 at 72 h. The pH of the fermented product was constant during storage at 4°C, which reflects the noted stability of organoleptic properties of the product. The stability was probably due to the buffering capacity of other compounds produced in the fermented product, such as acetic acid, lactic acid, and phenolic compounds. These results demonstrated that oat flour and honey supported the growth of *L. fermentum* PC1 and maintained viability during storage at 4°C, with levels above the recommended concentration of 10^6–10^7 CFU per ml (Shori, 2015).

Similarly, probiotic *L. casei* fermented with different oat substrates, including simple and germinated oat, had a final cell growth from 6.3 to 7.12 log CFU/ml (Herrera-Ponce et al., 2014). It has been shown that different fermentation factors including the percent of oats, sugar content, inoculum size, and fermentation time all influence the growth and stability of lactic acid bacteria in these products (Gupta et al., 2010). These workers optimized conditions and showed that with 5.5% oats, 1.25% sugar, and 5% inoculum (9.34 log CFU/ml) and a short fermentation time of 8 h, a high growth of 10.4 log CFU/ml *L. plantarum* ATCC 8014, was obtained; however,

TABLE 1 | Viability of *Lactobacillus fermentum* PC1 and pH value in the fermentation product.

Parameter	Fermentation and storage time	Viable count
Viable count (log CFU/ml)	0 h (day 0)	7.12 ± 0.04[a]
	24 h (day 1)	7.96 ± 0.05[b]
	48 h (day 2)	7.28 ± 0.03[c]
	72 h (day 3)	7.38 ± 0.01[d]
	storage 10-day at 4°C (day 13)	7.40 ± 0.03[d]
	storage 14-day at 4°C (day 17)	7.32 ± 0.06[c]
pH	0 h (day 0)	6.26 ± 0.02[a]
	24 h (day 1)	4.12 ± 0.01[b]
	48 h (day 2)	4.05 ± 0.01[c]
	72 h (day 3)	3.93 ± 0.02[d]
	storage 10-day at 4°C (day 13)	3.94 ± 0.01[d]
	storage 14-day at 4°C (day 17)	3.96 ± 0.01[d]

Results are presented as mean ± SD. Values in the column with different superscript letters (a–d) are significantly different ($p < 0.05$).

there was a reduction of viability of about 0.5 log CFU/ml at 14 days and 0.9 log CFU/ml at 21 days during storage at 4°C (Gupta et al., 2010). For the development of functional foods, not only high cell viability reached during fermentation is critical for maintaining the function of the probiotic and the desired level of acid production, but the stability of viable cells during storage is also important for maintaining the quality of the products. Our results demonstrated that this oat flour and honey fermented product has a great potential for the delivery of viable *L. fermentum* PC1 cells.

Survival of *L. fermentum* PC1 in Simulated Digestive Tract Conditions

The tolerance to gastrointestinal conditions is important for the function of probiotic strains in the gut. There are many different food matrices that have been investigated as probiotic carriers, but only a few studies have evaluated the effect of gastrointestinal conditions on the survival capacity of probiotics (Swieca et al., 2018). With the aim to improve the tolerance of *L. fermentum* PC1 under gastrointestinal conditions, we investigated the survival rate of *L. fermentum* PC1 in the fermented oat product using a standardized *in vitro* method for simulating conditions in the digestive tract (Minekus et al., 2014). The results indicate that both fermented products harvested at 72 h, and the product stored at 4°C for 14 days had significantly higher viable count and recovery rate as compared to control *L. fermentum* PC1 culture grown in MRS and resuspended in PBS (**Figure 1**). For the control *L. fermentum* PC1 48 h culture suspended in PBS, a viable count of 5.22 ± 0.28 log CFU/ml was obtained after simulated saliva and gastric conditions, while the subsequent intestinal simulated conditions resulted in no detectable viable cells (<3 log CFU/ml). In contrast, for *L. fermentum* PC1 fermented oat product, viable counts of 7.57 ± 0.05 log CFU/ml and 7.55 ± 0.03 log CFU/ml after exposure to simulated gastric and intestinal conditions were observed, respectively. For fermented oat product stored at 4°C for 14 days, viable counts of 7.48 ± 0.04 log CFU/ml and 7.46 ± 0.03 log CFU/ml after exposure to simulated gastric and intestinal conditions were observed, respectively (**Figure 1**).

Several studies have reported the improved survival rate of probiotic strains in food carriers. The protection effect depends on the probiotic strains, food matrices used, and fermentation conditions. For example, the inclusion of milk has been shown to significantly improve survival of probiotic *Lactobacilli* when exposed to simulated digestive tract conditions (Lo Curto et al., 2011). This is consistent with an earlier clinical study showing that drinking milk enhanced the survival of probiotic strains and raised the pH in the stomach (Conway et al., 1987). Another study used legumes with *L. plantarum* 299v and obtained a recovery rate of above 80% for lentils and around 40% for mung beans (Swieca et al., 2018). The difference in recovery rate is specific for the strains and the food matrices used. The high buffering capacity of oat flour and honey could probably be one important factor contributing to the high survival rate of the PC1, as previously shown for milk (Conway et al., 1987; Lo Curto et al., 2011). Moreover, sugar residues after fermentation could be another important factor that contributes to the survival rate noted in the present study, since it has been shown that survival of *Lactobacilli* in acidic environments is enhanced in the presence of metabolizable sugars (Corcoran et al., 2005).

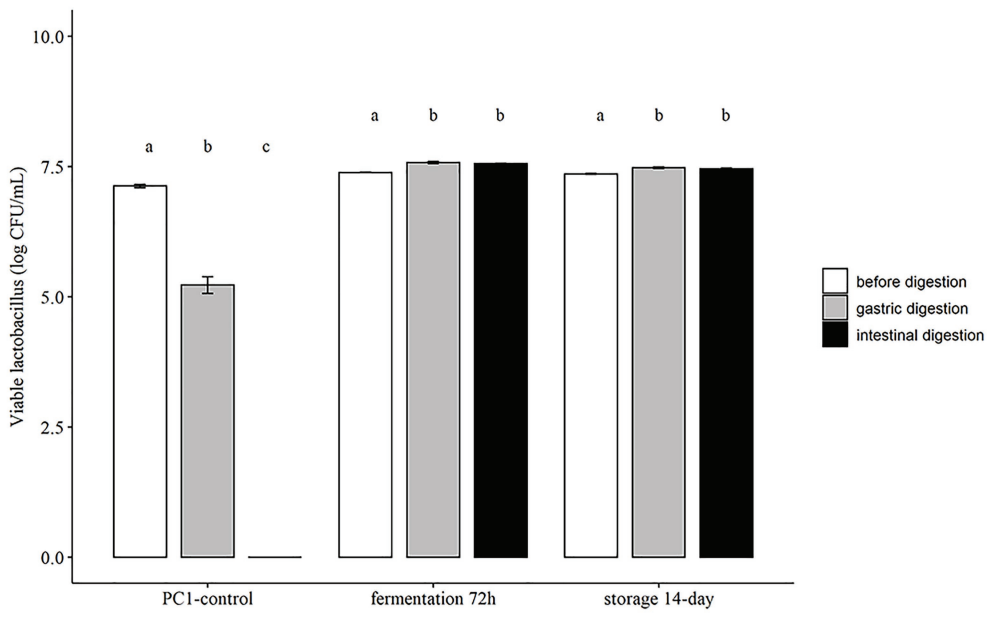

FIGURE 1 | Survival of *Lactobacillus fermentum* PC1 during exposure to simulated gastrointestinal conditions. Results are expressed as Log CFU/ml in mean ± SD. Values of *p* were calculated using *t*-test. The statistics are presented by labeling lowercase letter "a, b, c." The different superscripted letters indicate significant ($p < 0.05$) differences between each other.

Sugars and Organic Acids Content During Fermentation and Storage

The compositions of sugars and organic acids are important indicators of the metabolic state of probiotics during fermentation and storage. Since lactic acid is the major end-product of carbohydrate metabolism by lactic acid bacteria (Abdel-Rahman et al., 2013), the observed decrease in glucose and fructose and increase of lactic acid during fermentation were expected. In addition, we observed an increase in the concentration of acetic acid during fermentation (**Figure 2**). There was a significant decrease of glucose from 9.82 ± 0.03 g/L at 0 h to 6.65 ± 0.07 g/L at 24 h and a further decrease to 5.61 ± 0.07 g/L at the end of fermentation (72 h) with 5.38 ± 0.09 g/L after14 days of storage (**Figure 2**) at 4°C. The concentration of fructose decreased from 10.6 ± 0.03 g/L at 0 h to 9.39 ± 0.17 g/L after 24 h, with no significant decrease during further fermentation and storage. The concentration of lactic acid significantly increased from 0 g/L at 0 h to 2.04 ± 0.09 g/L at 24 h, and further increased to 2.85 ± 0.08 g/L after 72 h fermentation. There was a slight increase of lactic acid to 3.06 ± 0.11 g/L after 14 days of storage at 4°C (**Figure 2**). The concentration of acetic acid increased from 0 g/L at 0 h to 0.51 ± 0.04 g/L after 24 h and increased further to 0.62 ± 0.02 g/L by 3 days of fermentation and to 0.66 ± 0.03 g/L after storage at 4°C for 14 days (**Figure 2**). The observed sugar consumption and acid production were consistent with the growth of the *Lactobacillus* and decrease of pH value. The change of sugar and acids will contribute to the flavor and taste of the final product. Similar changes of sugar and organic acids were also observed in other lactic acid bacteria fermented products, such as fermented apple juice and coconut water beverage (Giri et al., 2018; Li et al., 2018). Similar to these studies, we also observed a substantial amount of residual glucose and fructose (>50%) in the fermented products, even after 3 days of fermentation. Moreover, a further decrease of glucose and an increase of lactic and acetic acids were observed in our product during storage at 4°C, which implies that there was ongoing metabolic activity of the *Lactobacillus* PC1 strain. Some studies indicated that the presence of residual sugars can assist in the continuous metabolic activity of the probiotics in fermented foods (Camargo Prado et al., 2015; Giri et al., 2018) and enhance the survival of *Lactobacilli* in acidic environments (Corcoran et al., 2005). This is consistent with the stable viability of the PC1 strain during storage and in simulated gastrointestinal conditions. This observation also suggests that metabolizable sugar could be one factor contributing to survival of the PC1 during storage and simulated gastrointestinal conditions.

Antioxidant Activity and Total Phenolic Acid Content During Fermentation and Storage

The health benefits of oats and honey have been associated with the presence of antioxidant capacity (Das et al., 2015; Martínez-Villaluenga and Peñas, 2017). Several studies have reported an enhanced antioxidant capacity and related bioactive compounds such as phenolic acids after fermentation (Hole et al., 2012; Călinoiu et al., 2019; Bei et al., 2020). In our study,

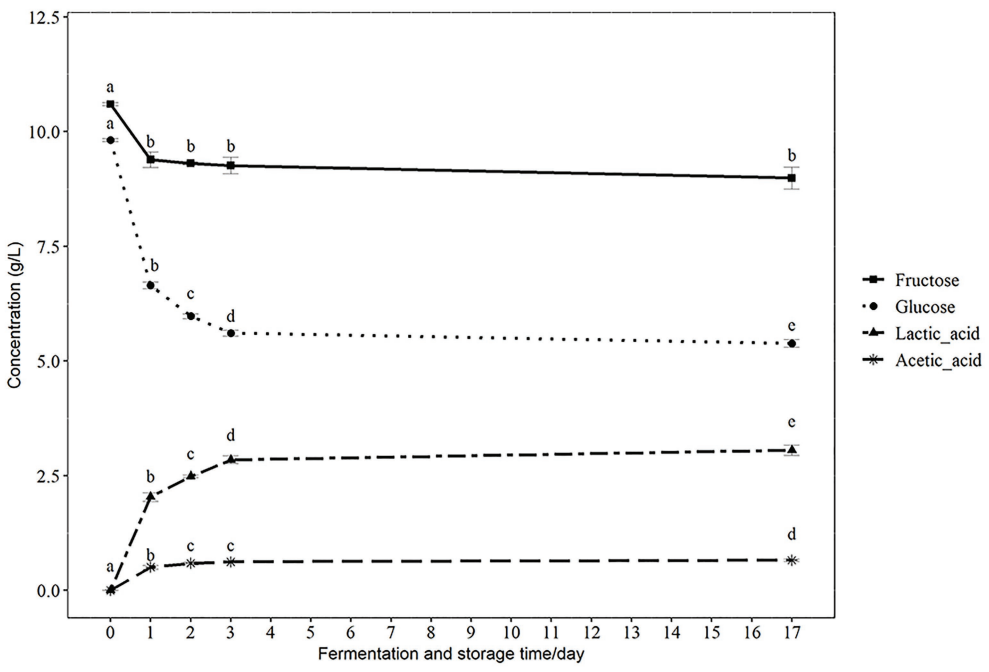

FIGURE 2 | The concentration of glucose, fructose, lactic acid, and acetic acid during fermentation and storage. Results are presented as g/L in mean ± SD. Values of *p* were calculated using *t*-test. The statistics are presented by labeling lowercase letter "a, b, c, d, e." The different superscripted letters indicate significant ($p < 0.05$) differences between each other.

80% aqueous methanol (Călinoiu et al., 2019) was used to extract the methanol soluble antioxidant and phenolic components in the fermented oat product. An increased antioxidant activity was shown using the Cu^{2+} reagent-based antioxidant assay and results expressed as nmol Trolox equivalents per mg sample (nmol TE/mg; **Figure 3A**). The antioxidant activity increased significantly in the fermented product after both 24 h (63.8 ± 2.76 nmol TE/mg) and 48 h (76.4 ± 4.51 nmol TE/mg), compared with that measured at 0 h (57.9 ± 3.52 nmol TE/mg). There was no significant increase at 72 h (76.9 ± 3.49 nmol TE/mg) as compared with 48 h (76.4 ± 4.51 nmol TE/mg). Interestingly, there was a significant increase in antioxidant activity during storage at 4°C, and it reached 95.7 ± 8.07 nmol TE/mg after 14 days of storage (**Figure 3A**), which further supports the suggestion that the probiotic strain was metabolically active during the storage period.

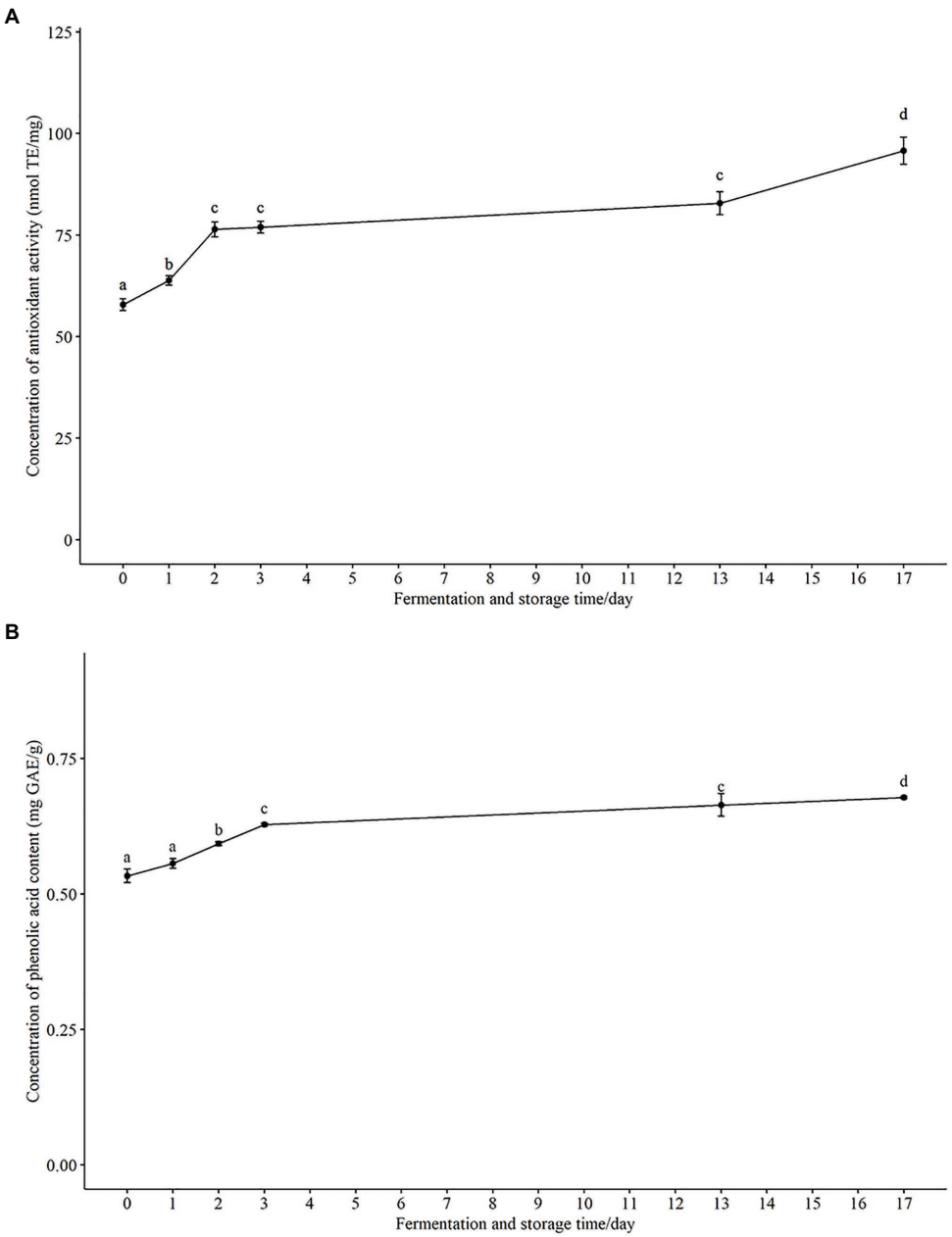

FIGURE 3 | The change of total antioxidant activity and phenolic acid content in fermented product during fermentation and storage. **(A)** Total antioxidant activity are expressed as nmol Trolox equivalents per mg sample (nmol TE/mg). **(B)** Total phenolic acids are expressed as mg gallic acid equivalents per g sample (mg GAE/g). Results are presented as mean ± SD. Values of p were calculated using t-test. The statistics are presented by labeling lowercase letter "a, b, c, d." The different superscripted letters indicate significant ($p < 0.05$) different between each other.

In agreement with the increase of total antioxidant capacity, we observed an increase in the total phenolic content from 0.534 ± 0.03 mg GAE/g at 0 h to 0.628 ± 0.01 mg GAE/g after 72 h fermentation (**Figure 3B**). The total phenolic content continued to increase to 0.678 ± 0.01 mg GAE/g during storage for 14 days at 4°C (**Figure 3B**) as did the total antioxidant activity. Phenolic compounds have been reported to be one of the most important natural antioxidants in oats (Soycan et al., 2019) and honey (Conway et al., 2010), consequently the observed increase of both total phenolic content and total antioxidant capacity would be anticipated. Pearson's correlation coefficient (r) between antioxidant activity and total phenolic content was 0.88. The strong positive correlation suggests that phenolic acid components present in the methanol extracts have a major contribution to the antioxidant activity of the fermented oat product. Similarly, solid-state fermentations of oats with fungi or other lactic acid bacteria have been shown to improve the phenolic composition and antioxidant activity of oats. For example, improved bioavailability of phenolic acids in barley and oats was observed during fermentation with eight probiotic strains (Hole et al., 2012). These workers showed that the improvement of phenolic acids was strain dependent and reported a dramatic increase of free phenolic acids (more than 25 folds) for three probiotic strains, *L. acidophilus*, *L. johnsonii*, and *L. reuteri* (Hole et al., 2012). An 83% increase of total phenolic content was observed in another solid-state yeast fermentation study (Călinoiu et al., 2019). Several probiotic fermentation studies have shown that the increase of antioxidant activity of fermented plant-based food is because there is increased release or synthesis of antioxidant compounds during fermentation (Hur et al., 2014; Călinoiu et al., 2019). The possible enzymes, such as glycoside hydrolase, cellulose, esterase, β-glucosidases, produced by strains during fermentation could enhance the availability of phenolics and other antioxidant compounds (Hole et al., 2012; Călinoiu et al., 2019). During fermentation, these enzymes could potentially release esterified and insoluble-bound phenols in a time-dependent manner (Călinoiu et al., 2019), because the enzyme production is dependent on fermentation time. Our data also indicated that the increased total phenolic content and antioxidant activity were dependent on fermentation time. In future studies, parameters including fermentation time could be optimized to enhance the total antioxidant capacity and total phenolic content. In addition, in order to identify the key enzymes in enhancing the availability of the phenolic content, it will be necessary to determine the changes of relevant enzyme. In short, our results support the finding that fermentation is an effective way to enhance the total antioxidant capacity of the probiotic product.

Changes in Phenolic Composition During Fermentation

Significantly enhanced total and specific phenolic compounds in oats during solid state fermentation have been reported in several studies (Călinoiu et al., 2019, Bei et al., 2020); however, there are limited studies of improvement of specific phenolic compounds in liquid state fermentation. To assess the effect of *L. fermentum* PC1 fermentation on the bioavailability of specific phenolic acids in the fermented oat product, the concentrations of 10 phenolic acids in the methanol soluble extract during fermentation and storage were analyzed by HPLC. As shown in **Table 2**, the phenolic composition was influenced by fermentation. Of the 10 identified phenolic compounds, the levels of gallic acid, catechin, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, and sinapic acid increased during fermentation, with the highest relative increase occurring after 72 h fermentation (gallic acid +58.65%, catechin +92.35%, vanillic acid +67.17%, caffeic acid +117.08, p-coumaric acid +197.87, ferulic acid +116.35, sinapic acid +49.20%). There were no significant changes in the concentration of 4-hydroxybenzoic acid, chlorogenic acid, and quercetin during fermentation. There were significant decreases in the concentration of 4-hydroxybenzoic acid (−10.37%), vanillic acid (−55.02%), caffeic acid (−21.41%), p-coumaric acid (−52.81%), and ferulic acid (−9.02%) during storage for 14 days at 4°C but not below the initial value noted for caffeic acid, p-coumaric acid, and ferulic acid in the control at 0 h. Since the production and activity of possible enzymes involved in the liberation of phenolic compounds is dependent on fermentation time, the changes of phenolic acid production could be explained by the changes of enzyme production and stability (Călinoiu et al., 2019). Moreover, the modulation of phenolic content is highly depended on the microorganisms used. Since the enzymes responsible for the metabolism of phenolic compounds may

TABLE 2 | Phenolic compounds analysis during fermentation and storage.

Phenolic compound	0 h	24 h	48 h	72 h	Storage 10-day	Storage 14-day
Gallic acid	2.93 ± 0.08^{a}	3.28 ± 0.12^{b}	3.62 ± 0.06^{c}	4.64 ± 0.24^{d}	4.81 ± 0.24^{d}	4.76 ± 0.35^{d}
4-Hydroxybenzoic acid	0.94 ± 0.08^{a}	0.91 ± 0.06^{a}	0.88 ± 0.08^{a}	0.90 ± 0.03^{a}	0.83 ± 0.05^{b}	0.81 ± 0.05^{b}
Chlorogenic acid	1.52 ± 0.23^{a}	1.49 ± 0.07^{a}	1.57 ± 0.10^{a}	1.55 ± 0.11^{a}	1.54 ± 0.02^{a}	1.53 ± 0.07^{a}
Catechin	1.27 ± 0.30^{a}	1.93 ± 0.27^{b}	2.22 ± 0.11^{b}	2.43 ± 0.23^{c}	2.37 ± 0.22^{c}	2.43 ± 0.23^{c}
Vanillic acid	0.34 ± 0.01^{a}	0.38 ± 0.07^{a}	0.47 ± 0.05^{b}	0.56 ± 0.04^{c}	0.48 ± 0.13^{b}	0.25 ± 0.06^{d}
Caffeic acid	0.39 ± 0.19^{a}	0.65 ± 0.01^{b}	0.73 ± 0.04^{c}	0.85 ± 0.06^{d}	0.68 ± 0.02^{e}	0.67 ± 0.02^{e}
p-Coumaric acid	0.45 ± 0.05^{a}	0.47 ± 0.02^{a}	1.24 ± 0.26^{b}	1.33 ± 0.16^{b}	0.82 ± 0.16^{c}	0.63 ± 0.07^{d}
Ferulic acid	0.32 ± 0.20^{a}	0.60 ± 0.01^{b}	0.58 ± 0.05^{b}	0.70 ± 0.08^{c}	0.61 ± 0.07^{c}	0.64 ± 0.11^{c}
Sinapic acid	1.42 ± 0.34^{a}	1.56 ± 0.13^{a}	1.82 ± 0.29^{a}	2.12 ± 0.28^{b}	2.15 ± 0.19^{b}	2.14 ± 0.20^{b}
Quercetin	5.08 ± 0.15^{a}	5.25 ± 0.11^{a}	5.37 ± 0.09^{a}	5.47 ± 0.18^{a}	5.43 ± 0.19^{a}	5.45 ± 0.17^{a}

Results are expressed as mean ± SD, Values in the same row followed by different superscript letters (a–e) indicate significant differences ($p < 0.05$) between days of fermentation and storage.

be only expressed in specific strains (Adebo and Gabriela Medina-Meza, 2020), it has been shown that the improvement of phenolic acids (caffeic, p-coumaric, ferulic, and sinapic acids) vary between different probiotic strains (Hole et al., 2012), and changes are associated with the bacterial feruloyl esterase in different strains. The decrease of phenolic acids during storage could be related to the decline of available nutrient and accumulation of waste compounds in the product (Călinoiu et al., 2019). The observed phenolic compounds in our study are mostly in line with other reports, but the concentrations of some phenolic acids differ from other findings. For example, ferulic acid was reported as the major component in several studies (Soycan et al., 2019; Bei et al., 2020), but the concentration of ferulic acid detected here was lower than the level reported in other studies. Since the phenolic composition varies between different oat products and extraction methods (Călinoiu et al., 2019), this could be due to different oat flour and extraction method used. In addition, the added honey (Cheung et al., 2019) also contributed to the phenolic composition of our product. Overall, it was demonstrated that the fermented samples had higher concentrations of several individual phenolic acids as compared with the 0 h non-fermented control.

Content of β-Glucan During Fermentation

Another main bioactive component in oats, β-glucan, has cholesterol-lowering effects at dietary intake levels of at least 3 g per day and may reduce the risk of cardiovascular disease (Ho et al., 2016). It has been reported that there was a loss of β-glucan during harsh processing, such as excessive heat and shearing (Zhu et al., 2016). Therefore, it was of interest in the present study to follow the β-glucan levels during the fermentation and storage to ensure the β-glucan was not lost. There was no change in the β-glucan content during the first 24 h of fermentation with 2.54 ± 0.09 g/100 g DW as compared to 0 h (2.54 ± 0.185 g/100 g DW). There was a slight decrease ($p > 0.05$) to 2.36 ± 0.135 g/100 g DW after 3 days of fermentation, and this level was maintained at 2.26 ± 0.270 g/100 g DW during storage at 4°C for 14 days (**Figure 4**). These results are in agreement with another study that investigated the effect of fermentation on β-glucans in oat sourdough (Lu et al., 2019). These workers reported that the total β-glucan content decreased slightly during fermentation and was stabilized when the lactic acid bacteria counts were almost stable. β-glucan is selectively utilized by bifidobacteria and Lactobacilli in the gut (Jaskari et al., 1998), and thereby produce short chain fatty acids (SCFA) which are linked to beneficial physiological effects (Simpson and Campbell, 2015). Consequently, it is important to retain stable levels of the β-glucan to ensure the fermented oat product has the capacity to support growth of potentially beneficial bacteria and the production of SCFAs.

CONCLUSION

In summary, it is shown that oat flour with added honey promoted the growth and maintained the survival of *L. fermentum* PC1 both during fermentation and storage. The viable count of PC1 was stable (7.32 log CFU/ml) during storage at 4°C for at least 14 days. The survival of the PC1 in the fermented oat exposed to simulated gastrointestinal conditions was significantly enhanced compared to control cells. Furthermore, it was apparent that the PC1 was metabolically active during storage at 4°C for at least 14 days since the content of sugars and acid production continued to change. Moreover, there were

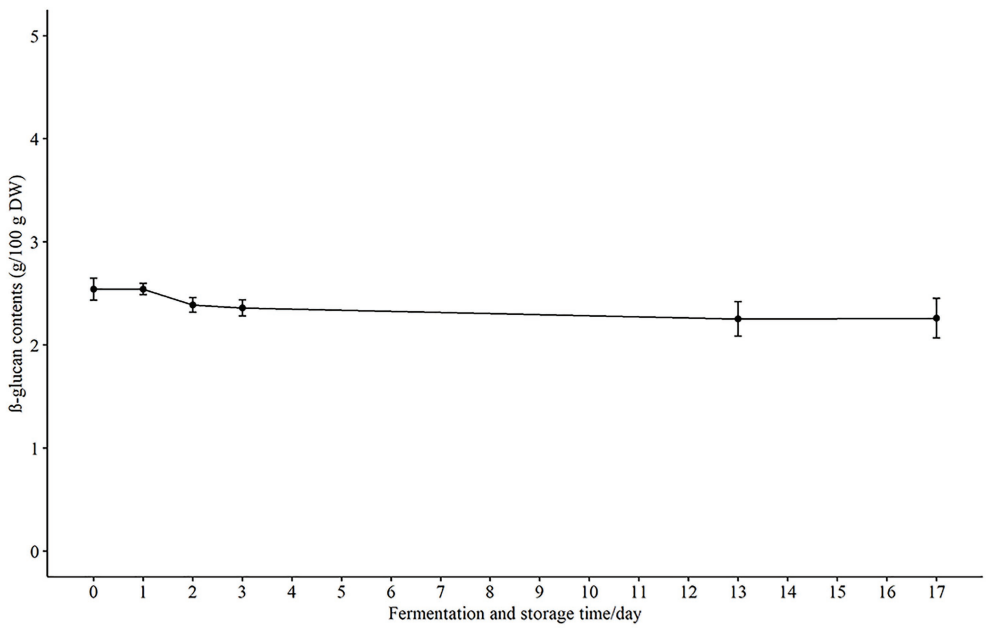

FIGURE 4 | The concentration of β-glucan in fermented product during fermentation and storage. Results are presented as g/100 g DW (dry weight) in mean ± SD.

improvements in antioxidant capacity and phenolic content and no significant decrease of β-glucan. The main phenolic components that were detected in higher amounts in the methanol extracts were gallic acid, catechin, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, and sinapic acid. Thus, this study demonstrated that fermentation of oat flour with added honey and *L. fermentum* PC1 could be a potentially valuable probiotic food with both improved levels of probiotic and bioactive components.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LC and DW have contributed equally to this work in participating in the design of the study, analysis, and interpretation of the data and drafted the manuscript. JS participated in interpretation of the data and revised the draft critically. PC participated in conception and design of the study, analysis, and interpretation of the data and revised the draft critically. All authors accepted and approved the submitted version.

FUNDING

This work was supported by Ageing Research Institute for Society and Education (ARISE), Nanyang Technological University, Singapore. DW was supported from the funding of China Scholarship Council (grant no. 201906240301).

ACKNOWLEDGMENTS

We sincerely thank Kaliyamoorthy Eganathan in SCELSE (Singapore Centre for Environmental Life Sciences Engineering) for helping us to run the sugars and organic acids analysis by HPLC-DAD.

REFERENCES

Abdel-Rahman, M. A., Tashiro, Y., and Sonomoto, K. (2013). Recent advances in lactic acid production by microbial fermentation processes. *Biotechnol. Adv.* 31, 877–902. doi: 10.1016/j.biotechadv.2013.04.002

Adebo, O. A., and Gabriela Medina-Meza, I. (2020). Impact of fermentation on the phenolic compounds and antioxidant activity of whole cereal grains: a mini review. *Molecules* 25:927. doi: 10.3390/molecules25040927

Al-Waili, N. S., Salom, K., Butler, G., and Al Ghamdi, A. A. (2011). Honey and microbial infections: a review supporting the use of honey for microbial control. *J. Med. Food* 14, 1079–1096. doi: 10.1089/jmf.2010.0161

Angelov, A., Yaneva-Marinova, T., and Gotcheva, V. (2018). Oats as a matrix of choice for developing fermented functional beverages. *J. Food Sci. Technol.* 55, 2351–2360. doi: 10.1007/s13197-018-3186-y

Bei, Q., Wu, Z., and Chen, G. (2020). Dynamic changes in the phenolic composition and antioxidant activity of oats during simultaneous hydrolysis and fermentation. *Food Chem.* 305:125269. doi: 10.1016/j.foodchem.2019.125269

Călinoiu, L. F., Cătoi, A. -F., and Vodnar, D. C. (2019). Solid-state yeast fermented wheat and oat bran as a route for delivery of antioxidants. *Antioxidants* 8:372. doi: 10.3390/antiox8090372

Camargo Prado, F., De Dea Lindner, J., Inaba, J., Thomaz-Soccol, V., Kaur Brar, S., and Soccol, C. R. (2015). Development and evaluation of a fermented coconut water beverage with potential health benefits. *J. Funct. Foods* 12, 489–497. doi: 10.1016/j.jff.2014.12.020

Cheung, Y., Meenu, M., Yu, X., and Xu, B. (2019). Phenolic acids and flavonoids profiles of commercial honey from different floral sources and geographic sources. *Int. J. Food Prop.* 22, 290–308. doi: 10.1080/10942912.2019.1579835

Conway, P. L., Gorbach, S. L., and Goldin, B. R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci.* 70, 1–12.

Conway, P. L., Stern, R., and Tran, L. (2010). *The value-adding potential of prebiotic components of Australian honey*. Rural Industries Research and Development Corporation.

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2005). Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Appl. Environ. Microbiol.* 71, 3060–3067. doi: 10.1128/AEM.71.6.3060-3067.2005

Das, A., Datta, S., Mukherjee, S., Bose, S., Ghosh, S., and Dhar, P. (2015). Evaluation of antioxidative, antibacterial and probiotic growth stimulatory activities of *Sesamum indicum* honey containing phenolic compounds and lignans. *LWT Food Sci. Technol.* 61, 244–250. doi: 10.1016/j.lwt.2014.11.044

Duru, K. C., Kovaleva, E., Danilova, I., and Belousova, A. (2019). Production and assessment of novel probiotic fermented oat flour enriched with isoflavones. *LWT* 111, 9–15. doi: 10.1016/j.lwt.2019.04.102

Esvaran, M., and Conway, P. L. (2016). Factors that influence the immunological adjuvant effect of *Lactobacillus fermentum* PC1 on specific immune responses in mice to orally administered antigens. *Vaccines* 4:24. doi: 10.3390/vaccines4030024

Esvaran, M., and Conway, P. L. (2019). *Lactobacillus fermentum* PC1 has the capacity to attenuate joint inflammation in collagen-induced arthritis in DBA/1 mice. *Nutrients* 11:785. doi: 10.3390/nu11040785

Gibson, S. A. W., and Conway, P. L. (1994). "Recovery of a probiotic organism from human Faces after Oral dosing" in *Human health: The contribution of microorganisms*. ed. A. W. Robards (London: Springer London), 119–143.

Giri, S. S., Sukumaran, V., Sen, S. S., and Park, S. C. (2018). Use of a potential probiotic, *Lactobacillus* casei L4, in the preparation of fermented coconut water beverage. *Front. Microbiol.* 9:1976. doi: 10.3389/fmicb.2018.01976

Gupta, S., and Abu-Ghannam, N. (2012). Probiotic fermentation of plant based products: possibilities and opportunities. *Crit. Rev. Food Sci. Nutr.* 52, 183–199. doi: 10.1080/10408398.2010.499779

Gupta, S., Cox, S., and Abu-Ghannam, N. (2010). Process optimization for the development of a functional beverage based on lactic acid fermentation of oats. *Biochem. Eng. J.* 52, 199–204. doi: 10.1016/j.bej.2010.08.008

Herrera-Ponce, A., Nevárez-Morillón, G., Ortega-Rívas, E., Pérez-Vega, S., and Salmerón, I. (2014). Fermentation adaptability of three probiotic *Lactobacillus* strains to oat, germinated oat and malted oat substrates. *Lett. Appl. Microbiol.* 59, 449–456. doi: 10.1111/lam.12302

Ho, H. V. T., Sievenpiper, J. L., Zurbau, A., Blanco Mejia, S., Jovanovski, E., Au-Yeung, F., et al. (2016). The effect of oat β-glucan on LDL-cholesterol, non-HDL-cholesterol and apoB for CVD risk reduction: a systematic review and meta-analysis of randomised-controlled trials. *Br. J. Nutr.* 116, 1369–1382. doi: 10.1017/S000711451600341X

Hole, A. S., Rud, I., Grimmer, S., Sigl, S., Narvhus, J., and Sahlstrøm, S. (2012). Improved bioavailability of dietary phenolic acids in whole grain barley and oat groat following fermentation with probiotic *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri*. *J. Agric. Food Chem.* 60, 6369–6375. doi: 10.1021/jf300410h

Hur, S. J., Lee, S. Y., Kim, Y. -C., Choi, I., and Kim, G. -B. (2014). Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chem.* 160, 346–356. doi: 10.1016/j.foodchem.2014.03.112

Jaskari, J., Kontula, P., Siitonen, A., Jousimies-Somer, H., Mattila-Sandholm, T., and Poutanen, K. (1998). Oat beta-glucan and xylan hydrolysates as selective

substrates for Bifidobacterium and *Lactobacillus* strains. *Appl. Microbiol. Biotechnol.* 49, 175–181. doi: 10.1007/s002530051155

Li, Z., Teng, J., Lyu, Y., Hu, X., Zhao, Y., and Wang, M. (2018). Enhanced antioxidant activity for apple juice fermented with *Lactobacillus plantarum* ATCC14917. *Molecules* 24:51. doi: 10.3390/molecules24010051

Lo Curto, A., Pitino, I., Mandalari, G., Dainty, J. R., Faulks, R. M., and John Wickham, M. S. (2011). Survival of probiotic *Lactobacilli* in the upper gastrointestinal tract using an in vitro gastric model of digestion. *Food Microbiol.* 28, 1359–1366. doi: 10.1016/j.fm.2011.06.007

Lu, J., Shan, L., Xie, Y., Min, F., Gao, J., Guo, L., et al. (2019). Effect of fermentation on content, molecule weight distribution and viscosity of β-glucans in oat sourdough. *Int. J. Food Sci. Technol.* 54, 62–67. doi: 10.1111/ijfs.13902

Martínez-Villaluenga, C., and Peñas, E. (2017). Health benefits of oat: current evidence and molecular mechanisms. *Curr. Opin. Food Sci.* 14, 26–31. doi: 10.1016/j.cofs.2017.01.004

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct.* 5, 1113–1124. doi: 10.1039/C3FO60702J

O'Riordan, K., Andrews, D., Buckle, K., and Conway, P. (2001). Evaluation of microencapsulation of a Bifidobacterium strain with starch as an approach to prolonging viability during storage. *J. Appl. Microbiol.* 91, 1059–1066. doi: 10.1046/j.1365-2672.2001.01472.x

Plant, L. J., and Conway, P. L. (2002). Adjuvant properties and colonization potential of adhering and non-adhering *Lactobacillus* spp. following oral administration to mice. *FEMS Immunol. Med. Microbiol.* 34, 105–111. doi: 10.1111/j.1574-695X.2002.tb00610.x

Plant, L., Lam, C., Conway, P. L., and O'Riordan, K. (2003). Gastrointestinal microbial community shifts observed following oral administration of a *Lactobacillus fermentum* strain to mice. *FEMS Microbiol. Ecol.* 43, 133–140. doi: 10.1111/j.1574-6941.2003.tb01052.x

Ranadheera, C. S., Vidanarachchi, J. K., Rocha, R. S., Cruz, A. G., and Ajlouni, S. (2017). Probiotic delivery through fermentation: dairy vs. non-dairy beverages. *Fermentation* 3:67. doi: 10.3390/fermentation3040067

Şanlier, N., Gökcen, B. B., and Sezgin, A. C. (2019). Health benefits of fermented foods. *Crit. Rev. Food Sci. Nutr.* 59, 506–527. doi: 10.1080/10408398.2017.1383355

Shori, A. B. (2015). The potential applications of probiotics on dairy and non-dairy foods focusing on viability during storage. *Biocatal. Agric. Biotechnol.* 4, 423–431. doi: 10.1016/j.bcab.2015.09.010

Simpson, H. L., and Campbell, B. J. (2015). Review article: dietary fibre-microbiota interactions. *Aliment. Pharmacol. Ther.* 42, 158–179. doi: 10.1111/apt.13248

Soycan, G., Schär, M. Y., Kristek, A., Boberska, J., Alsharif, S. N. S., Corona, G., et al. (2019). Composition and content of phenolic acids and avenanthramides in commercial oat products: are oats an important polyphenol source for consumers? *Food Chem.* 3:100047. doi: 10.1016/j.fochx.2019.100047

Swieca, M., Kordowska-Wiater, M., Pytka, M., Gawlik-Dziki, U., Bochnak, J., Zlotek, U., et al. (2018). *Lactobacillus plantarum* 299V improves the microbiological quality of legume sprouts and effectively survives in these carriers during cold storage and in vitro digestion. *PLoS One* 13:e0207793. doi: 10.1371/journal.pone.0207793

Verspreet, J., Damen, B., Broekaert, W. F., Verbeke, K., Delcour, J. A., and Courtin, C. M. (2016). A critical look at prebiotics within the dietary fiber concept. *Annu. Rev. Food Sci. Technol.* 7, 167–190. doi: 10.1146/annurev-food-081315-032749

Wu, H., Rui, X., Li, W., Xiao, Y., Zhou, J., and Dong, M. (2018). Whole-grain oats (*Avena sativa* L.) as a carrier of lactic acid bacteria and a supplement rich in angiotensin I-converting enzyme inhibitory peptides through solid-state fermentation. *Food Funct.* 9, 2270–2281. doi: 10.1039/C7FO01578J

Zhu, F., Du, B., and Xu, B. (2016). A critical review on production and industrial applications of beta-glucans. *Food Hydrocoll.* 52, 275–288. doi: 10.1016/j.foodhyd.2015.07.003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 December 2020
doi: 10.3389/fmicb.2020.597946

Isolation of Novel Probiotic *Lactobacillus* and *Enterococcus* Strains From Human Salivary and Fecal Sources

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Probiotics are non-pathogenic microorganisms that can interact with the gastrointestinal microbiota. They have numerous beneficial health effects that include enhancement of the host immune response, antiallergic, antimicrobial, anti-cancer, and anti-inflammatory properties. Probiotics are capable of restoring the impaired microbiome of a dysbiotic gut. They can be isolated from different environments. However, it is frequently suggested that probiotics for human use should come from human sources. The objective of this study was to isolate and characterize novel probiotic strains from the saliva and feces of healthy human individuals. To meet the criteria for probiotic attributes, the isolates were subjected to numerous standard morphological and biochemical tests. These tests included Gram staining, catalase tests, antibiotic susceptibility testing, hemolytic and antagonistic evaluation, tolerance tests involving temperature, NaCl levels, pH and bile salts, adherence ability assays, and genotypic characterization involving 16S rRNA gene sequencing. From 26 saliva and 11 stool samples, 185 microbial strains were isolated. Based on morphological and biochemical characteristics, 14 potential probiotic candidates were selected and identified genotypically. The new strains belonged to *Lactobacillus fermentum, Enterococcus faecium*, and *Enterococcus hire*. The selected strains were non-hemolytic, showed high tolerance to low pH and bile salts, and strong adherence abilities. Furthermore, the strains displayed a wide range of antimicrobial activities, particularly against antibiotic-resistant pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA). Moreover, five of the selected isolates demonstrated antiproliferative features against human colon cancer cell line (Caco-2). The results of this investigation confirm the diversity of microbial populations in the human gut and saliva, and since these strains are of human origin, they will highly likely display maximal activities in food and drugs set for human use. Hence, the new strains of this study require additional *in vivo* experiments to assess their health-promoting effects.

Keywords: probiotics, saliva, feces, *Lactobacillus*, *Enterococcus*, microbiome, lactic acid bacteria

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *26 August 2020*
Accepted: *11 November 2020*
Published: *04 December 2020*

Citation:
Bazireh H, Shariati P, Azimzadeh Jamalkandi S, Ahmadi A and Boroumand MA (2020) Isolation of Novel Probiotic Lactobacillus and Enterococcus Strains From Human Salivary and Fecal Sources. Front. Microbiol. 11:597946. doi: 10.3389/fmicb.2020.597946

INTRODUCTION

Probiotics are live microorganisms that can confer health benefits to the host when consumed in adequate amounts FAO/WHO (2002). In fact, probiotics have recently been developed that can balance and restore the human gut microbiome inflicted with dysbiosis (Kumar et al., 2020). Many probiotics are lactic acid-producing bacteria (LAB) that are Gram-positive and catalase-negative. The two most common genera of probiotics are *Lactobacillus* and *Bifidobacterium,* which have been shown to have beneficial roles in human health.

The use of probiotics not just as supplements but as actual treatment strategies for various diseases has now become more prevalent and the current focus of attention in the scientific and medical communities. The multidimensional effects of probiotics are currently being evaluated in many fields of medicine that include infectious diseases (Anwar et al., 2020; d'Ettorre et al., 2020; Silva et al., 2020; Tan et al., 2020), the immune system (Gill et al., 2000; Dargahi et al., 2020), chronic diseases such as cancer (Haghshenas et al., 2014; Nami et al., 2015), cardiovascular (Ettinger et al., 2014; Gómez-Guzmán et al., 2015; Daliri et al., 2017), neurodegenerative (Westfall et al., 2017), inflammatory diseases (Plaza-Díaz et al., 2017), and diabetes (Kocsis et al., 2020).

Accordingly, this has culminated in the search for new bacterial strains with numerous inherent attributes that can be of potential use in the treatment of many ailments and disorders. For example, the localized use of the probiotic *Lactobacillus plantarum* ATCC 10241 strain in a burn model was found to interfere with *Pseudomonas aeruginosa*, stimulating phagocytosis of this pathogen by tissue phagocytes, decreasing apoptosis, and thereby improving tissue repair (Valdéz et al., 2005). In recent years with the emergence of antibiotic resistance, a lot of emphasis has been placed on investigating probiotics and their products as alternatives to antibiotics. The antagonistic activity of probiotics against pathogens is brought about by a series of mechanisms that include, competitive exclusion of pathogens, boosting the function of the intestinal barrier, and producing effective antimicrobial compounds such as peptides (Fijan, 2016; Besser et al., 2019). Hence, numerous *Lactobacillus* strains have been found to inhibit the growth of many different types of multi-drug resistant bacterial pathogens, such as MRSA (methicillin resistant *Staphylococcus aureus*), *Streptococcus mutans*, *Escherichia coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Shigella* spp. and *Clostridium difficile* (McFarland, 2015; Kumar et al., 2016; Kang et al., 2017; Chen et al., 2019; Nami et al., 2019b).

In another study, the cholesterol removal capacity of *L. plantarum* YS5 *in vitro* was shown to reduce cholesterol levels by 84%. Moreover, probiotic supplementation was found to decrease serum total cholesterol, low density lipoprotein cholesterol, and triglyceride levels in male Wistar rats (Nami et al., 2019a). In other research involving colon cancer, the administration of *Lactobacillus acidophilus* ATCC 314 and *Lactobacillus fermentum* NCIMB 5221 in the murine colon cancer model was found to reduce or stop the growth of tumors, by stimulating an antitumor immune response (Kahouli et al., 2017). Many studies have shown that certain specific probiotics exhibit anticarcinogenic activities and contribute to the prevention of colon cancer through host-dependent mechanisms. One such mechanism involves the production of metabolites such as SCFAs, acetate, propionate and butyrate by a number of probiotic *Lactobacillus*, *Bifidobacterium,* and *Streptococcus* strains, showing positive effects on immune and epithelial cells (Ganapathy et al., 2013; Singh et al., 2014; Gao et al., 2017; Drago, 2019).

Probiotics can be found in many environments such as dairy products, fermented, food and humans. However, the use of probiotics of human origin for use in humans is frequently proposed (Sanders, 2008; Kumar et al., 2020).

The aim of this study is to identify novel indigenous bacterial strains from healthy human individuals that can be used as potential probiotics for the treatment and prevention of various human ailments. In order to be recognized as potential probiotics, bacterial strains that are isolated from various sources must meet specific criteria. For this purpose, a series of standard tests are usually carried out to identify and characterize potential probiotic strains. These tests include evaluating the ability to survive under harsh conditions, e.g., low pH, the presence of antibacterial activity, ability to adhere to epithelial cells, demonstrating non-hemolytic activity, and lacking antibiotic resistance genes.

MATERIALS AND METHODS

Materials, Reagents and Strains

Standard strains were purchased from the Iranian Research Organization for Science and Technology (IROST), and culture media were obtained from Ibresco, Zist Kavosh Iranian Co, Iran. All antibiogram disks were provided by Padtan Teb Co. (Iran) including gentamycin, cefixime, penicillin, chloramphenicol, streptomycin, erythromycin, ampicillin, trimethoprim, kanamycin, vancomycin, rifampin, azithromycin, and clindamycin. Also, molecular detection was carried out using the PCR master mix kit (Ampliqon, Denmark), and primers (synthesized by Taq Copenhagen Co, Denmark). For cell culture experiments, reagents were obtained from DNAbiotech Co. (Iran).

Sampling

Twenty six saliva and 11 stool samples were collected from healthy human individuals. People were informed regarding the study, and written consent forms were provided. This study was approved by the ethics committee at the National Institute of Genetic engineering and Biotechnology (NIGEB, Tehran, Iran) and registered as IR.NIGEB.EC.1398.12.3.B. Samples were transported to the laboratory on ice and were immediately diluted with peptone water, spread onto de Man-Rogosa-Sharpe (MRS) agar medium and Brain Heart Infusion (BHI) agar, then incubated for 48–72 h at 37°C under aerobic and microaerophilic (by using an anaerobic jar) conditions.

Biochemical and Morphological Characterization

Morphological characterization was carried out using the Gram staining technique, and biochemical characterization was performed using the catalase test and analysis of carbohydrate fermentation profiles. Physiological tests included the ability to grow in the presence of NaCl [3% and 4.5% (w/v)], and also at temperatures of 15°C and 45°C. All catalase-negative and Gram-positive bacilli or cocci, the morphology of which was similar to LAB bacteria were classified as potential probiotic strains.

Hemolytic Activity

Fresh bacterial cultures were streaked onto blood agar media [containing 5–10% sheep blood (Zist Royesh Co, Iran)] and incubated for 24 h at 37°C. The isolates were then examined for the presence of clear zones surrounding the colonies. Clear zones are considered as beta hemolysis, greenish zones as alpha hemolysis and the absence of zones indicating no hemolysis is known as gamma hemolysis. Colonies showing beta or alpha hemolysis were excluded, and only those with gamma hemolysis were selected (Halder et al., 2017).

Survival Under Low pH

In order to determine the acid tolerance of the bacterial isolates, a procedure was carried out in accordance with standard protocols, but with some minor modifications (Vernazza et al., 2006; Haghshenas et al., 2016). Briefly, fresh overnight bacterial cells were harvested by centrifugation and inoculated at 1% (v/v) into MRS broth (pH 3). The cultures were incubated for 3 h at 37°C. Thereafter, culture samples were removed at 0 and 3 h, and spread onto MRS agar plates, which were then incubated at 37°C. Survival rate was measured at 0 and 3 h after incubation using the colony count procedure.

Bile Salt Tolerance

This test was conducted according to the method by Nami et al. (2019b), but with some minor changes. In brief, overnight bacterial cultures were inoculated at 1% (v/v) into both MRS broth media (control) and MRS broth containing 0.3% (w/v) oxgall (Ibresco Co, Iran). They were both incubated for 4 h at 37°C, and the optical density (OD) of the cultures was then measured at 600 nm. Subsequently, the percentage of growth inhibition was determined with the following formula:

$$\text{Inhibition\%} = \left(\text{Growth in control} - \text{Growth in oxgall} / \text{Growth in control}\right) \times 100 \quad (1)$$

Antagonistic Activity Against Pathogens

To detect the LAB inhibitory properties against chosen pathogens, the well diffusion assay method was used (Alkalbani et al., 2019; Chen et al., 2019). Briefly, bacterial isolates cultured at 37°C for 24–48 h were centrifuged for 10 min at 10,000 rpm, and the resulting supernatants were then separated and used against ten pathogenic bacterial and fungal strains including, *S. aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, *P. aeruginosa* ATCC 27853, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Escherichia coli* (*E. coli*) ATCC 25922, *S. mutans* ATCC 35668, *Listeria monocytogenes* ATCC 13932, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, and *Candida albicans* ATCC 10231. After 24 h of incubation, the inhibition zones around the wells were measured. Each test was conducted in triplicate.

Antibiotic Susceptibility Test

The antibiotic susceptibility test was conducted using the disk diffusion assay method. Fresh overnight cultures of bacterial isolates were spread onto MRS or BHI agar plates, and 13 antibiogram disks were then carefully placed on the agar plates, which were subsequently incubated at 37°C for 24 h. The antibiotic disks consisted of gentamycin (10 μg), cefixime (5 μg), penicillin (10 μg), chloramphenicol (30 μg), streptomycin (10 μg), erythromycin (15 μg), ampicillin (10 μg), trimethoprim (5 μg), kanamycin (30 μg), vancomycin (30 μg), rifampin (5 μg), azithromycin (15 μg), and clindamycin (2 μg). Finally, results were reported according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Kook et al., 2019).

Adhesion Ability

The human colon carcinoma cell line (Caco-2; kindly provided by NIGEB) was grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM; DNAbiotech Co, Iran) supplemented with 10% FBS and 1% Penicillin-Streptomycin, at 37°C with a 5% CO_2 atmosphere. The medium was changed every other day until the cell confluency of 70–80% was reached. The cells were then trypsinized, counted (4×10^5 cells/mL) and transferred to a 24-well dish. The absorbance of the freshly prepared bacterial cultures was adjusted to 0.5 McFarland using DMEM. Then, 100 microliters of bacterial suspension were added to each well followed by incubation at 37°C for 2 h. Afterward, cells were washed twice with phosphate buffer saline (PBS), fixed with methanol and stained with crystal violet for 5 min. Adherent cell numbers were counted as outlined previously by (Fernández et al., 2003).

Molecular Identification

The bacterial isolates that fulfilled the selection criteria for probiotics were finally chosen as probiotic candidates to be identified genotypically using the 16S rRNA gene amplification method.

The PCR reaction mixture with a total volume of 25 microliters, consisted of 10 pmol primers, and the PCR master mix reaction mixture. The following universal primers; 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) were used in the reaction.

PCR program in the thermal cycler (peQlab, United States) was comprised of initial denaturation at 95°C for 10 min followed by 35 cycles containing the second denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min and 30 s, followed by a final extension step at 72°C for 10 min.

PCR products were detected and visualized by agarose gel electrophoresis (1% w/v) and subsequently sequenced. The resulting Sanger sequencing data were employed by the

basic local alignment search tool (BLAST) to obtain sequence similarities. Thereafter, the sequences were registered in the NCBI[1] database and assigned with accession numbers.

Biofilm Production

The potential ability of probiotic strains to form biofilm was investigated, as previously reported by Pérez Ibarreche et al. (2014). After 24 h of incubation at 37°C, the OD of the isolates was measured at 570 nm using an ELISA microplate reader (Biotech, United States). Comparison of the strains with the negative control (MRS and PBS), revealed the strains competency in biofilm formation. Each test was conducted in three experiments and the final cut- off was considered as non-biofilm formation (OD $\leq$ ODc [ODc: the OD of the control]), week biofilm formation (ODc $<$ OD $\leq 2 \times$ ODc), modest biofilm formation ($2 \times$ ODc $<$ OD $\leq 4 \times$ ODc), and strong Biofilm formation ($4 \times$ ODc $<$ OD; ODc is the optical density of the control; Borges et al., 2012).

Auto-Aggregation

The auto-aggregation test evaluates the ability of isolates to adhere to the intestine, exerting antipathogenic effects (Krausova et al., 2019). This test was performed using the method by Xu et al. (2009). Fresh cultures of bacterial isolates (grown for 16–18 h) were washed twice with PBS, and the optical densities of the resulting bacterial suspensions were adjusted to 0.5 McFarland at 600 nm. They were subsequently incubated for 2 h at 37°C, thereafter the upper phase was removed, and its OD was measured. Finally, the auto-aggregation percentage was determined by using the following formula (A0 = Initial OD, At = OD after 2 h).

$$\text{Auto} - \text{aggregation}\ (\%) = (\text{OD A0} - \text{OD At/A0}) \times 100$$

Hydrophobicity

To further assess the adhesion abilities of the probiotic isolates, the hydrophobicity of the isolates was measured using the microbial adhesion to hydrocarbons (MATH) method, as described by Vinderola et al. (2004). In short, an overnight culture of the isolates was washed twice using PBS, and their optical densities were then adjusted to 0.5–0.6 at 600 nm (A0). One milliliter of xylene was added to each suspension and vortexed vigorously for 1 min. Then the mixture was incubated at 37°C for 1 h. After incubation and phase separation, the aqueous phase was carefully removed to measure its absorbance (At). Hydrophobicity percentage was calculated with the formula presented below.

$$\text{Hydrophobicity}\ (\%) = (1 - \text{At/A0}) \times 100$$

MTT Assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to show the cytotoxic effects of bacterial culture-free supernatants on CaCo-2 cell lines as pointed out by (Chen et al., 2017). In short, 10^4 cells were seeded in 96 microtiter plates and were allowed to attach to the bottom of the plate. Then, different concentrations (25 and 100 microliters) of the fresh bacterial culture-free supernatants were added to each well. After 24, 48, and 72 h of incubation at 37°C with a 5% CO_2 atmosphere, MTT solution was added to each well and the resulting mixtures were incubated for another 3–4 h. The solution in every single well was then collected following the addition of 100 microliters of dimethylsulfoxide (DMSO) to each well. Finally, the OD was measured using an ELISA microplate reader at 570 nm. Cell viability was determined according to the following formula:

$$\text{Cell viability}\ (\%) = \left(\text{OD treat/OD control}\right) \times 100$$

Statistical Analysis

Statistical analyses were carried out using one-way analysis of variance (ANOVA) and SPSS software version 25. Each test was performed in triplicate.

RESULTS

Biochemical and Morphological Test Results

As shown in **Table 1** all the strains were Gram-positive and catalase-negative, and were able to grow in the presence of 3% (w/v), 4.5% NaCl (w/v) and at the high temperature of 45°C, while nine strains were not able to grow at 15°C. Sugar fermentation patterns confirmed that the rod-shaped isolates were likely to be *Lactobacillus* strains whereas the cocci belonged to *Enterococcus* genus.

Hemolytic Activity Results

In terms of hemolytic activity, three strains exhibiting hemolytic activity (beta or alpha hemolysis) were excluded, and the rest, which showed non-hemolytic activity, were used for further experiments.

Survival Under Low pH Conditions Results

Among the 185 isolates screened for low pH tolerance, 43 exhibited tolerance to pH 3. The colonies of the potential acid-tolerant isolates were then counted at 0 and 3 h after incubation in MRS agar at pH 3. Strains SA 135, ST 80, and SA 151 demonstrated relatively the highest rate of survival after 3 h of incubation. The results are shown in **Table 2**.

Bile Salt Tolerance Results

Following the assessment of bile salt tolerance for 4 h, isolates ST 13, SA 151, and ST 172 were shown to be the most resistant, with growth inhibition capabilities ranging from $3.21 \pm 0.01\%$ to $10.71 \pm 0.03\%$, however, isolates SA 171, SA 109, and SA 179 exhibited the least resistance, ranging from $39.56 \pm 0.02\%$ to $27.85 \pm 0.03\%$. In general, nearly all the strains showed above the 50% tolerance ability (**Figure 1**).

[1] http://www.ncbi.nlm.nih.gov/GeneBank

TABLE 1 | Results of the morphological and biochemical tests carried out for selected isolates.

Strains	SA 151	SA 135	SA 171	SA 139	SA 109	SA 110	SA 12	SA 16	ST 80	ST 13	ST 67	ST 126	ST 172	ST 179
Cell morphology	rod	rod	rod	rod	rod	rod	rod	rod	cocci	cocci	cocci	cocci	cocci	cocci
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth in presence of NaCl 3%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in presence of NaCl 4.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 15°C	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrate fermentation														
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Manitol	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L–Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L–xylose	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Sorbitol	+	+	+	+	+	+	+	+	+	–	–	–	–	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Antagonicity Test Results

The isolated strains were assessed for antimicrobial activity against ten types of pathogens. The results are indicated in **Table 3**. Certain isolates had an inhibitory impact on the selected pathogens. The four isolates, SA 151, SA 135, ST 80, and SA 139 were able to moderately constrain a wide array of pathogens. Seven isolates including SA 171, SA 12, SA 109, SA 110, SA 16, ST 13, and ST 126 were able to inhibit at least four types of pathogens. Six isolates (SA 151, SA 135, ST 80, SA 109, SA 110, and SA 16) could inhibit the MRSA even though none inhibited the *S. enterica* strain.

TABLE 2 | Acid tolerance and survival rate of selected isolates under acidic conditions.

Strains	0 h CFU/ml	3 h CFU/ml	Survival rate (%)
SA 151	6.17 ± 0.1	6.01 ± 0.03	97.4%
SA 135	7.38 ± 0.01	7.33 ± 0.03	99.32%
ST 80	6.51 ± 0.01	6.46 ± 0.01	99.23%
SA 139	6.47 ± 0.1	6.17 ± 0.1	95.36%
SA 171	6.47 ± 0.1	5.60 ± 0.1	86.55%
SA 12	7.8 ± 0.06	7.3 ± 0.3	93.58%
SA 109	6.54 ± 0.06	5.17 ± 0.1	79.05%
SA 110	7.09 ± 0.08	5.87 ± 0.02	82.79%
SA 16	6.47 ± 0.03	5.54 ± 0.06	85.62%
ST 13	7.17 ± 0.1	6.17 ± 0.1	86.05%
ST 67	6.49 ± 0.01	6.30 ± 0.2	97.07%
ST 126	6.47 ± 0.1	5.30 ± 0.2	81.91%
ST 172	7.14 ± 0.03	5.65 ± 0.04	79.13%
ST 179	6.90 ± 0.05	5.47 ± + 0.1	79.27%

Values are mean ± standard deviation of triplicates.

Evaluation of Antibiotic Susceptibility

As shown in **Table 4**, antibiotic susceptibility tests using the disk diffusion method indicated that all the strains were resistant to kanamycin and streptomycin, except for SA 109, which was sensitive to streptomycin; nevertheless, each of the isolates was sensitive to chloramphenicol and ampicillin. No antibiotic resistance patterns were reported for erythromycin and clindamycin except intermediate susceptibility in (SA 139, SA 12, and ST 126) and (ST 80 and ST 179), respectively. Strains were resistant to gentamycin (n = 5), cefixime (n = 6), penicillin (n = 3), trimethoprim (n = 9), vancomycin (n = 8), rifampin (n = 6), and azithromycin (n = 1). Resistance rate, calculated via the number of antibiotic resistance of each strain to the whole number of tested antibiotics, varied from 23.07% (represented by SA 151, ST 110, and ST 16) to 46.15% (demonstrated by SA 139, ST 13).

Adhesion Ability Results

An essential criterion for the selection of a probiotic is the ability to adhere to mucosal surfaces and epithelial cells, to allow its survival and colonization of the human gut. Hence the adhesion ability of probiotic candidates was examined using the Caco-2 cell line. Isolates SA 135, SA 171, SA 139, SA 12, ST 126, ST 172, ST 179, and ST 67 were able to adhere firmly to Caco-2 cell line while ST 80, SA 151, ST 109, ST 110, ST 16, and ST 13 showed moderate adhesion ability (**Table 5**).

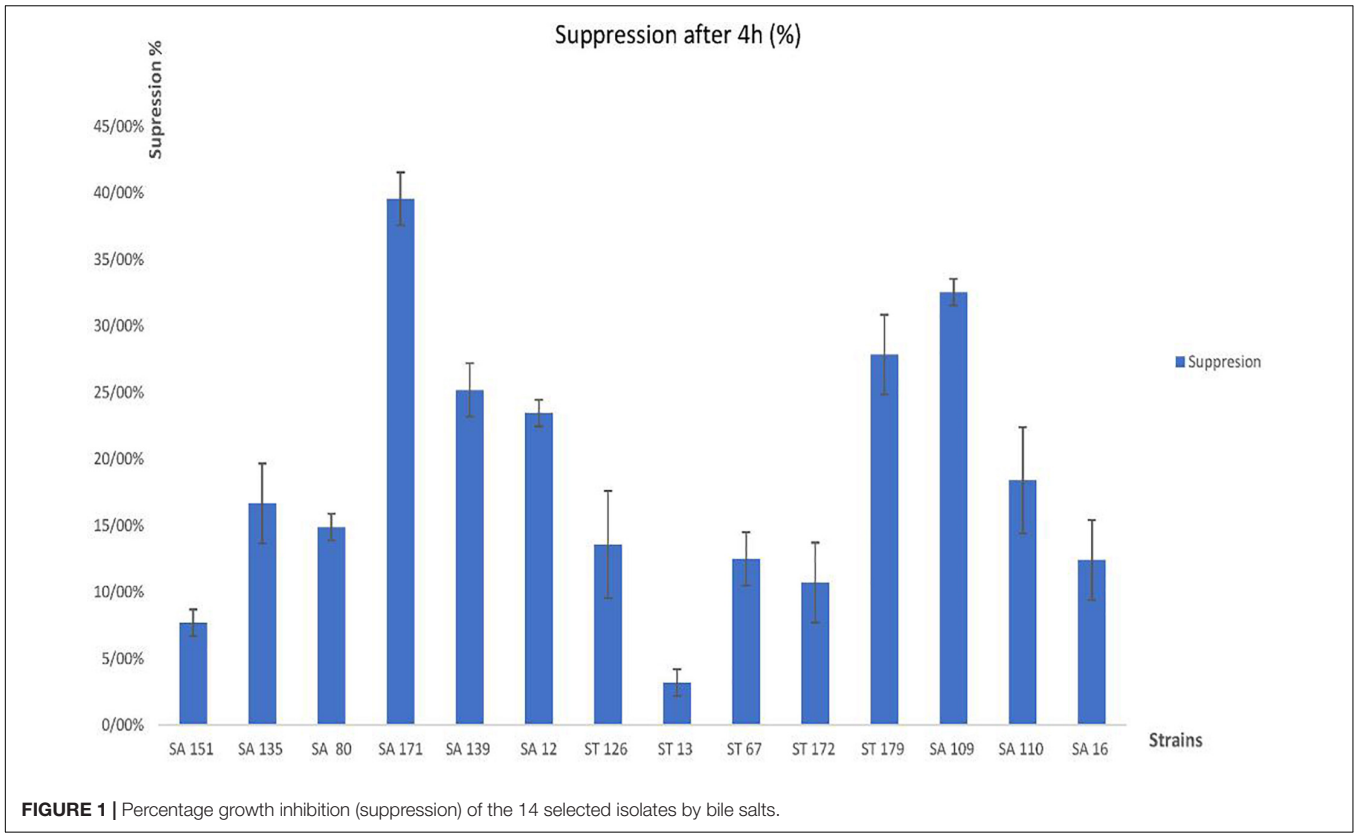

FIGURE 1 | Percentage growth inhibition (suppression) of the 14 selected isolates by bile salts.

TABLE 3 | Antimicrobial activities against ten pathogens.

Strains	*S. aureus (mm)*	*L. monocytogenes (mm)*	*B. cereus (mm)*	*S. mutans (mm)*	*E. coli (mm)*	*P. aeruginosa (mm)*	*C. albicans (mm)*	*E. faecalis (mm)*	MRSA *(mm)*	*S. enterica (mm)*
SA 151	15	13	15	10.5	–	15.5	15.5	11	20	–
SA 135	15	15	16	15	–	17	20	–	11	–
ST 80	12	16	18	11	–	15	22	–	17	–
SA 139	–	10	13	15	10	13	16	10	–	–
SA 171	18	–	15	15	–	20	19	–	–	–
SA 12	–	–	14	12	–	10	12	12	–	–
SA 109	–	–	10	12	–	15	21	–	30	–
SA 110	–	–	10	10	–	12	20	–	25	–
SA 16	–	–	12	12	–	14	15	–	21	–
ST 13	–	14	10	–	14	12	–	10	–	–
ST 67	–	13	9	–	–	–	–	10	–	–
ST 126	–	13	13	–	15	17	–	15	–	–
ST 172	–	–	–	–	–	10	–	10	–	–
ST 179	–	10	–	–	–	–	–	–	–	–

The zone diameter values above are the average of two experiments and presented in millimeter (mm).

16s rRNA Sequencing and Phylogenetic Tree Results

Fourteen selected candidates were investigated for molecular characterization using the Sanger sequencing method and the BLAST tool. The Sanger sequencing data analysis and the resulting phylogenetic tree revealed that the isolates belong to *L. fermentum*, *Enterococcus faecium,* and *Enterococcus hirae* strains. All the isolates' names and accession numbers can be found in **Table 6**. The phylogenetic tree was constructed by MEGAX software using the bootstrap method (**Figure 2**).

Biofilm Production Results

All isolates showed strong biofilm formation except isolates SA 135, ST 13, ST 67, and ST 126, which only showed modest biofilm production.

TABLE 4 | Antibiotic susceptibility test results.

Strains	Gentamycin	Cefixime	Penicillin	Chloramphenicol	Streptomycin	Erythromycin	Ampicillin	Trimethoprim	Kanamycin	Vancomycin	Rifampin	Azithromycin	Clindamycin
SA 151	S	S	S	S	R	S	S	S	R	R	S	S	S
SA 135	R	R	S	S	R	S	S	R	R	R	S	S	S
ST 80	R	R	S	S	R	S	S	R	R	R	S	S	I
SA 171	S	S	R	S	R	S	S	S	R	R	S	S	S
SA 139	R	R	S	S	R	I	S	R	R	S	R	I	S
SA 109	S	R	S	S	S	S	S	R	R	R	S	S	S
SA 110	S	S	S	S	R	S	S	S	R	R	S	S	S
SA 12	R	I	S	S	R	I	S	R	R	S	R	I	S
SA 16	S	S	S	S	R	S	S	S	R	R	S	S	S
ST 13	S	R	R	S	R	S	S	R	R	S	R	S	S
ST 67	S	I	R	S	R	S	S	R	R	S	R	S	S
ST 126	S	S	R	S	R	I	S	R	R	S	R	S	S
ST 172	S	I	S	S	R	S	S	R	R	S	R	R	S
ST 179	R	R	S	S	R	S	S	S	R	R	S	I	I

R, Resistant; S, Sensitive; and I, Intermediate.

Auto-Aggregation and Hydrophobicity Results

Isolates were examined for adherence to hydrophobic surfaces, e.g., using xylene and the auto-aggregation method. As shown in **Table 7**, the isolates hydrophobicity extended from 0% to 69.68 ± 0.01% while auto-aggregation ranged from 2.23 ± 0.002% to 33.43 ± 0.007%. Maximum percentage of hydrophobicity to xylene was demonstrated by SA 135, followed by SA 151. In contrast, the highest auto-aggregation rate was observed in ST 179 and ST 13, respectively.

MTT Assay Results

The top five isolates that exhibited relatively good probiotic properties were utilized for the MTT assay on Caco-2 cell line. As shown in **Figure 3**, the cytotoxic activities varied from 38% to 89% at 25% (v/v) concentration after 24 h of incubation, while boosting the concentration of supernatants to 100%, nearly all the cells were killed during the incubation period. By increasing the incubation period and the concentration of the supernatants, cytotoxic activity had fallen to 7% in the SA 171 isolate after 72 h of incubation. In general, isolates SA 171, ST 80 revealed the best cytotoxicity after 24 h of incubation.

DISCUSSION

Over the past decades, research in probiotics has gained a surge of interest because of their multiple health benefits and market demands. Many investigations have been undertaken to isolate new promising probiotic species from the human gut and salivary microbiota (Kiliç and Karahan, 2010; Vijayabharathi et al., 2012; Terai et al., 2015), but continuous research is required due to their species-specific features.

Probiotics used by humans are usually isolated from different environments that include dairy and non-dairy sources. However, probiotics that are isolated from human or animal intestines have certain characteristics that differ from those isolated from dairy products. For example, probiotics isolated from the human gut are usually more resistant to high bile salt concentrations and low pH levels. Furthermore, they possess higher adhering abilities when compared to those of dairy-isolated probiotics. Thus, non-diary probiotics are highly likely to be exploited in people who suffer from lactose intolerance (Sornplang and Piyadeatsoontorn, 2016; Sardana et al., 2018).

Traditional probiotics that have long been used globally, only cover a small spectrum of microorganisms. With the advent of next-generation sequencing, a greater understanding of the gut microbiome is revealing an immense number of new microorganisms with unknown characteristics that could have potential healing properties. The extensive research that is currently demonstrating the multidimensional benefits of the gut microbiome on human health will inevitably culminate in the identification and development of new microbial strains with novel therapeutic properties valuable to human health and the pharmaceutical industry. Accordingly, these new potential probiotic strains are referred to as "next-generation probiotics" (O'Toole et al., 2017).

TABLE 5 | The adhesion ability of the selected isolates.

Strains	Adhesion	Strains	Adhesion
SA 135	Strong	ST 67	Strong
SA 171	Strong	ST 80	Moderate
SA 139	Strong	SA 151	Moderate
SA 12	Strong	SA 109	Moderate
ST 126	Strong	SA 110	Moderate
ST 172	Strong	SA 16	Moderate
ST 179	Strong	ST 13	Moderate

TABLE 6 | Candidate probiotics identified based on percentage similarity of the 16s rRNA sequence to those available in the GenBank database.

Accession Number	Name	Similarity (%)	Strains
ST 80	84.44%	*Enterococcus faecium*	MT815471
SA 151	90.43%	*Lactobacillus fermentum*	MN128866
SA 135	98.30%	*Lactobacillus fermentum*	MN475882
SA 12	98.65%	*Lactobacillus fermentum*	MN475960
SA 139	98.93%	*Lactobacillus fermentum*	MN128688
ST 13	98.97%	*Enterococcus faecium*	MN475959
SA 109	99.00%	*Lactobacillus fermentum*	MN475903
ST 172	99.04%	*Enterococcus faecium*	MN128647
SA 171	99.15%	*Lactobacillus fermentum*	MN475879
SA 110	99.20%	*Lactobacillus fermentum*	MN475967
ST 179	99.35%	*Enterococcus hirae*	MN147877
SA 16	99.43%	*Lactobacillus fermentum*	MN475920
ST 126	99.71%	*Enterococcus faecium*	MN148088
ST 67	99.78%	*Enterococcus faecium*	MN475904

This study seeks new probiotic strains, hence, lactic acid bacteria were isolated from the gut and saliva of healthy human individuals. In total, 185 isolated were tested for probiotic properties.

Before approving any probiotic for its health benefits and use in the food industry, its safety must be evaluated by *in vitro* and *in vivo* studies. The two main experimental tests that are carried out in this regard are the hemolysis and antibiotic resistance tests (Oh and Jung, 2015). None of the selected strains in this study showed beta-hemolytic activity.

The ability to tolerate harsh conditions, e.g., low pH, gastric juice, and bile salts, are the main contributing factors in the selection of good probiotic candidates (Kandylis et al., 2016). One of the key features in the selection of probiotics is acid tolerance, since they must be able to survive under the low pH conditions of the gastric juice in the stomach. In this study, the survival rate in acidic and bile salt circumstances vastly varied from one strain to another, suggesting a strain-specific pattern. Isolates SA 135, SA 151, and ST 80 showed maximum survival ability in the presence of acidic and bile salt conditions. The results of this study are in agreement with those of previous research (Chou and Weimer, 1999; Zago et al., 2011).

In our study, the inhibitory effects of LAB supernatants were found against a variety of pathogens including *S. aureus*, *P. aeruginosa*, MRSA, *E. coli*, *S. mutans*, *L. monocytogenes*, *B. cereus*, *E. faecalis,* and *C. albicans*. Our results differ somewhat from the published studies, whereby the LAB could not affect Gram-negative pathogens (Zommiti et al., 2018). These findings emphasize the importance of the selected strains in our studies, as they tend to show broad-spectrum antimicrobial activities, particularly against antibiotic-resistant microorganisms such as MRSA and the fungus, *C. albicans*. Although the broad antimicrobial effects of LABs are most often the result of organic acid production, the activity of antimicrobial peptides and other metabolites that may be produced by such strains cannot be ruled out (Kivanç et al., 2011; Somashekaraiah et al., 2019).

Another significant feature of probiotics is their ability to colonize the gut, which can be evaluated by the Caco-2 cell adhesion assay (Kozak et al., 2016), auto-aggregation, and hydrophobicity tests (Collado et al., 2008). In this research, the probiotic candidates showed a diverse adhesion model, demonstrating moderate to strong adhesion patterns. Regarding hydrophobicity and auto-aggregation, the highest

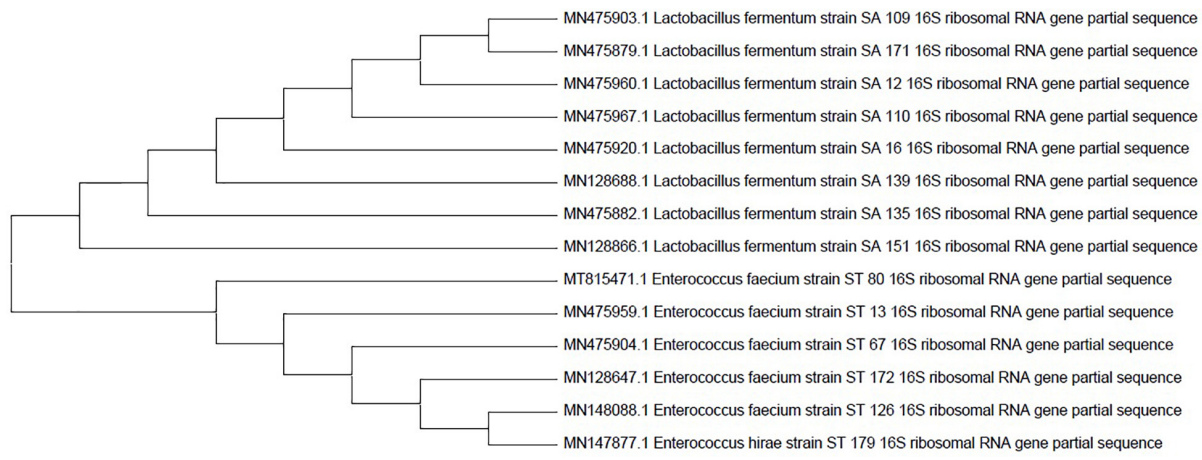

FIGURE 2 | Phylogenetic tree of the 14 probiotic isolates based on 16s rRNA sequences. The tree was constructed by the MEGAX software using the bootstrap method.

TABLE 7 | Percentage of cell surface hydrophobicity of candidate probiotic strains.

Strains	Auto-aggregation (%)	Hydrophobicity (%)	Strains	Auto-aggregation (%)	Hydrophobicity (%)
SA 151	16.23 ± 0.002	55.84 ± 0.08	SA 16	15.77 ± 0.004	27.12 ± 0.007
ST 80	12.89 ± 0.002	20.69 ± 0.003	SA 110	6.29 ± 0.002	11.51 ± 0.04
SA 135	13.13 ± 0.004	69.68 ± 0.01	ST 13	17.39 ± 0.002	1.45 ± 0.01
SA 12	7.62 ± 0.006	41.14 ± 0.003	ST 172	11.30 ± 0.002	0
SA 171	2.23 ± 0.002	11.48 ± 0.007	ST 179	33.43 ± 0.007	6.44 ± 0.01
SA 139	11.81 ± 0.002	0	ST 67	13.82 ± 0.007	5.26 ± 0.01
SA 109	14.79 ± 0.005	32.24 ± 0.002	ST 126	10.57 ± 0.007	3 ± 0.01

Values are shown in mean ± Standard deviation.

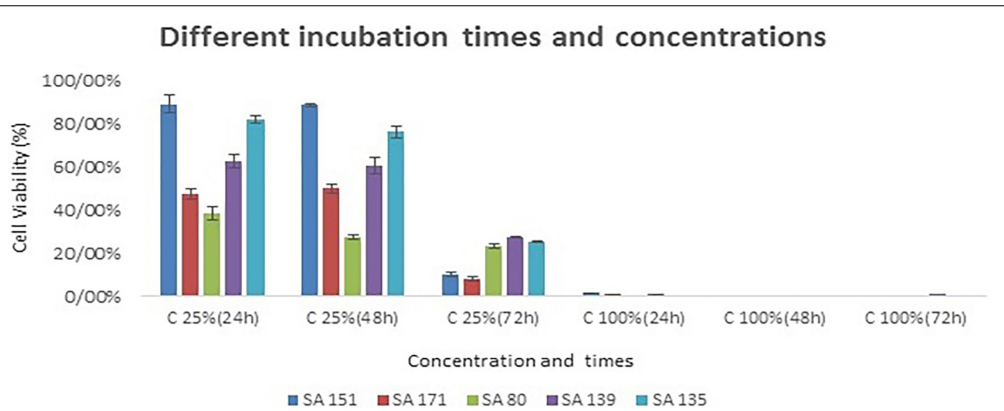

FIGURE 3 | The cytotoxic effects of bacterial culture free supernatants on the CaCo-2 cell line at different supernatant concentrations and incubation periods. The five probiotic candidates that were assessed included SA 151, SA 171, SA139 and SA 135.

rate of hydrophobicity and auto-aggregation were observed in SA 151, SA 135, and in ST 179, ST 13, respectively. The adhesion results demonstrate the ability of the selected strains to adhere to the epithelial cells and thus colonize the gut. Adhesion is a very important trait of a suitable and prevailing probiotic, as it prevents the colonization of the gastrointestinal tract by pathogenic bacteria (Abushelaibi et al., 2017; Somashekaraiah et al., 2019).

As a key feature, a good probiotic candidate should not possess or acquire any antibiotic resistance genes. Consistent with the literature, this research found that nearly all isolates were resistant to kanamycin and streptomycin, except for SA 109, which showed sensitivity to streptomycin (Kook et al., 2019). A possible explanation for these results may be the overuse of antibiotics in Iran. Notably, some were also found to be resistant to vancomycin, which is in accordance with previous reports that have shown vancomycin resistance as an intrinsic trait of LAB, such as *Lactobacillus, Leuconostoc,* and *Pediococcus*. In fact, many *Lactobacillus* strains, including *L. fermentum* are routinely used in the food industry. Vancomycin resistance in this group of bacteria is chromosomally encoded and is non-transferable and non-inducible (Swenson et al., 1990; Tynkkynen et al., 1998; Sharma et al., 2014).

Another important characteristic of the potential probiotic candidates in this study is their anti-cancer properties. Five of the selected isolates demonstrated antiproliferative activities against the human colon cancer cell line (Caco-2). Their culture supernatants inhibited the growth of cancerous cells by up to 7% after 72 h of incubation at the concentration of 25%. Our study confirms a previous finding by Lee et al. (2019), who demonstrated the antiproliferative effects of the culture-free supernatant (CFS) of a *L. fermentum* strain against colorectal cancer (CRC). It was shown that the CFS induces apoptosis, thereby inhibiting cell growth in CRC lines. The antiproliferative activity of *L. fermentum* is brought about by preventing NF-κB signaling. They showed that the *Lactobacillus* CFS is capable of inducing cell death, and thus has the potential to be used as a powerful multitarget anti-cancer chemotherapeutic agent (Lee et al., 2019).

In a previous study by Wei et al. (2019), it was suggested that exopolysaccharide (EPS) extracted from *L. fermentum* YL-11 could inhibit Caco-2 and HT-29 cell lines by up to 45.6 ± 6.1%. They proved that EPS could act as an antiproliferative agent in these two types of CRC cell lines (Wei et al., 2019).

Overall, we were able to select 14 potential probiotics with multifaceted probiotic attributes. These strains were subsequently characterized and identified genotypically, and were found to belong to the *Lactobacillus* and *Enterococcus* genus. By considering the unique characteristics of these indigenous probiotic strains, they can be of great benefit to the pharmaceutical, cosmetic, and food industries. Furthermore, this study also proved that the human gut and saliva can act as suitable sources of novel probiotics with desirable functional properties.

CONCLUSION

Given the favorable probiotic attributes in our isolates, the following conclusion can be drawn that saliva and feces are two suitable and potential sources for isolating novel probiotics strains of human origin. Since the isolates of this study, in particular SA 151, ST 80, and SA 135, showed relatively good antipathogenic activity, survival in harsh conditions, biofilm production, and reasonable adhesion, they could, therefore, be viewed as promising "next generation" probiotic candidates, useful to the pharmaceutical industry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The study involving human participants was reviewed and approved by the Ethics Committee at the National Institute of Genetic engineering and Biotechnology (NIGEB, Tehran, Iran), and was registered as IR.NIGEB.EC.1398.12.3.B. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PS contributed to the original idea, conceptualization, supervision and project administration. HB and PS contributed to the design, methodology and implementation of research. PS and HB contributed to the writing and original draft preparation of the manuscript. PS contributed to the full revision and editing of the manuscript. HB, PS, SAJ, AA, and MB contributed to the analysis of the results and to the writing and editing of the manuscript.

FUNDING

This work was financially supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB).

ACKNOWLEDGMENTS

We would like to thank the National Institute of Genetic Engineering and Biotechnology (NIGEB) for financial assistance and provision of facilities. We also thank all the volunteers who participated in this study and those who have helped us during this study.

REFERENCES

Abushelaibi, A., Al-Mahadin, S., El-Tarabily, K., Shah, N. P., and Ayyash, M. (2017). Characterization of potential probiotic lactic acid bacteria isolated from camel milk. *LWT Food Sci. Technol.* 79, 316–325. doi: 10.1016/j.lwt.2017.01.041

Alkalbani, N. S., Turner, M. S., and Ayyash, M. M. (2019). Isolation, identification, and potential probiotic characterization of isolated lactic acid bacteria and *in vitro* investigation of the cytotoxicity, antioxidant, and antidiabetic activities in fermented sausage. *Microb. Cell Fact* 18:188. doi: 10.1186/s12934-019-1239-1

Anwar, F., Altayb, H. N., Al-Abbasi, F. A., Al-Malki, A. L., Kamal, M. A., and Kumar, V. (2020). Antiviral effects of probiotic metabolites on COVID-19. *J. Biomol. Struct. Dyn.* 1–10. doi: 10.1080/07391102.2020.1775123

Besser, M., Terberger, J., Weber, L., Ghebremedhin, B., Naumova, E. A., Arnold, W. H., et al. (2019). Impact of probiotics on pathogen survival in an innovative human plasma biofilm model (hpBIOM). *J. Transl. Med.* 17:243. doi: 10.1186/s12967-019-1990-4

Borges, S., Silva, J., and Teixeira, P. (2012). Survival and biofilm formation by Group B *Streptococci* in simulated vaginal fluid at different pHs. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* 101, 677–682. doi: 10.1007/s10482-011-9666-y

Chen, C. C., Lai, C. C., Huang, H. L., Huang, W. Y., Toh, H. S., Weng, T. C., et al. (2019). Antimicrobial activity of *Lactobacillus* species against carbapenem-resistant *Enterobacteriaceae*. *Front. Microbiol.* 10:789. doi: 10.3389/fmicb.2019.00789

Chen, Z. Y., Hsieh, Y. M., Huang, C. C., and Tsai, C. C. (2017). Inhibitory effects of probiotic *Lactobacillus* on the growth of human colonic carcinoma cell line HT-29. *Molecules* 22:107. doi: 10.3390/molecules22010107

Chou, L. S., and Weimer, B. (1999). Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci.* 82, 23–31. doi: 10.3168/jds.S0022-0302(99)75204-5

Collado, M. C., Meriluoto, J., and Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *Eur. Food Res. Technol.* 226, 1065–1073. doi: 10.1007/s00217-007-0632-x

Daliri, E. B. M., Lee, B. H., and Oh, D. H. (2017). Current perspectives on antihypertensive probiotics. *Probiot. Antimicrob. Proteins* 9, 91–101. doi: 10.1007/s12602-016-9241-y

Dargahi, N., Johnson, G., and Apostolopoulos, V. (2020). *Streptococcus thermophilus* alters the expression of genes associated with innate and adaptive immunity in human peripheral blood mononuclear cells. *PLoS One* 15:e0228531. doi: 10.1371/journal.pone.0228531

d'Ettorre, G., Ceccarelli, G., Marazzato, M., Campagna, G., Pinacchio, C., Alessandri, F., et al. (2020). Challenges in the management of SARS-CoV2 infection: the role of oral bacteriotherapy as complementary therapeutic strategy to avoid the progression of COVID-19. *Front. Med.* 7:389. doi: 10.3389/fmed.2020.00389

Drago, L. (2019). Probiotics and colon cancer. *Microorganisms* 7:66. doi: 10.3390/microorganisms7030066

Ettinger, G., MacDonald, K., Reid, G., and Burton, J. P. (2014). The influence of the human microbiome and probiotics on cardiovascular health. *Gut Microb.* 5, 719–728. doi: 10.4161/19490976.2014.983775

FAO/WHO (2002). *Guidelines for the Evaluation of Probiotics in Food*. Rome: FAO.

Fernández, M. F., Boris, S., and Barbés, C. (2003). Probiotic properties of human *Lactobacilli* strains to be used in the gastrointestinal tract. *J. Appl. Microbiol.* 94, 449–455. doi: 10.1046/j.1365-2672.2003.01850.x

Fijan, S. (2016). "Antimicrobial effect of probiotics against common pathogens," in *Probiotics and Prebiotics in Human Nutrition and Health*, eds V. Rao and L. G. Rao (London: InTechopen), doi: 10.5772/63141

Ganapathy, V., Thangaraju, M., Prasad, P. D., Martin, P. M., and Singh, N. (2013). Transporters and receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. *Curr. Opin. Pharmacol.* 13, 869–874. doi: 10.1016/j.coph.2013.08.006

Gao, C., Ganesh, B. P., Shi, Z., Shah, R. R., Fultz, R., Major, A., et al. (2017). Gut microbe-mediated suppression of inflammation-associated colon carcinogenesis by luminal histamine production. *Am. J. Pathol.* 187, 2323–2336. doi: 10.1016/j.ajpath.2017.06.011

Gill, H. S., Rutherfurd, K. J., Prasad, J., and Gopal, P. K. (2000). Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Br. J. Nutr.* 83, 167–176. doi: 10.1017/s0007114500000210

Gómez-Guzmán, M., Toral, M., Romero, M., Jiménez, R., Galindo, P., Sánchez, M., et al. (2015). Antihypertensive effects of probiotics *Lactobacillus* strains in spontaneously hypertensive rats. *Mol. Nutr. Food Res.* 59, 2326–2336. doi: 10.1002/mnfr.201500290

Haghshenas, B., Haghshenas, M., Nami, Y., Khosroushahi, A. Y., Abdullah, N., Barzegari, A., et al. (2016). Probiotic assessment of *Lactobacillus plantarum* 15HN and *Enterococcus mundtii* 50H isolated from traditional dairies microbiota. *Adv. Pharm. Bull.* 6:37. doi: 10.15171/APB.2016.007

Haghshenas, B., Nami, Y., Abdullah, N., Radiah, D., Rosli, R., and Khosroushahi, A. Y. (2014). Anti-proliferative effects of *Enterococcus* strains isolated from fermented dairy products on different cancer cell lines. *J. Funct. Foods* 11, 363–374. doi: 10.1016/j.jff.2014.10.002

Halder, D., Mandal, M., Chatterjee, S. S., Pal, N. K., and Mandal, S. (2017). Indigenous probiotic *Lactobacillus* isolates presenting antibiotic like activity against human pathogenic bacteria. *Biomedicines* 5:31. doi: 10.3390/biomedicines5020031

Kahouli, I., Malhotra, M., Westfall, S., Alaoui-Jamali, M. A., and Prakash, S. (2017). Design and validation of an orally administrated active *L. fermentum-L. acidophilus* probiotic formulation using colorectal cancer Apc $^{Min/+}$ mouse model. *Appl. Microbiol. Biotechnol.* 101, 1999–2019. doi: 10.1007/s00253-016-7885-x

Kandylis, P., Pissaridi, K., Bekatorou, A., Kanellaki, M., and Koutinas, A. A. (2016). Dairy and non-dairy probiotic beverages. *Curr. Opin. Food Sci.* 7, 58–63. doi: 10.1016/j.cofs.2015.11.012

Kang, M.-S., Lim, H.-S., Oh, J.-S., Lim, Y.-J., Wuertz-Kozak, K., Harro, J. M., et al. (2017). Antimicrobial activity of *Lactobacillus salivarius* and *Lactobacillus fermentum* against *Staphylococcus aureus*. *Pathog. Dis.* 75:ftx009. doi: 10.1093/femspd/ftx009

Kiliç, G. B., and Karahan, A. G. (2010). Identification of lactic acid bacteria isolated from the fecal samples of healthy humans and patients with dyspepsia, and determination of their pH, bile, and antibiotic tolerance properties. *J. Mol. Microbiol. Biotechnol.* 18, 220–229. doi: 10.1159/000319597

Kivanç, M., Yilmaz, M., and Çakir, E. (2011). Isolation and identification of lactic acid bacteria from boza, and their microbial activity against several reporter strains. *Turkish J. Biol.* 35, 313–324. doi: 10.3906/biy-0906-67

Kocsis, T., Molnár, B., Németh, D., Hegyi, P., Szakács, Z., Bálint, A., et al. (2020). Probiotics have beneficial metabolic effects in patients with type 2 diabetes mellitus: a meta-analysis of randomized clinical trials. *Sci. Rep.* 10:11787. doi: 10.1038/s41598-020-68440-1

Kook, S.-Y., Chung, E.-C., Lee, Y., Lee, D. W., and Kim, S. (2019). Isolation and characterization of five novel probiotic strains from Korean infant and children faeces. *PLoS One* 14:e0223913. doi: 10.1371/journal.pone.0223913

Kozak, K., Charbonneau, D., Sanozky-Dawes, R., and Klaenhammer, T. (2016). Characterization of bacterial isolates from the microbiota of mothers' breast milk and their infants. *Gut Microb.* 6, 341–351. doi: 10.1080/19490976.2015.1103425

Krausova, G., Hyrslova, I., and Hynstova, I. (2019). *In Vitro* evaluation of adhesion capacity, hydrophobicity, and auto-aggregation of newly isolated potential probiotic strains. *Fermentation* 5:100. doi: 10.3390/fermentation5040100

Kumar, M., Dhaka, P., Vijay, D., Vergis, J., Mohan, V., Kumar, A., et al. (2016). Antimicrobial effects of *Lactobacillus plantarum* and *Lactobacillus acidophilus* against multidrug-resistant enteroaggregative *Escherichia coli*. *Int. J. Antimicrob. Agents* 48, 265–270. doi: 10.1016/j.ijantimicag.2016.05.014

Kumar, R., Sood, U., Gupta, V., Singh, M., Scaria, J., and Lal, R. (2020). Recent advancements in the development of modern probiotics for restoring human gut microbiome *Dysbiosis*. *Indian J. Microbiol.* 60, 12–25. doi: 10.1007/s12088-019-00808-y

Lee, J.-E., Lee, J., Kim, J. H., Cho, N., Lee, S. H., Park, S. B., et al. (2019). Characterization of the anti-cancer activity of the probiotic bacterium *Lactobacillus fermentum* using 2D vs. 3D culture in colorectal cancer cells. *Biomolecules* 9:557. doi: 10.3390/biom9100557

McFarland, L. (2015). Probiotics for the primary and secondary prevention of *C. difficile* infections: a meta-analysis and systematic review. *Antibiotics* 4, 160–178. doi: 10.3390/antibiotics4020160

Nami, Y., Haghshenas, B., Haghshenas, M., Abdullah, N., and Khosroushahi, A. Y. (2015). The Prophylactic effect of probiotic *Enterococcus lactis* IW5 against different human cancer cells. *Front. Microbiol.* 6:1317. doi: 10.3389/fmicb.2015.01317

Nami, Y., Vaseghi Bakhshayesh, R., Manafi, M., and Hejazi, M. A. (2019a). Hypocholesterolaemic activity of a novel autochthonous potential probiotic *Lactobacillus plantarum* YS5 isolated from yogurt. *LWT Food Sci. Technol.* 111, 876–882. doi: 10.1016/j.lwt.2019.05.057

Nami, Y., Vaseghi Bakhshayesh, R., Mohammadzadeh Jalaly, H., Lotfi, H., Eslami, S., and Hejazi, M. A. (2019b). Probiotic properties of *Enterococcus* isolated from artisanal dairy products. *Front. Microbiol.* 10:300. doi: 10.3389/fmicb.2019.00300

Oh, Y. J., and Jung, D. S. (2015). Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from omegisool, a traditionally fermented milletalcoholic beverage in Korea. *LWT Food Sci. Technol.* 63, 437–444. doi: 10.1016/j.lwt.2015.03.005

O'Toole, P. W., Marchesi, J. R., and Hill, C. (2017). Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat. Microbiol.* 2:17057. doi: 10.1038/nmicrobiol.2017.57

Pérez Ibarreche, M., Castellano, P., and Vignolo, G. (2014). Evaluation of anti-Listeria meat borne *Lactobacillus* for biofilm formation on selected abiotic surfaces. *Meat Sci.* 96, 295–303. doi: 10.1016/j.meatsci.2013.07.010

Plaza-Díaz, J., Ruiz-Ojeda, F. J., Vilchez-Padial, L. M., and Gil, A. (2017). Evidence of the anti-inflammatory effects of probiotics and synbiotics in intestinal chronic diseases. *Nutrients* 9:555. doi: 10.3390/nu9060555

Sanders, M. E. (2008). Probiotics: definition, sources, selection, and uses. *Clin. Infect. Dis.* 46, S58–S61. doi: 10.1086/523341

Sardana, R. K., Chhikara, N., Tanwa, B., and Pangh, A. (2018). Dietary impact on esophageal cancer in humans: a review. *Food Funct.* 9, 1967–1977. doi: 10.1039/c7fo01908d

Sharma, P., Tomar, S. K., Goswami, P., Sangwan, V., and Singh, R. (2014). Antibiotic resistance among commercially available probiotics. *Food Res. Int.* 57, 176–195. doi: 10.1016/j.foodres.2014.01.025

Silva, D. R., Sardi, J. C. O., de Pitangui, N. S., Roque, S. M., da Silva, A. C. B., and Rosalen, P. L. (2020). Probiotics as an alternative antimicrobial therapy: current reality and future directions. *J. Funct. Foods* 73:104080. doi: 10.1016/j.jff.2020.104080

Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., et al. (2014). Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* 40, 128–139. doi: 10.1016/j.immuni.2013.12.007

Somashekaraiah, R., Shruthi, B., Deepthi, B. V., and Sreenivasa, M. Y. (2019). Probiotic properties of lactic acid bacteria isolated from neera: a naturally fermenting coconut palm nectar. *Front. Microbiol.* 10:1382. doi: 10.3389/fmicb.2019.01382

Sornplang, P., and Piyadeatsoontorn, S. (2016). Probiotic isolates from unconventional sources: a review. *J. Anim. Sci. Technol.* 58:26. doi: 10.1186/s40781-016-0108-2

Swenson, J. M., Facklam, R. R., and Thornsberry, C. (1990). Antimicrobial susceptibility of vancomycin-resistant *Leuconostoc*, *Pediococcus*, and *Lactobacillus* species. *Antimicrob. Agents Chemother.* 34, 543–549. doi: 10.1128/AAC.34.4.543

Tan, G. S. E., Tay, H. L., Tan, S. H., Lee, T. H., Ng, T. M., and Lye, D. C. (2020). Gut microbiota modulation: implications for infection control and antimicrobial stewardship. *Adv. Ther.* 37, 4054–4067. doi: 10.1007/s12325-020-01458-z

Terai, T., Okumura, T., Imai, S., Nakao, M., Yamaji, K., Ito, M., et al. (2015). Screening of probiotic candidates in human oral bacteria for the prevention of dental disease. *PLoS One* 10:e0128657. doi: 10.1371/journal.pone.0128657

Tynkkynen, S., Singh, K. V., and Varmanen, P. (1998). Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (van) genes. *Int. J. Food Microbiol.* 41, 195–204. doi: 10.1016/S0168-1605(98)00051-8

Valdéz, J. C., Peral, M. C., Rachid, M., Santana, M., and Perdigón, G. (2005). Interference of *Lactobacillus plantarum* with *Pseudomonas aeruginosa* in vitro and in infected burns: the potential use of probiotics in wound treatment. *Clin. Microbiol. Infect.* 11, 472–479. doi: 10.1111/j.1469-0691.2005.01142.x

Vernazza, C. L., Gibson, G. R., and Rastall, R. A. (2006). Carbohydrate preference, acid tolerance and bile tolerance in five strains of *Bifidobacterium*. *J. Appl. Microbiol.* 100, 846–853. doi: 10.1111/j.1365-2672.2006.02832.x

Vijayabharathi, R., Palanisamy, B. D., Manoharan, R. K., Sathyabama, S., Bruntha, P., and Priyadarisini, V. B. (2012). Screening for probiotic properties of strains isolated from feces of various human groups. *Artic. J. Microbiol.* 50, 603–612. doi: 10.1007/s12275-012-2045-1

Vinderola, C. G., Medici, M., and Perdigón, G. (2004). Relationship between interaction sites in the gut, hydrophobicity, mucosal immunomodulating capacities and cell wall protein profiles in indigenous and exogenous bacteria. *J. Appl. Microbiol.* 96, 230–243. doi: 10.1046/j.1365-2672.2004.02158.x

Wei, Y., Li, F., Li, L., Huang, L., and Li, Q. (2019). Genetic and biochemical characterization of an exopolysaccharide with in vitro antitumoral activity produced by *Lactobacillus fermentum* YL-11. *Front. Microbiol.* 10:2898. doi: 10.3389/fmicb.2019.02898

Westfall, S., Lomis, N., Kahouli, I., Dia, S. Y., Singh, S. P., and Prakash, S. (2017). Microbiome, probiotics and neurodegenerative diseases: deciphering the gut brain axis. *Cell. Mol. Life Sci.* 74, 3769–3787. doi: 10.1007/s00018-017-2550-9

Xu, H., Jeong, H. S., Lee, H. Y., and Ahn, J. (2009). Assessment of cell surface properties and adhesion potential of selected probiotic strains. *Lett. Appl. Microbiol.* 49, 434–442. doi: 10.1111/j.1472-765X.2009.02684.x

Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suàrez, V., Vinderola, G., et al. (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiol.* 28, 1033–1040. doi: 10.1016/j.fm.2011.02.009

Zommiti, M., Cambronel, M., Maillot, O., Barreau, M., Sebei, K., Feuilloley, M., et al. (2018). Evaluation of probiotic properties and safety of *Enterococcus faecium* isolated from artisanal Tunisian Meat "Dried Ossban.". *Front. Microbiol.* 9:1685. doi: 10.3389/fmicb.2018.01685

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 28 January 2021
doi: 10.3389/fmicb.2021.631254

Role of Probiotics, Prebiotics, and Synbiotics in the Elderly: Insights Into Their Applications

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Elderly people are an important part of the global population who suffer from the natural processes of senescence, which lead to changes in the gut microbiota composition. These modifications have a great impact on their quality of life, bringing a general putrefactive and inflammatory status as a consequence. Some of the most frequent conditions related to this status are constipation, undernutrition, neurodegenerative diseases, susceptibility to opportunistic pathogens, and metabolic disbalance, among others. For these reasons, there is an increasing interest in improving their quality of life by non-invasive treatments such as the consumption of probiotics, prebiotics, and synbiotics. The aim of the present mini-review is to describe the benefits of these functional supplements/food according to the most recent clinical and pre-clinical studies published during the last decade. In addition, insights into several aspects we consider relevant to improve the quality of future studies are provided.

Keywords: microbiota, elderly, probiotic, prebiotic, synbiotic, health

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *19 November 2020*
Accepted: *04 January 2021*
Published: *28 January 2021*

Citation:
Ale EC and Binetti AG (2021) Role of Probiotics, Prebiotics, and Synbiotics in the Elderly: Insights Into Their Applications. Front. Microbiol. 12:631254. doi: 10.3389/fmicb.2021.631254

INTRODUCTION

Worldwide, elderly people (aged 65 or older) represent 12.4% of the global population (Bedani et al., 2016). According to the European Union, the share of people aged 80 years or above is projected to have a two-and-a-half-fold increase between 2019 and 2100, from 5.8 to 14.6% (Eurostat Statistics Explained, 20201[1]).

This rapid evolution has a significant impact on national public health institutes, social services, and health care systems. Consequently, elderly people are gaining increasing interest since they suffer from chronic health conditions that affect their quality of life, leading to a high demand of health services in general (Bedani et al., 2016). For this reason, new options for preserving their health have been investigated, with being functional food a potential option. In this context, probiotics, prebiotics, and synbiotics are worth studying since the scientific evidence about their beneficial effects on gut microbiota homeostasis is constantly increasing. While probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), the term prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). From their combination, the term synbiotics arose, which is defined as "a mixture comprising live microorganisms and substrate(s) selectively

[1]https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Population_structure_and_ageing&oldid=502987

utilized by host microorganisms that confers a health benefit on the host" (Swanson et al., 2020).

Several phenomena take place during aging, among them, a low-level systemic inflammation during immunosenescence was described by Guigoz et al. (2008). The term "senescence" refers mainly to non-pathological (biological and physiological) processes dependent on age, while the term "aging" refers to physiological and pathological changes (Rowe, 1997; Troen, 2003). In other words, cellular senescence refers to a permanent state of cell cycle arrest that occurs under different stress factors. Therefore, it works as a cellular defense mechanism that prevents cell damage, and it occurs during different physiological (and sometimes pathological) processes, such as tissue remodeling, cancer, injury, and aging (Calcinotto et al., 2019). During immunosenescence, a global reduction in the ability to cope with a wide range of stressors occurs, with a concomitant progressive increase toward a pro-inflammatory status, a process that seems to be mediated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB factor; Salminen et al., 2008). In addition, inflammatory responses may be caused by the leakage of the intestinal barrier, allowing microbial and/or microbial components to filtrate (Shalim et al., 2019). On the other hand, modifications of the T-cell repertoire have been associated with an increase in morbidity caused by infectious diseases (Vasto et al., 2006), and a low activity of natural killer (NK) cells has been reported as well (Jing et al., 2007).

The gut microbiota of elderly subjects also suffer a gradual shift toward a reduced bacterial diversity: a decline in beneficial microorganisms and an increase of facultative anaerobic bacteria. In general, lower levels of *Firmicutes* (mainly *Clostridium* cluster XIVa and *Faecalibacterium prausnitzii*) and *Actinobacteria* (mainly bifidobacteria), together with increased populations of *Proteobacteria*, have been found when comparing with adults (Salazar et al., 2017). Elderly people may also have reduced dentition and chewing strength, together with a loss of appetite, which can lead to a limited variety of food ingredients that support the limited microbial diversity (O'Toole and Claesson, 2010). These changes are responsible for a decrease in short chain fatty acids (SCFA) production and shift from a predominantly saccharolytic metabolism (normally observed in adults) toward a predominantly putrefactive metabolism (Woodmansey et al., 2004). SCFAs are volatile fatty acids produced by the gut microbiota in the large bowel from food components that are unabsorbed/undigested in the small intestine. They exert beneficial health effects, such as protection against pathogens and shaping the gut environment, apart from presenting anti-inflammatory properties (Ríos-Covián et al., 2016). Furthermore, they have been associated with the upregulation of the anti-inflammatory cytokines *in vitro*, together with the induction of $CD4^+CD25^+$ Treg cells (Asarat et al., 2016).

In this context, considering that the inflammatory status of this group is highly modulated by the gut microbiota (Guigoz et al., 2008) and that external factors such as diet and lifestyle are crucial for this modulation (O'Toole and Claesson, 2010), functional food turns to be an attractive target to study.

In the present review, we intend to revise the latest studies about the application of probiotics, prebiotics, and synbiotics (solely or in different food matrices) on elderly subjects and the effects these strategies have on their health and the quality of life in general. Besides, some guidelines we consider useful for the development of future products aimed at this part of the population are provided.

GUT MICROBIOTA COMPOSITION OF THE ELDERLY

According to O'Toole and Jeffery (2015), the composition of microbiota does not suddenly alter at a certain age, but it is a gradual process dependent on several factors, such as gender, location, diet, lifestyle, physical activity, immune system functionality, and the use of medication (O'Toole and Jeffery, 2015; Komanduri et al., 2019). In general, a reduced microbial diversity has been observed, with *Bacteroides* and *Firmicutes* as the most dominant phyla (Claesson et al., 2011; Biagi et al., 2012; Odamaki et al., 2016). Many studies have reported a decline in viable counts of *Bacteroides* with increased age, together with reduced diversity within this genus (Bartosch et al., 2004; Woodmansey et al., 2004; Woodmansey, 2007). This may have a direct impact on digestion since bacteria from this genus are believed to play an important role in the digestion of polysaccharides in the colon (Flint et al., 2012). Furthermore, a decrease in starch and sucrose metabolism, galactose and pyruvate metabolism, and glycolysis/gluconeogenesis has been found using shotgun sequencing, changes that were accompanied by a loss of fibrolytic microorganisms belonging to *Eubacterium*, *Bifidobacterium*, and *Faecalibacterium* genera (Rampelli et al., 2013b). A rise in facultative anaerobes and proteolytic bacteria, such as fusobacteria, propionibacteria, and clostridia, has been reported as well, suggesting a trend toward putrefaction of the large bowel (Woodmansey, 2007). Another characteristic widely observed for this part of the population is a decline in the levels and diversity of bifidobacteria (Woodmansey et al., 2004; Arboleya et al., 2016), possibly leading to a reduced immune responsiveness and an increased susceptibility to gastrointestinal infections (Woodmansey, 2007). In some cases, reduced levels of *Clostridium cluster* XIVa and *Faecalibacterium* were described (O'Toole and Claesson, 2010; Salazar et al., 2013, 2019).

Among the elderly, age seems to be an important factor that determines the microbiota composition, as observed by Salazar et al. (2019). In this study, the levels of *Akkermansia* and *Lactobacillus* for a subgroup of elderly (>80 years old) were significantly higher than those observed in adult (<50 years old) and the younger elderly (50–80 years old) groups, respectively. In this sense, Biagi et al. (2010) found comparable diversity values of the gut microbiota between the elderly and young adults, while centenarians stood out as a separate population, with *Bacteroidetes* and *Firmicutes* still dominating the gut microbiota. However, some changes in the relative proportion of *Firmicutes* subgroups were observed in comparison with the younger adults, with a decrease in the *Clostridium* cluster XIVa, as observed elsewhere (Bartosch et al., 2004; Zwielehner et al., 2009). In addition, the authors described

an increase in bacilli, a rearrangement of the *Clostridium cluster* IV (lower levels of *F. prausnitzii* in centenarians than in the younger elderly) and increased *Proteobacteria*. This last group contains many "pathobionts" bacteria, which, under some circumstances (e.g., inflammation), might induce pathology (Biagi et al., 2010). Some members of this group are *Helicobacter hepaticus*, segmented filamentous bacteria, *Escherichia coli*, and *Enterococcus faecalis* (Jochum and Stecher, 2020). Regarding SCFA production, several butyrate producers were found in lower amounts in centenarians than in other age groups, indicating a general decrease in SCFA levels with age (Salazar et al., 2013, 2019).

PROBIOTICS, PREBIOTICS, AND SYNBIOTICS: POTENTIAL APPLICATIONS

Bifidobacteria and lactobacilli have been widely considered health-promoting constituents of the microbiota (Bedani et al., 2016). Different strains of these genera were lately used as probiotics and proved to have many health benefits within the elderly, such as microbiota modulation, improvement of bowel movements, control of opportunistic bacteria, positive effects on mental conditions, stimulation of the innate immune system, increased vitamin intake, among other effects detailed in **Supplementary Table S1** according to clinical and pre-clinical trials. Although the impact of prebiotics and synbiotics has been studied to a lesser extent, there are some recent clinical studies indicating positive health benefits as well. Several attempts to isolate probiotic strains from elderly people have been reported; for example, Silvi et al. (2003) isolated *Limosilactobacillus fermentum* and *Bifidobacterium longum* strains from elderly people (aged 65–87 years, Italy) as part of an EU-funded project, whose final objective was the future application of the isolated strains to design appropriate functional foods for the elderly. Similarly, Park et al. (2015) isolated *L. fermentum* as the most frequent species in fecal samples from longevity (>80 years) populations in Korea, highlighting the potential relevance of this particular species for the formulation of probiotic food or supplements for seniors.

Effects Demonstrated on Elderly Subjects by Clinical Trials

Most of the latest research carried out in this field implied clinical trials addressed to healthy elderly people (**Supplementary Table S1**). In general, the application of commercial probiotics (one strain or a cocktail) was the most chosen strategy among them. The effects observed indicate that the consumption of probiotics may positively impact the gut microbiota by increasing the levels of bifidobacteria or modifying subpopulations of lactobacilli (Nagata et al., 2011; Akatsu et al., 2013; Ostan et al., 2015). Furthermore, probiotics were associated with the ability to promote interactions between key constituents of the microbiota and the host epithelium (Eloe-Fadrosh et al., 2015), enhance the immunity response (Finamore et al., 2019; Yamamoto et al., 2019; Wang et al., 2020) and improve bowel movements (Nagata et al., 2015; Inoue et al., 2018; Aoyagi et al., 2019). Other health benefits were related to their ability to revert age-related increase of opportunistic pathogens, such as *Clostridium difficile*, involved in antibiotic-associated diarrhea that impact on nutrition and inflammatory status, exerting an important role in pathophysiological processes. In the elderly, *C. difficile*-associated diarrhea was linked with a reduction on the number of bifidobacteria (Hopkins and MacFarlane, 2002); for this reason, therapies based on the use of probiotics to correct the microbiota imbalance would be promising (Rampelli et al., 2013a). In this direction, Nagamine et al. (2019) reported that probiotics could reduce *Clostridium difficile* infection (CDI) among elderly patients who underwent proximal femoral fracture surgery, but at this moment, there is not enough information about their mechanisms of action, and the current guidelines do not recommend their administration (McDonald et al., 2018). Notwithstanding the promising results, other studies reported controversial ones, most of them having no significant results (Mallina et al., 2018; Sofian et al., 2019).

Extra-Intestinal Effects

When considering the extra-intestinal positive effects, probiotics were able to enhance the oral health of elderly by the control of *Candida* and hyposalivation, common problems among this group (Ishikawa et al., 2014; Kraft-Bodi et al., 2015; Lee et al., 2019). Other reports have also associated probiotic consumption with positive effects on respiratory tract infections by reducing the duration (Guillemard et al., 2010; Fujita et al., 2013) or accelerating the healing process in patients with acute distal radius fracture (Lei et al., 2016). Although probiotics showed potential effects on bone metabolism in different mouse models (Roberts et al., 2019, 2020), further clinical trials are required to assess this effect on the elderly.

Recently, brain-gut axis has received special attention, since the application of some probiotics on elderly subjects improved mental conditions, such as anxiety and depression, when combined with a 12-week resistance-training program which consisted of classes comprising a warm-up session, resistance training, and a cool-down session (Inoue et al., 2018). Probiotics also promoted mental flexibility and alleviated stress (Kim et al., 2020). Particularly, they were recommended for the treatment of different health conditions as the silent systematic inflammation and neuroinflammation that are frequently observed in the early stage of Alzheimer's disease (Leblhuber et al., 2018). These authors reported positive results that indicate a probiotic supplement influenced by gut bacteria composition (increased levels *F. prausnitzii*), tryptophan metabolism and immune response. In this case, in spite of its limitations (a small sample size and the absence of placebo control), it was suggested that the increase of *F. praunitzii* could mitigate the cerebral accumulation of β-amyloid and lipopolysaccharides, which are overproduced during the pathogenesis of Alzheimer's disease.

Other effects that probiotics exert on the elderly were associated with augmented levels of vitamins in the blood. For example, Valentini et al. (2015) reported increased

concentration of vitamin B12 and folate in serum, accompanied by a reduction in plasma homocysteine in subjects that received a commercial probiotic supplementation. The lack of one of these vitamins may cause megaloblastic anemia and a series of neurological and mental symptoms in the elderly, as both vitamins play a crucial role in the cognitive function (Lokk, 2003). These increases were positively correlated with the change in fecal bifidobacterial concentrations for the subjects with low-grade inflammation. The authors suggested that, since the decrease in homocysteine levels was clinically relevant, the probiotic could provide protective effects against some aging-associated conditions (such as cardiovascular or neurological diseases). However, several limitations can be mentioned: It was an open label study without a placebo group, and it used biological instead of clinical endpoints.

On the other hand, the use of synbiotics (combination of probiotics and prebiotics) has also demonstrated similar beneficial effects on the gut microbiota (**Supplementary Table S1**). For example, synbiotics proved to increase the number of bifidobacteria and lactobacilli, improve the stool frequency and mucosal integrity, increase butyrate production, diminish pro-inflammatory response, and enhance lipid metabolism (Björklund et al., 2012; Granata et al., 2013; Macfarlane et al., 2013). In addition, synbiotics significantly decreased metabolic syndrome prevalence, several cardiovascular risk factors and markers of insulin resistance in elderly patients (Cicero et al., 2020). Regarding prebiotics, some clinical trials demonstrated that they have positive effects on the gut microbiota composition and immune responses as well (Walton et al., 2012; Alfa et al., 2017).

To sum up, **Figure 1** shows, as a graphical representation, the physiological, nutritional and immune targets of intervention generally identified for elderly people and the possible effects of these functional supplements on this population.

Effects on Different Mouse Models

Recently, there have been several studies about the health benefits that probiotics exert on the elderly using different pre-clinical (*in vivo*) models, as summarized in **Supplementary Table S1**. In general, the most selected model for studying the advanced age is C57BL/6J mice, aged 18 months or more (Flurkey et al., 2007). With this murine model, along with other *in vitro* assays, Ahmadi et al. (2020) demonstrated that a probiotic cocktail prevented mice into undergoing a high-fat diet from microbiota dysbiosis, leaky gut, inflammation, and metabolic and physical dysfunctions. In this direction, Jeong et al. (2016) obtained similar results for a probiotic strain of the species *Levilactobacillus brevis* since the treatment was effective in modulating the gut microbiota, inhibited the expression of inflammatory markers, enhanced colonic tight junctions, and ameliorated colitis and memory impairment. Similarly, Vemuri et al. (2019) demonstrated that a probiotic strain of *Lactobacillus acidophilus* increased the abundances of beneficial bacteria, such as *Akkermansia* spp. and *Lactobacillus* spp., and enhanced the levels of butyrate while downregulating the production of inflammatory cytokines. An interesting result observed by Lee et al. (2016) indicated a probiotic strain provided by female C57BL/6J mice with healthy skin, active folliculogenesis, and hair growth, together with immunomodulation. Probiotics supplementation has also been associated with a positive impact on oxidative stress and inflammation in peripheral tissues in this strain of mice (Ni et al., 2019).

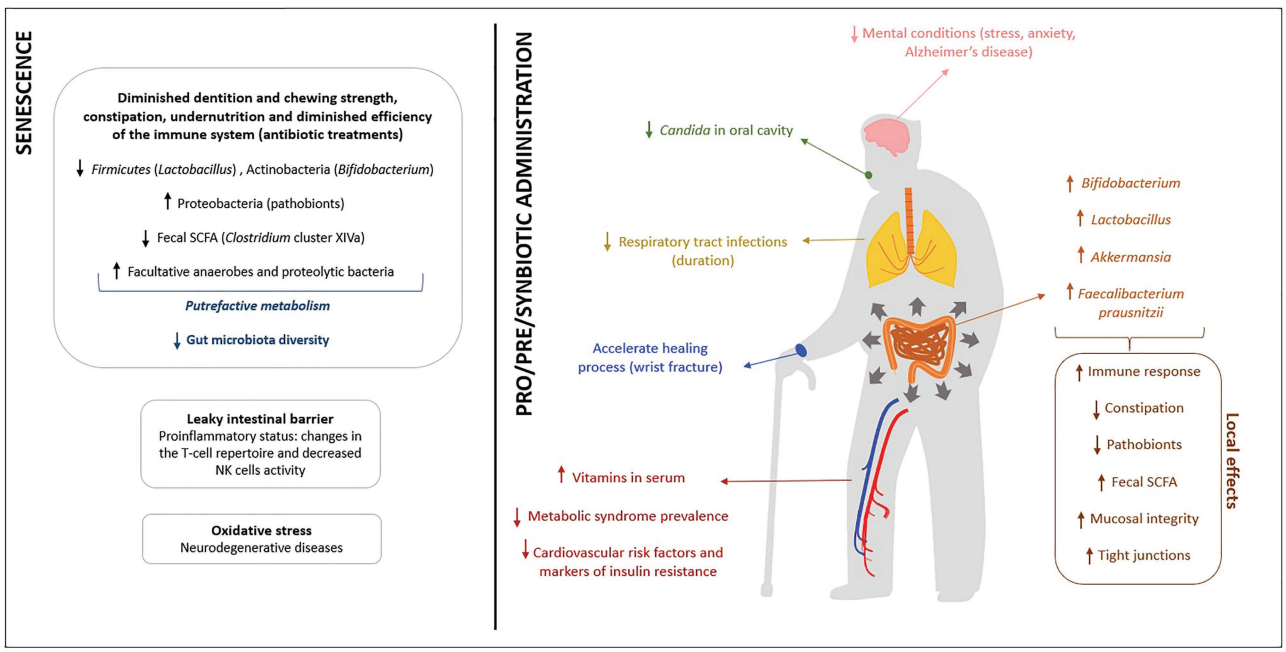

FIGURE 1 | Summary of the main health effects attributed to probiotics,prebiotics and synbiotics in the elderly.

As shown in **Supplementary Table S1**, there are other murine models that were successfully applied, one of them consisting of using D-galactose to induce premature senescence on Sprague Dawley rats. The results suggest probiotics ameliorated aging-induced metabolic diseases, pathogens growth, microbiota dysbiosis, oxidative stress, inflammation, and alteration of gut metabolites (Hor et al., 2019a,b; Lew et al., 2020). In other works, the BALB/c strain was used, with or without D-galactose injection. Improvement of immunological markers (Molina et al., 2016), modulation of microbiota and protective effects on oxidative stress induced by D-galactose (Zhang et al., 2017) were reported with this model.

Finally, there are some other murine models used for specific studies. This is the case of Yang et al. (2020), who used SAMP8 mice to study the potential of probiotics to treat deficits of the microbiota-gut-brain axis and cognitive function in aging. On the other hand, transgenic B6 mice were used to analyze the effects probiotics have on Alzheimer's disease, showing promising results on the glucose metabolism (improved glucose uptake) and on the disease progression (Bonfili et al., 2020).

PARAMETERS TO CONSIDER FOR FUTURE STUDIES

From the analysis of the information provided in the present mini-review, guidelines to address future studies regarding the role of probiotics, prebiotics and synbiotics in aging could be proposed. Without focusing on specific age groups, the minimum criteria that apply exclusively to probiotic strains for their use in foods and dietary supplements were recently revised (Binda et al., 2020). Similar principles could be considered for the administration of probiotics, prebiotics, or synbiotics to elderly people with special focus on their particular needs.

Special attention should be payed when designing the experiences to ensure the reliability of the clinical trial itself and the correct publication of the results, which should be based on recognized guidelines, as the Good Clinical Practice guidelines of the International Council for Harmonization, ICH-GCP,[2] and The Consolidated Standards of Reporting Trials.[3] Some of the factors to consider are (i) the choice of adequate controls, (ii) blinding, (iii) design, and (iv) the selection of the elderly population sample (health or disease status, male or female). In order to diminish the variability among studies, researchers should be aware of the effect different ages could cause, since significant differences between young elderly people (65–80 years old), those aged >80 years and centenarians, have been previously reported (Biagi et al., 2010; Salazar et al., 2019). For this reason, homogenous groups are recommended to avoid skewing the results obtained. On balance, nutritional strategies for the elderly should be addressed from a holistic point of view considering their special nutritional needs, the high susceptibility to disease, and the frequent medicine (mainly antibiotics) intake, as a whole (Salazar et al., 2017).

The location where the research takes place has an important influence (elderly living in their homes, in a hospitalized environment, developed or developing countries, etc), since parameters such as diet and lifestyle are of great relevance (O'Toole and Jeffery, 2015; Salazar et al., 2017). In addition, the matrix in which probiotics, prebiotics, or synbiotics are delivered should be chosen carefully because it may have an impact on the overall results. Diminished dentition, chewing strength, and constipation are essential factors to consider when choosing the matrix. The dose, administration schedule, and duration of intervention must be contemplated as well, as too long treatments might be difficult to be followed in practice, this affecting the health effects expected. Therefore, functional food for seniors, containing probiotics, prebiotics, or synbiotics, should cover all these aspects and should be available in the market as more personalized treatments than products for the public in general.

CONCLUSION

The present work suggests that modifying the gut microbiota of the elderly population by the intake of functional food/supplements as probiotics, prebiotics, or synbiotics may be an effective and non-invasive strategy to counteract the natural consequences of aging, in most cases affected by the extended use of antibiotics, providing a better quality of life. At the same time, these functional products may be suitable, affordable, and economical to most elderly people. However, several concerns should be considered when future studies are addressed, to obtain not only reliable results but also treatments feasible to be applied and practical to be followed.

AUTHOR CONTRIBUTIONS

EA and AB conceptualized and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Universidad Nacional del Litoral (UNL).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.631254/full#supplementary-material

[2]https://ichgcp.net

[3]http://www.consort-statement.org/consort-2010

REFERENCES

Ahmadi, S., Wang, S., Nagpal, R., Wang, B., Jain, S., Razazan, A., et al. (2020). A human-origin probiotic cocktail ameliorates aging-related leaky gut and inflammation via modulating the microbiota/taurine/tight junction axis. *JCI Insight* 5:e132055. doi: 10.1172/jci.insight.132055

Ahmed, M., Prasad, J., Gill, H., Stevenson, L., and Gopal, P. (2007). Impact of consumption of different levels of *Bifidobacterium lactis* HN019 on the intestinal microflora of elderly human subjects. *J. Nutr. Health Aging* 11, 26–31.

Akatsu, H., Iwabuchi, N., Xiao, J. Z., Matsuyama, Z., Kurihara, R., Okuda, K., et al. (2013). Clinical effects of probiotic *Bifidobacterium longum* BB536 on immune function and intestinal microbiota in elderly patients receiving enteral tube feeding. *J. Parenter. Enter. Nutr.* 37, 631–640. doi: 10.1177/0148607112467819

Alfa, M. J., Strang, D., Tappia, P. S., Graham, M., Van Domselaar, G., Forbes, J. D., et al. (2017). A randomized trial to determine the impact of a digestion resistant starch composition on the gut microbiome in older and mid-age adults. *Clin. Nutr.* 37, 797–807. doi: 10.1016/j.clnu.2017.03.025

Allen, S. J., Wareham, K., Wang, D., Bradley, C., Sewell, B., Hutchings, H., et al. (2013). A high-dose preparation of lactobacilli and bifidobacteria in the prevention of antibiotic-associated and *Clostridium difficile* diarrhea in older people admitted to hospital: a multicentre, randomised, double-blind, placebo-controlled, parallel arm trial. *Health Technol. Assess.* 17, 1–140. doi: 10.3310/hta17570

Aoyagi, Y., Amamoto, R., Park, S., Honda, Y., Shimamoto, K., Kushiro, A., et al. (2019). Independent and interactive effects of habitually ingesting fermented milk products containing *Lactobacillus casei* strain shirota and of engaging in moderate habitual daily physical activity on the intestinal health of older people. *Front. Microbiol.* 10:1477. doi: 10.3389/fmicb.2019.01477

Arboleya, S., Watkins, C., Stanton, C., and Ross, R. P. (2016). Gut bifidobacteria populations in human health and aging. *Front. Microbiol.* 7:1204. doi: 10.3389/fmicb.2016.01204

Asarat, M., Apostolopoulos, V., Vasiljevic, T., and Donkor, O. (2016). Short-chain fatty acids regulate cytokines and Th17/Treg cells in human peripheral blood mononuclear cells *in vitro*. *Immunol. Investig.* 45, 205–222. doi: 10.3109/08820139.2015.1122613

Bartosch, S., Fite, A., Macfarlane, G. T., and McMurdo, M. E. T. (2004). Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.* 70, 3575–3581. doi: 10.1128/AEM.70.6.3575-3581.2004

Bartosch, S., Woodmansey, E. J., Paterson, J. C. M., McMurdo, M. E. T., and Macfarlane, G. T. (2005). Microbiological effects of consuming a synbiotic containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and oligofructose in elderly persons, determined by real-time polymerase chain reaction and counting of viable bacteria. *Clin. Infect. Dis.* 40, 28–37. doi: 10.1086/426027

Bedani, R., Isay Saad, S. M., and Sivieri, K. (2016). "Potential benefits of probiotics, prebiotics, and synbiotics on the intestinal microbiota of the elderly" in *Probiotics, prebiotics, Synbiotics: Bioactive foods in health*. eds. R. R. Watson and V. R. Preedy (London, UK: Academic Press, Elsevier), 525–538.

Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C., and Brigidi, P. (2012). Ageing of the human metaorganism: the microbial counterpart. *Age (Dordr.)* 34, 247–267. doi: 10.1007/s11357-011-9217-5

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., et al. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One* 5:e10667. doi: 10.1371/journal.pone.0010667

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., et al. (2020). Criteria to qualify microorganisms as "probiotic" in foods and dietary supplements. *Front. Microbiol.* 11:1662. doi: 10.3389/fmicb.2020.01662

Björklund, M., Ouwehand, A. C., Forssten, S. D., Nikkilä, J., Tiihonen, K., Rautonen, N., et al. (2012). Gut microbiota of healthy elderly NSAID users is selectively modified with the administration of *Lactobacillus acidophilus* NCFM and lactitol. *Age (Dordr.)* 34, 987–999. doi: 10.1007/s11357-011-9294-5

Bonfili, L., Cecarini, V., Gogoi, O., Berardi, S., Scarpona, S., Angeletti, M., et al. (2020). Gut microbiota manipulation through probiotics oral administration restores glucose homeostasis in a mouse model of Alzheimer's disease. *Neurobiol. Aging* 87, 35–43. doi: 10.1016/j.neurobiolaging.2019.11.004

Butler, C. C., Lau, M., Gillespie, D., Owen-Jones, E., Lown, M., Wootton, M., et al. (2020). Effect of probiotic use on antibiotic administration among care home residents: a randomized clinical trial. *JAMA* 324, 47–56. doi: 10.1001/jama.2020.8556

Calcinotto, A., Kohli, J., Zagato, E., Pellegrini, L., Demaria, M., and Alimonti, A. (2019). Cellular senescence: aging, cancer, and injury. *Physiol. Rev.* 99, 1047–1078. doi: 10.1152/physrev.00020.2018

Cicero, A. F. G., Fogacci, F., Bove, M., Giovannini, M., and Borghi, C. (2020). Impact of a short-term synbiotic supplementation on metabolic syndrome and systemic inflammation in elderly patients: a randomized placebo-controlled clinical trial. *Eur. J. Nutr.* doi: 10.1007/s00394-020-02271-8 [Epub ahead of print]

Claesson, M. J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., De Weerd, H., Flannery, E., et al. (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc. Natl. Acad. Sci. U. S. A.* 108, 4586–4591. doi: 10.1073/pnas.1000097107

Eloe-Fadrosh, E. A., Brady, A., Crabtree, J., Drabek, E. F., Ma, B., Mahurkar, A., et al. (2015). Functional dynamics of the gut microbiome in elderly people during probiotic consumption. *mBio* 6, e00231–e00215. doi: 10.1128/mBio.00231-15

Finamore, A., Roselli, M., Donini, L. M., Brasili, D. E., Rami, R., Carnevali, P., et al. (2019). Supplementation with *Bifidobacterium longum* Bar33 and *Lactobacillus helveticus* Bar13 mixture improves immunity in elderly humans (over 75 years) and aged mice. *Nutrition* 63–64, 184–192. doi: 10.1016/j.nut.2019.02.005

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., and Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* 3, 289–306. doi: 10.4161/gmic.19897

Flurkey, K., Currer, J. M., and Harrison, D. E. (2007). "The mouse in aging research" in *The mouse in biomedical research. 2nd Edn.* eds. J. G. Fox, S. Barthold, M. Davisson, C. Newcomer, F. Quimby and A. Smith (Burlington, MA: American College Laboratory Animal Medicine, Elsevier), 637–672.

Fonollá, J., Gracián, C., Maldonado-Lobón, J. A., Romero, C., Bédmar, A., Carrillo, J. C., et al. (2017). Effects of *Lactobacillus coryniformis* K8 CECT5711 on the immune response to influenza vaccination and the assessment of common respiratory symptoms in elderly subjects: a randomized controlled trial. *Eur. J. Nutr.* 58, 83–90. doi: 10.1007/s00394-017-1573-1

Fujita, R., Iimuro, S., Shinozaki, T., Sakamaki, K., Uemura, Y., Takeuchi, A., et al. (2013). Decreased duration of acute upper respiratory tract infections with daily intake of fermented milk: a multicenter, double-blinded, randomized comparative study in users of day care facilities for the elderly population. *Am. J. Infect. Control* 41, 1231–1235. doi: 10.1016/j.ajic.2013.04.005

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: the international scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14, 491–502. doi: 10.1038/nrgastro.2017.75

Granata, M., Brandi, G., Borsari, A., and Gasbarri, R., and Di Gioia, D. (2013). Synbiotic yogurt consumption by healthy adults and the elderly: the fate of bifidobacteria and LGG probiotic strain. *Int. J. Food Sci. Nutr.* 64, 162–168. doi: 10.3109/09637486.2012.718742

Guigoz, Y., Doré, J., and Schiffrin, E. J. (2008). The inflammatory status of old age can be nurtured from the intestinal environment. *Curr. Opin. Clin. Nutr. Metab. Care* 11, 13–20. doi: 10.1097/MCO.0b013e3282f2bfdf

Guillemard, E., Tondu, F., Lacoin, F., and Schrezenmeir, J. (2010). Consumption of a fermented dairy product containing the probiotic lactobacillus casei DN-114001 reduces the duration of respiratory infections in the elderly in a randomised controlled trial. *Br. J. Nutr.* 103, 58–68. doi: 10.1017/S0007114509991395

Hatakka, K., Ahola, A. J., Yli-Knuuttila, H., Richardson, M., Poussa, T., Meurman, J. H., et al. (2007). Probiotics reduce the prevalence of oral *Candida* in the elderly a randomized controlled trial. *J. Dent. Res.* 86, 125–130. doi: 10.1177/154405910708600204

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate

use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hopkins, M. J., and MacFarlane, G. T. (2002). Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. *J. Med. Microbiol.* 51, 448–454. doi: 10.1099/0022-1317-51-5-448

Hor, Y. Y., Lew, L. C., Jaafar, M. H., Lau, A. S. Y., Ong, J. S., Kato, T., et al. (2019a). *Lactobacillus* sp. improved microbiota and metabolite profiles of aging rats. *Pharmacol. Res.* 146:104312. doi: 10.1016/j.phrs.2019.104312

Hor, Y. Y., Ooi, C. H., Khoo, B. Y., Choi, S. B., Seeni, A., Shamsuddin, S., et al. (2019b). *Lactobacillus* strains alleviated aging symptoms and aging-induced metabolic disorders in aged rats. *J. Med. Food* 22, 1–13. doi: 10.1089/jmf.2018.4229

Ibrahim, F., Ruvio, S., Granlund, L., Salminen, S., Viitanen, M., and Ouwehand, A. C. (2010). Probiotics and immunosenescence: cheese as a carrier. *FEMS Immunol. Med. Microbiol.* 59, 53–59. doi: 10.1111/j.1574-695X.2010.00658.x

Inoue, T., Kobayashi, Y., Mori, N., Sakagawa, M., Xiao, J. Z., Moritani, T., et al. (2018). Effect of combined bifidobacteria supplementation and resistance training on cognitive function, body composition and bowel habits of healthy elderly subjects. *Benefic. Microbes* 9, 843–853. doi: 10.3920/BM2017.0193

Ishikawa, K. H., Mayer, M. P. A., Miyazima, T. Y., Matsubara, V. H., Silva, E. G., Paula, C. R., et al. (2014). A multispecies probiotic reduces oral *Candida* colonization in denture wearers. *J. Prosthodont.* 24, 194–199. doi: 10.1111/jopr.12198

Jeong, J. J., Kim, K. A., Hwang, Y. J., Han, M. J., and Kim, D. H. (2016). Anti-inflammaging effects of *Lactobacillus brevis* OW38 in aged mice. *Benefic. Microbes* 7, 707–718. doi: 10.3920/BM2016.0016

Jing, Y., Gravenstein, S., Rao Chaganty, N., Chen, N., Lyerly, K. H., Joyce, S., et al. (2007). Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood. *Exp. Gerontol.* 42, 719–732. doi: 10.1016/j.exger.2007.01.009

Jochum, L., and Stecher, B. (2020). Label or concept – what is a pathobiont? *Trends Microbiol.* 28, 789–792. doi: 10.1016/j.tim.2020.04.011

Kim, C. -S., Cha, L., Sim, M., Jung, S., Chun, W. Y., Baik, H. W., et al. (2020). Probiotic supplementation improves cognitive function and mood with changes in gut microbiota in community-dwelling older adults: a randomized, double-blind, placebo-controlled, multicenter trial. *J. Gerontol. A Biol. Sci. Med. Sci.* 76, 32–40. doi: 10.1093/gerona/glaa090

Kobayashi, Y., Kuhara, T., Oki, M., and Xiao, J. Z. (2019). Effects of *Bifidobacterium breve* a1 on the cognitive function of older adults with memory complaints: a randomised, double-blind, placebo-controlled trial. *Benefic. Microbes* 10, 511–520. doi: 10.3920/BM2018.0170

Komanduri, M., Gondalia, S., Scholey, A., and Stough, C. (2019). The microbiome and cognitive aging: a review of mechanisms. *Psychopharmacology* 236, 1559–1571. doi: 10.1007/s00213-019-05231-1

Kraft-Bodi, E., Jørgensen, M. R., Keller, M. K., Kragelund, C., and Twetman, S. (2015). Effect of probiotic bacteria on oral *Candida* in frail elderly. *J. Dent. Res.* 94, 181S–186S. doi: 10.1177/0022034515595950

Lahtinen, S. J., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., et al. (2012). Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM® modifies subpopulations of fecal lactobacilli and *Clostridium difficile* in the elderly. *Age (Dordr.)* 34, 133–143. doi: 10.1007/s11357-011-9208-6

Lahtinen, S. J., Tammela, L., Korpela, J., Parhiala, R., Ahokoski, H., Mykkänen, H., et al. (2009). Probiotics modulate the *Bifidobacterium* microbiota of elderly nursing home residents. *Age (Dordr.)* 31, 59–66. doi: 10.1007/s11357-008-9081-0

Leblhuber, F., Steiner, K., Schuetz, B., Fuchs, D., and Gostner, J. M. (2018). Probiotic supplementation in patients with Alzheimer's dementia - an explorative intervention study. *Curr. Alzheimer Res.* 15, 1106–1113. doi: 10.2174/1389200219666180813144834

Lee, X., Vergara, C., and Lozano, C. P. (2019). Severity of *Candida*-associated denture stomatitis is improved in institutionalized elders who consume *Lactobacillus rhamnosus* SP1. *Aust. Dent. J.* 64, 229–236. doi: 10.1111/adj.12692

Lee, J., Yang, W., Hostetler, A., Schultz, N., Suckow, M. A., Stewart, K. L., et al. (2016). Characterization of the anti-inflammatory *Lactobacillus reuteri* BM36301 and its probiotic benefits on aged mice. *BMC Microbiol.* 16:69. doi: 10.1186/s12866-016-0686-7

Lei, M., Hua, L. M., and Wang, D. W. (2016). The effect of probiotic treatment on elderly patients with distal radius fracture: a prospective double-blind, placebo-controlled randomised clinical trial. *Benefic. Microbes* 7, 631–637. doi: 10.3920/BM2016.0067

Lew, L. C., Hor, Y. Y., Jaafar, M. H., Lau, A. S. Y., Khoo, B. Y., Sasidharan, S., et al. (2020). Effects of potential probiotic strains on the fecal microbiota and metabolites of D-galactose-induced aging rats fed with high-fat diet. *Probiotics Antimicrob. Proteins* 12, 545–562. doi: 10.1007/s12602-019-09545-6

Liu, Y. -P., Liu, X., and Dong, L. (2012). Lactulose plus live binary *Bacillus subtilis* in the treatment of elders with functional constipation. *Zhonghua yi xue za zhi* 92, 2961–2964. doi: 10.3760/cma.j.issn.0376-2491.2012.42.003

Lokk, J. (2003). News and views on folate and elderly persons. *J. Gerontol. A Biol. Sci. Med. Sci.* 58, M354–M361. doi: 10.1093/gerona/58.4.M354

Macfarlane, S., Cleary, S., Bahrami, B., Reynolds, N., and Macfarlane, G. T. (2013). Synbiotic consumption changes the metabolism and composition of the gut microbiota in older people and modifies inflammatory processes: a randomised, double-blind, placebo-controlled crossover study. *Aliment. Pharmacol. Ther.* 38, 804–816. doi: 10.1111/apt.12453

Makino, S., Ikegami, S., Kume, A., Horiuchi, H., Sasaki, H., and Orii, N. (2010). Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. *Br. J. Nutr.* 104, 998–1006. doi: 10.1017/S000711451000173X

Mallina, R., Craik, J., Briffa, N., Ahluwalia, V., Clarke, J., and Cobb, A. G. (2018). Probiotic containing *Lactobacillus casei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* (ACTIMEL) for the prevention of *Clostridium difficile* associated diarrhea in the elderly with proximal femur fractures. *J. Infect. Public Health* 11, 85–88. doi: 10.1016/j.jiph.2017.04.001

Mañé, J., Pedrosa, E., Lorén, V., Gassull, M. A., Espadaler, J., Cuñé, J., et al. (2011). A mixture of *Lactobacillus plantarum* CECT 7315 and CECT 7316 enhances systemic immunity in elderly subjects. A dose-response, double-blind, placebo-controlled, randomized pilot trial. *Nutr. Hosp.* 26, 228–235. doi: 10.3305/nh.2011.26.1.5112

Maneerat, S., Lehtinen, M. J., Childs, C. E., Forssten, S. D., Alhoniemi, E., Tiphaine, M., et al. (2013). Consumption of *Bifidobacterium lactis* bi-07 by healthy elderly adults enhances phagocytic activity of monocytes and granulocytes. *J. Nutr. Sci.* 2, 1–10. doi: 10.1017/jns.2013.31

Manzoni, M. S. J., Rossi, E. A., Pauly-Silveira, N. D., Pinto, R. A., Roselino, M. N., Carlos, I. Z., et al. (2017). Consumption effect of a synbiotic beverage made from soy and yacon extracts containing *Bifidobacterium animalis* ssp. *lactis* BB-12 on the intestinal polyamine concentrations in elderly individuals. *Food Res. Int.* 99, 495–500. doi: 10.1016/j.foodres.2017.06.005

McDonald, L. C., Gerding, D. N., Johnson, S., Bakken, J. S., Carroll, K. C., Coffin, S. E., et al. (2018). Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin. Infect. Dis.* 66, 987–994. doi: 10.1093/cid/ciy149

Molina, V., Médici, M., Villena, J., Font, G., and Pía Taranto, M. (2016). Dietary supplementation with probiotic strain improves immune-health in aged mice. *Open J. Immunol.* 06, 73–78. doi: 10.4236/oji.2016.63008

Moro-Garcia, M. A., Alonso-Arias, R., Baltadjieva, M., Benitez, C. F., Barrial, M. A. F., Ruisánchez, E. D., et al. (2013). Oral supplementation with *Lactobacillus delbrueckii* subsp. *bulgaricus* 8481 enhances systemic immunity in elderly subjects. *Age (Dordr.)* 35, 1311–1326. doi: 10.1007/s11357-012-9434-6

Moroti, C., Souza Magri, L., De Rezende Costa, M., Cavallini, D. C. U., and Sivieri, K. (2012). Effect of the consumption of a new symbiotic shake on glycemia and cholesterol levels in elderly people with type 2 diabetes mellitus. *Lipids Health Dis.* 11, 1–8. doi: 10.1186/1476-511X-11-29

Nagamine, T., Matsumoto, Y., and Nakamura, M. (2019). Combination probiotics may prevent *Clostridium difficile* infection among elderly patients undergoing an orthopedic surgerya. *Biosci. Microbiota. Food Health* 38, 31–33. doi: 10.12938/bmfh.18-009

Nagata, S., Asahara, T., Ohta, T., Yamada, T., Kondo, S., Bian, L., et al. (2011). Effect of the continuous intake of probiotic-fermented milk containing *Lactobacillus casei* strain Shirota on fever in a mass outbreak of norovirus gastroenteritis and the faecal microflora in a health service facility for the aged. *Br. J. Nutr.* 106, 549–556. doi: 10.1017/S000711451100064X

Nagata, S., Asahara, T., Wang, C., Suyama, Y., Chonan, O., Takano, K., et al. (2015). The effectiveness of *Lactobacillus* beverages in controlling infections

among the residents of an aged care facility: a randomized placebo-controlled double-blind trial. *Ann. Nutr. Metab.* 68, 51–59. doi: 10.1159/000442305

Ni, Y., Yang, X., Zheng, L., Wang, Z., Wu, L., Jiang, J., et al. (2019). *Lactobacillus* and *Bifidobacterium* improves physiological function and cognitive ability in aged mice by the regulation of gut microbiota. *Mol. Nutr. Food Res.* 63, 1–14. doi: 10.1002/mnfr.201900603

O'Toole, P. W., and Claesson, M. J. (2010). Gut microbiota: changes throughout the lifespan from infancy to elderly. *Int. Dairy J.* 20, 281–291. doi: 10.1016/j.idairyj.2009.11.010

O'Toole, P. W., and Jeffery, I. B. (2015). Gut microbiota and aging. *Science* 350, 1214–1215. doi: 10.1126/science.aac8469

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J., et al. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol.* 16:90. doi: 10.1186/s12866-016-0708-5

Ostan, R., Béné, M. C., Spazzafumo, L., Pinto, A., Donini, L. M., Pryen, F., et al. (2015). Impact of diet and nutraceutical supplementation on inflammation in elderly people. Results from the RISTOMED study, an open-label randomized control trial. *Clin. Nutr.* 35, 812–818. doi: 10.1016/j.clnu.2015.06.010

Park, J. -S., Shin, E., Hong, H., Shin, H. -J., Cho, Y. -H., Ahn, K. -H., et al. (2015). Characterization of *Lactobacillus fermentum* PL9988 isolated from healthy elderly Korean in a longevity village. *J. Microbiol. Biotechnol.* 25, 1510–1518. doi: 10.4014/jmb.1505.05015

Rampelli, S., Candela, M., Severgnini, M., Biagi, E., Turroni, S., Roselli, M., et al. (2013a). A probiotics-containing biscuit modulates the intestinal microbiota in the elderly. *J. Nutr. Health Aging* 17, 166–172. doi: 10.1007/s12603-012-0372-x

Rampelli, S., Candela, M., Turroni, S., Biagi, E., Collino, S., Franceschi, C., et al. (2013b). Functional metagenomic profiling of intestinal microbiome in extreme ageing. *Aging (Albany NY)* 5, 902–912. doi: 10.18632/aging.100623

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., De los Reyes-Gavilán, C. G., and Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* 7:185. doi: 10.3389/fmicb.2016.00185

Roberts, J., Darby, T., Fernandes, L., Jones, R., and Drissi, H. (2019). Probiotic supplements accelerate bone repair and prevent systemic bone loss following femoral fracture (FS09-04-19). *Curr. Dev. Nutr.* 3:nzz044.FS09-04-19. doi: 10.1093/cdn/nzz044.FS09-04-19

Roberts, J. L., Liu, G., Darby, T. M., Fernandes, L. M., Diaz-Hernandez, M. E., Jones, R. M., et al. (2020). *Bifidobacterium adolescentis* supplementation attenuates fracture-induced systemic sequelae. *Biomed. Pharmacother.* 132:110831. doi: 10.1016/j.biopha.2020.110831

Rowe, J. W. (1997). The new gerontology. *Science* 278, 367–367. doi: 10.1126/science.278.5337.367

Salazar, N., Arboleya, S., Fernández-Navarro, T., de los Reyes-Gavilán, C. G., Gonzalez, S., and Gueimonde, M. (2019). Age-associated changes in gut microbiota and dietary components related with the immune system in adulthood and old age: a cross-sectional study. *Nutrients* 11, 1–11. doi: 10.3390/nu11081765

Salazar, N., López, P., Valdés, L., Margolles, A., Suárez, A., Patterson, Á. M., et al. (2013). Microbial targets for the development of functional foods accordingly with nutritional and immune parameters altered in the elderly. *J. Am. Coll. Nutr.* 32, 399–406. doi: 10.1080/07315724.2013.827047

Salazar, N., Valdés-Varela, L., González, S., Gueimonde, M., and de los Reyes-Gavilán, C. G. (2017). Nutrition and the gut microbiome in the elderly. *Gut Microbes* 8, 82–97. doi: 10.1080/19490976.2016.1256525

Salminen, A., Huuskonen, J., Ojala, J., Kauppinen, A., Kaarniranta, K., and Suuronen, T. (2008). Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. *Ageing Res. Rev.* 7, 83–105. doi: 10.1016/j.arr.2007.09.002

Shalim, C. P., Yoewono, A., Utomo, Y., and Kuswardhani, R. T. (2019). The role of probiotic supplementation on the immune system in elderly. *Int. J. Med. Biomed. Stud.* 3, 250–255. doi: 10.32553/ijmbs.v3i8.492

Silvi, S., Verdenelli, M. C., Orpianesi, C., and Cresci, A. (2003). EU project Crownalife: functional foods, gut microflora and healthy ageing: isolation and identification of *Lactobacillus* and *Bifidobacterium* strains from faecal samples of elderly subjects for a possible probiotic use in functional foods. *J. Food Eng.* 56, 195–200. doi: 10.1016/S0260-8774(02)00249-2

Sofian, M., Eghbal, E., Ghaznavi-Rad, E., Ramezani, A., and Mohaghegh, P. (2019). The effect of probiotic yogurt on the frequency of *Clostridium difficile* in old hospitalized patients. *J. Arak Uni. Med. Sci.* 22, 52–65. doi: 10.32598/JAMS.22.4.50

Swanson, K. S., Gibson, G. R., Hutkins, R., Reimer, R. A., Reid, G., Verbeke, K., et al. (2020). The international scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. *Nat. Rev. Gastroenterol. Hepatol.* 17, 687–701. doi: 10.1038/s41575-020-0344-2

Tannock, G. W., Tiong, I. S., Priest, P., Munro, K., Taylor, C., Richardson, A., et al. (2011). Testing probiotic strain *Escherichia coli* Nissle 1917 (Mutaflor) for its ability to reduce carriage of multidrug-resistant *E. coli* by elderly residents in long-term care facilities. *J. Med. Microbiol.* 60, 366–370. doi: 10.1099/jmm.0.025874-0

Toda, T., Kosaka, H., Terai, M., Mori, H., Benno, Y., and Yamori, Y. (2005). Effects of fermented milk with *Lactococcus lactis* subsp. *cremoris* FC on defecation frequency and fecal microflora in healthy elderly volunteers. *Nippon Shokuhin Kagaku Kogaku Kaishi* 52, 243–250. doi: 10.3136/nskkk.52.243

Troen, B. R. (2003). The biology of aging. *Mt Sinai J. Med.* 70, 3–22.

Valentini, L., Pinto, A., Bourdel-Marchasson, I., Ostan, R., Brigidi, P., Turroni, S., et al. (2015). Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota - the "RISTOMED project": randomized controlled trial in healthy older people. *Clin. Nutr.* 34, 593–602. doi: 10.1016/j.clnu.2014.09.023

Van Puyenbroeck, K., Hens, N., Coenen, S., Michiels, B., Beunckens, C., Molenberghs, G., et al. (2012). Efficacy of daily intake of *Lactobacillus casei* Shirota on respiratory symptoms and influenza vaccination immune response: a randomized, double-blind, placebo-controlled trial in healthy elderly nursing home residents. *Am. J. Clin. Nutr.* 95, 1165–1171. doi: 10.3945/ajcn.111.026831

Vasto, S., Malavolta, M., and Pawelec, G. (2006). Age and immunity. *Immun. Ageing* 3:2. doi: 10.1186/1742-4933-3-2

Vemuri, R., Gundamaraju, R., Shinde, T., Perera, A. P., Basheer, W., Southam, B., et al. (2019). *Lactobacillus acidophilus* DDS-1 modulates intestinal-specific microbiota, short-chain fatty acid and immunological profiles in aging mice. *Nutrients* 11, 1–23. doi: 10.3390/nu11061297

Vulevic, J., Drakoularakou, A., Yaqoob, P., Tzortzis, G., and Gibson, G. R. (2008). Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *Am. J. Clin. Nutr.* 88, 1438–1446. doi: 10.3945/ajcn.2008.26242

Vulevic, J., Juric, A., Walton, G. E., Claus, S. P., Tzortzis, G., Toward, R. E., et al. (2015). Influence of galacto-oligosaccharide mixture (B-GOS) on gut microbiota, immune parameters and metabonomics in elderly persons. *Br. J. Nutr.* 114, 586–595. doi: 10.1017/S0007114515001889

Walton, G. E., van den Heuvel, E. G. H. M., Kosters, M. H. W., Rastall, R. A., Tuohy, K. M., and Gibson, G. R. (2012). A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *Br. J. Nutr.* 107, 1466–1475. doi: 10.1017/S0007114511004697

Wang, P., Yin, X., Chen, G., Li, L., Le, Y., Xie, Z., et al. (2020). Perioperative probiotic treatment decreased the incidence of postoperative cognitive impairment in elderly patients following non-cardiac surgery: a randomised double-blind and placebo-controlled trial. *Clin. Nutr.* 40, 64–71. doi: 10.1016/j.clnu.2020.05.001

Woodmansey, E. J. (2007). Intestinal bacteria and ageing. *J. Appl. Microbiol.* 102, 1178–1186. doi: 10.1111/j.1365-2672.2007.03400.x

Woodmansey, E. J., McMurdo, M. E. T., Macfarlane, G. T., and Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Appl. Environ. Microbiol.* 70, 6113–6122. doi: 10.1128/AEM.70.10.6113-6122.2004

Yamamoto, Y., Saruta, J., Takahashi, T., To, M., Shimizu, T., Hayashi, T., et al. (2019). Effect of ingesting yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 on influenza virus-bound salivary IgA in elderly residents of nursing homes: a randomized controlled trial. *Acta Odontol. Scand.* 77, 517–524. doi: 10.1080/00016357.2019.1609697

Yang, X., Yu, D., Xue, L., Li, H., and Du, J. (2020). Probiotics modulate the microbiota–gut–brain axis and improve memory deficits in aged SAMP8 mice. *Acta Pharm. Sin. B* 10, 475–487. doi: 10.1016/j.apsb.2019.07.001

Zaharoni, H., Rimon, E., Vardi, H., Friger, M., Bolotin, A., and Shahar, D. R. (2011). Probiotics improve bowel movements in hospitalized elderly

patients - the proage study. *J. Nutr. Health Aging* 15, 215–220. doi: 10.1007/s12603-010-0323-3

Zhang, J., Zhao, X., Jiang, Y., Zhao, W., Guo, T., Cao, Y., et al. (2017). Antioxidant status and gut microbiota change in an aging mouse model as influenced by exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibetan kefir. *J. Dairy Sci.* 100, 6025–6041. doi: 10.3168/jds.2016-12480

Zhao, J., Tian, F., Yan, S., Zhai, Q., Zhang, H., and Chen, W. (2018). *Lactobacillus plantarum* CCFM10 alleviating oxidative stress and restoring the gut microbiota in d-galactose-induced aging mice. *Food Funct.* 9, 917–924. doi: 10.1039/C7FO01574G

Zwielehner, J., Liszt, K., Handschur, M., Lassl, C., Lapin, A., and Haslberger, A. G. (2009). Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV in institutionalized elderly. *Exp. Gerontol.* 44, 440–446. doi: 10.1016/j.exger.2009.04.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 09 February 2021
doi: 10.3389/fmicb.2021.630572

In vitro Selection of Probiotics for Microbiota Modulation in Normal-Weight and Severely Obese Individuals: Focus on Gas Production and Interaction With Intestinal Epithelial Cells

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The intestinal microbiota plays important roles in the maintenance of health. Strategies aiming at its modulation, such as probiotics, have received a deal of attention. Several strains have been studied in different *in vitro* models; however, the correlation of results obtained with the *in vivo* data has been limited. This questions the usefulness of such *in vitro* selection models, traditionally relying on over-simplified tests, not considering the influence of the accompanying microbiota or focusing on microbiota composition without considering functional traits. Here we assess the potential of six *Bifidobacterium*, *Lactobacillus* and *Lacticaseibacillus* strains in an *in vitro* model to determine their impact on the microbiota not just in terms of composition but also of functionality. Moreover, we compared the responses obtained in two different population groups: normal-weight and severely obese subjects. Fecal cultures were conducted to evaluate the impact of the strains on specific intestinal microbial groups, on the production of short-chain fatty acids, and on two functional responses: the production of gas and the interaction with human intestinal epithelial cells. The response to the different probiotics differed between both human groups. The addition of the probiotic strains did not induce major changes on the microbiota composition, with significant increases detected almost exclusively for the species added. Higher levels of gas production were observed in cultures from normal-weight subjects than in the obese population, with some strains being able to significantly reduce gas production in the latter group. Moreover, in obese subjects all the *Bifidobacterium* strains tested and *Lacticaseibacillus rhamnosus* GG were able to

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *17 November 2020*
Accepted: *18 January 2021*
Published: *09 February 2021*

Citation:
Nogacka AM, de los Reyes-Gavilán CG, Arboleya S, Ruas-Madiedo P, Martínez-Faedo C, Suarez A, He F, Harata G, Endo A, Salazar N and Gueimonde M (2021) In vitro Selection of Probiotics for Microbiota Modulation in Normal-Weight and Severely Obese Individuals: Focus on Gas Production and Interaction With Intestinal Epithelial Cells. Front. Microbiol. 12:630572. doi: 10.3389/fmicb.2021.630572

modify the response of the intestinal cells, restoring values similar to those obtained with the microbiotas of normal-weight subjects. Our results underline the need for the screening and selection of probiotics in a target-population specific manner by using appropriate *in vitro* models before enrolling in clinical intervention trials.

Keywords: *in vitro* model, gut microbiota, probiotics, gas production, severe obesity, *Bifidobacterium*, *Lactobacillus*, SCFA

INTRODUCTION

Recent studies have underlined the important role of the gastrointestinal microbiota (GIM) in the maintenance of host's health (Thursby and Juge, 2017). Alterations on this GIM, the so-called "dysbiosis," have been identified in several diseases (Duvallet et al., 2017). To this regard, obesity is not an exception and both compositional and functional differences between normal-weight (NW) subjects and obese patients have been repeatedly reported (Gomes et al., 2018; Vallianou et al., 2019). Although studies with severely obese individuals (OB) are scarce, accumulating evidence indicates the existence of dysbiosis in this group as well (Aron-Wisnewsky et al., 2019; Cani, 2019; Nogacka et al., 2020a). As it could be expected from the GIM differences, and very likely also due to the different dietary intakes, the fecal levels of short-chain fatty acids (SCFA) in OB subjects is also modified with regard to NW individuals (Kim et al., 2019; Nogacka et al., 2020a). These SCFA are important mediators on the GIM-host interaction, playing important roles on host's health (Ríos-Covián et al., 2016).

The GIM represents a potential target for strategies focused on health maintenance and improvement, with the use of probiotics constituting a promising approach to this end. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Several probiotic strains are being used in different functional products and a vast array of *in vitro* tests have been carried out to screen different strains. Most often, the process has been based in classical tests, such as tolerance to simulated gastrointestinal transit, adhesion to intestinal epithelial cells, co-culture with pathogens or immune cells, etc. However, in these *in vitro* tests, frequently the effect of the accompanying GIM has not been taken into account. For assessing the impact on the microbiota, several fecal culture models and simulators of the gastrointestinal tract have been used (Williams et al., 2015; von Martels et al., 2017), with compositional changes in the GIM being the main outcome in most of these studies, without considering potential functional changes. Moreover, in most cases this *in vitro* screening for potential probiotic strains was not driven by the impact on a specific target population. However, several studies have reported a high inter-individual variability in the response to probiotics, pointing out to the need for a population-specific selection (van Baarlen et al., 2011; Grześkowiak et al., 2012; Arboleya et al., 2013a,b). As a result of the use of these *in vitro* models, the correlation between *in vitro* and *in vivo* has often been limited (Vinderola et al., 2017). This lack of correlation may be partly explained by the use of over-simplified tests that do not consider the influence of the accompanying GIM, or when considering it, focusing only on its composition regardless of functional traits. The final consequence is that in spite of the huge amount of promising *in vitro* studies carried out with hundreds of microbial strains, only for a very limited number of them efficacy has been finally demonstrated in human intervention trials.

Different models of GIM-host interaction, such as co-cultivation of fecal samples or isolated GIM from different population groups or added with pro/prebiotics, with epithelial and/or immune cells, have been used (Arboleya et al., 2015; Nogacka et al., 2018, 2020a; Richards et al., 2019). Interestingly, several of these studies have demonstrated differences in the response induced by microbiotas from different population groups (Nogacka et al., 2018, 2020a) and between isolated GIM or among GIM added with probiotics or prebiotics (Arboleya et al., 2015; Nogacka et al., 2020b). These suggest that the inter-population differences on the gut microbiota may partly explain the high variability observed in the response to probiotics and the low correlation between *in vivo* and *in vitro* data.

In this work we explored the impact of different probiotic strains employing previously developed *in vitro* models, which take into account the GIM and that allow assessing the impact of the strains not just in terms of composition but also in terms of functionality on the GIM. This was achieved by monitoring, in real-time, the production of gas in fecal cultures and the interaction with HT29 intestinal epithelial cells, in addition to the study of the microbial composition and SCFA production, in two human population groups; NW and OB individuals.

MATERIALS AND METHODS

Strains and Culture Conditions

Four *Bifidobacterium* strains (*Bifidobacterium animalis* subsp *lactis* IF20/1 [IPLA20020], *Bifidobacterium bifidum* TMC3108, *B. bifidum* TMC3115 and *Bifidobacterium longum* IF14/11 [IPLA20022] as well as two lactobacilli (*Lactobacillus gasseri* BM7/10 [IPLA20212] and *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) GG [ATCC53103]) (Zheng et al., 2020) were used in this study. Frozen stocks were reactivated weekly in MRS agar (Biokar Diagnostics, Beauvois, France) supplemented with 0.25% (w/v) L-cysteine (MRSc; Sigma Chemical Co., St. Louis, MO, United States) by 48 h incubation at 37°C in an anaerobic chamber MG500 (Don Whitley Scientific, West Yorkshire, United Kingdom) under 80% (v/v) N_2, 10% (v/v) CO_2, and 10% H_2 atmosphere. Two overnight passages in MRSc broth before batch culture experiments were performed. The microbial suspensions for fecal cultures were obtained by inoculating (1%

v/v) fresh culture medium, incubating overnight under anaerobic conditions, centrifuging and washing twice the bacterial cells with PBS and adjusting to a final concentration of 1×10^{10} CFU/mL.

Volunteers and Fecal Sample Collection

Fecal samples were obtained from nine healthy NW adults (BMI < 25 kg/m^2) and six OB volunteers (BMI ≥ 40 kg/m^2) recruited at the Digestive and Endocrinology and Nutrition Services, respectively, of the Asturias Central University Hospital (HUCA, Asturias, Spain). The mean age of the volunteers was 40 ± 9 and 44 ± 9 years for NW and OB subjects, respectively. All participants followed an unrestricted diet and had not taken antibiotics during the previous 6 months. The study was approved by the Bioethical Committee of CSIC and from the Regional Ethics Committee for Clinical Research of the Principality of Asturias in compliance with the Declaration of Helsinki of 1964, last revised in 2013. An informed written consent was obtained from each volunteer. Samples were collected and immediately introduced into anaerobic jars (Anaerocult A System, Merck, Darmstadt, Germany) for transportation to the laboratory within 1 h and stored at −80°C until use.

Fecal Batch Cultures

Fecal samples were thawed at 37°C under anaerobic conditions. Then the samples were diluted 1/10 (w/v) with pre-reduced PBS and homogenized in a Lab-Blender 400 stomacher (Seward Medical, London, United Kingdom) at full-speed for 5 min and used as inocula for the fecal culture experiments. Carbohydrate-free basal medium (CFBM) (Al-Tamimi et al., 2006) was prepared and reduced overnight in anaerobic chamber one day before the batch fecal experiment. Pre-reduced CFBM was inoculated (10% v/v) with the fecal homogenate described above and then distributed into 100 mL bottles of the ANKOM RF system (ANKOM Technology, United States). The fecal cultures were allowed to stabilize overnight at 37°C in anaerobic conditions.

In brief, seven independent pH-free batch fermentations were performed for each human donor. We used as a carbon source 0.3% (w/v) of the fructooligosaccharide 1-kestose (β-Food Science Co. Ltd., Japan) which in previous experiments demonstrated to be more fermentable than other fructooligosaccharides not just by bifidobacteria and other intestinal anaerobes but also by lactobacilli (Nogacka et al., 2020b). Bacterial strains were added at a final concentration of 1×10^8 CFU/mL to fecal cultures in bottles. A bottle was left without probiotic added to be used as control. The fecal cultures were then incubated under anaerobic conditions at 37°C for 24 h. Samples (1 mL) were taken in duplicate at time 0 before incubation (time 0; basal conditions) and after 24 h of incubation. These samples were centrifuged at full speed for 10 min and supernatants and pellets were stored separately at −20°C until analyses.

pH and Gas Monitoring in Fecal Cultures

The pH of the cultures was determined with a pHmeter (SensION + PH3, HACH, Barcelona, Spain) and was considered as an indicator of the progression of fermentation. The cumulative gas produced along the different fermentations was monitored in real-time by using the ANKOM RF system. This system provides the increases in pressure (psi) which can be converted to mL of gas produced using the Ideal Gas Equation:

$$V = Vj \cdot Ppsi \cdot 0.068004084 \quad (1)$$

where: V = gas volume at 39°C in mL, Vj = headspace of digestion jar (Glass Bottle) in mL, Ppsi = cumulative pressure recorded by Gas Monitor System software.

The data of gas production were fitted to modified-Gompertz equation, a model frequently used to fit data of bacterial, plant growth, tumor proliferation and gas production (Ware and Power, 2017), by using the formula:

$$y = A \times \exp\left\{-\exp\left[\frac{\mu \times e}{A}(\lambda - t) + 1\right]\right\} \quad (2)$$

In which variables: "A" represents the upper asymptote (mL), "μ" is the rate of gas production (mL/h) and "λ" is the time lag before exponential phase (h).

Microbiota Composition and SCFA Quantification

DNA was extracted from the bacterial pellets by using the QIAamp DNA Stool Mini kit (Qiagen GmbH, Hilden, Germany) as previously described (Nogacka et al., 2020a) and the isolated DNA was stored at −20°C until use. The absolute levels of relevant intestinal microbial groups (*Bacteroides-Prevotella-Porphyromonas* group, *Lactobacillus*-group, *Akkermansia*, *Clostridium* cluster XIVa, *Bifidobacterium* and *Faecalibacterium* genera) as well as total bacteria were determined at 0 and 24 h of fermentation by qPCR using previously described primers and conditions (Valdés et al., 2017). Variations in the levels of the species *B. longum, B. bifidum*, *B. animalis*, *Bifidobacterium adolescentis* and *Bifidobacterium catenulatum* were assessed as described elsewhere (Salazar et al., 2015; Arboleya et al., 2020). In order to investigate microbial changes as regards to the basal microbiota, the data were expressed as the log-ratio of the Fold Change before (time 0) and after 24 h incubation with different probiotic strains.

The analysis of SCFA was performed by Gas Chromatography (GC) in the fecal culture supernatants (CS) in order to determine the molar concentrations of three main compounds: acetic, propionic and butyric acids. The remaining SCFA, namely iso-butyric and iso-valeric acids were also quantified and summed up (BCFA) for further analysis. Briefly, 0.25 mL of the culture supernatants were mixed with 0.3 mL methanol, 0.05 mL of an internal standard solution (2-ethylbutyric 1.05 mg/mL), and 0.05 mL of 20% formic acid. This mixture was centrifuged and the supernatant was used for quantification of SCFA by GC as described previously (Nogacka et al., 2018). Samples were analyzed in triplicate. Increments in molar concentration of SCFA with respect to the time 0 were calculated for each fermentation batch with the different probiotic strains tested.

Monitoring the Interaction of Intestinal Microbiotas and Fecal Culture Supernatants Supplemented With the Probiotics With HT29 Cells

We also aimed at evaluating the impact of the addition of probiotic strains on the interaction between the gut microbiota and enterocytes. To this end, we purified the microbiotas of the volunteers as described by Nogacka and co-workers (2018) and added them with the probiotic strains to be tested. In order to prevent acidification, which could damage the HT29 cells, these microbial mixtures were inactivated by UV light (Nogacka et al., 2018) and the interaction with confluent HT29 cells monolayers was evaluated by using a real-time cell analyser (RTCA-DP) xCelligence apparatus (ACEA Bioscience Inc., San Diego, CA, United States). Variations in HT29 cell monolayer trans-epithelial resistance (due to changes in morphology and/or attachment of the epithelial cells) during exposure to the microbiotas and CS were assessed. The culture conditions and the maintenance of the intestinal epithelial cell line HT29 (ECACC 91072201) is detailed in a previous work where the functional model was developed (Nogacka et al., 2018). For this functional assessment, each strain was mixed with the isolated microbiota in a bacterial proportion 1:1. Then, a ratio 10:1 of the total bacteria (6.5×10^7 cells/mL) with respect to the epithelial cell was added in McCoy's medium.

The functional assessment of the fecal CS on the behavior of HT29 cells monolayers was assessed by using filtered CS collected after 24 h of the fecal culture (pH adjusted to 7.55 ± 0.05) and diluted at 40% with McCoy's medium. Additionally, several controls consisting on basal microbiota, and McCoy's medium without bacteria or fecal supernatants added, were included in each experiment. Each sample was tested by duplicate using two independent E-plates. The monitoring was followed for every 10 min under standard incubation conditions. CI values recorded were normalized by the time of the sample addition and by the control sample, as previously described (Valdés et al., 2015). For statistical comparison purposes the "Area Under the Curve" (AUC), representing the CI values along 10 h of incubation for each sample, was calculated as explained in Nogacka et al. (2018).

Statistical Analyses

Unless otherwise specified, all experimental data are reported as mean ± standard deviation. Statistical analysis of results was performed using the software SPSS v.25 (SPSS Inc., Chicago, United States). Data were compared for the effect caused on the parameters analyzed by the addition of different probiotic strains in fecal cultures from each population cohort (NW and OB) at the end of fermentation (24 h). For variables with a normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene test), one way ANOVA followed by post-hoc DMS comparison were conducted. In the remaining cases (variables showing non-normal distribution), a Kruskal-Wallis test followed by a post-hoc Dunn's test of pairwise comparisons were applied when necessary. A significant p-value of 0.05 was used for the interpretation of results. For two-group comparisons between OB and NW subjects, a two-tailed Student's t-test or Mann-Whitney's U test was conducted for the evaluation of data by parametric or non-parametric contrast, respectively.

RESULTS

Effect of the Probiotic Strains on pH and Gas Production in Fecal Cultures

Drops of pH observed in fecal cultures were similar between the two human populations studied, OB and NW subjects, with values ranging from 1.42 to 1.78 pH units (**Table 1**). However, the response to the different probiotics showed noticeable differences between both groups. Whilst in the OB group no statistically differences were observed among treatments (control or the different probiotic strains), in NW subjects the strains *B. bifidum* TMC3115, *B. longum* IF14/11 and *L. gasseri* BM7/10 induced significantly (p-value <0.05) higher decreases of pH than those found in the control culture. The opposite trend was observed for gas production; thus, whilst in OB subjects some strains were able to significantly (p-value <0.05) reduce gas production in comparison to the control, no significant differences among treatments were detected in fecal cultures of the NW group (**Table 1**). Interestingly, in all the conditions tested (control and the different probiotics) fecal cultures of NW subjects shower higher gas production ability than cultures of OB individuals, although no statistical significant differences were found between cultures of both groups. In addition, the inter-individual variability was higher in NW than in the OB group, the later subjects resulting quite homogeneous in terms of gas production in fecal cultures. The kinetic parameters analyzed confirm this observation, with lower production rates in OB than in NW subjects (**Table 1**).

Effect of the Probiotic Strains Addition to Fecal Cultures on the Intestinal Microbiota

The probiotic strains tested induced changes in the microbiota of fecal cultures, mostly linked to increases in the administered bacterial group or species (**Figure 1** and **Supplementary Table 1**). As it could be expected, the addition of *Lactobacillus*/*Lacticaseibacillus* strains increased the levels of lactobacilli in the fecal culture, whereas the administration of *Bifidobacterium* strains increased bifidobacteria, and specifically the species used, regardless of the human population group. Regarding the other intestinal bacterial groups assessed, no effect of the addition of the probiotics, neither in cultures of OB nor in those of NW subjects, was observed for the levels of *Akkermansia*, Enterobacteriaceae, *Bacteroides*-group, *Faecalibacterium* or total bacteria (**Supplementary Table 1**). As with regard to *Clostridium* cluster XIVa the levels were not affected by the probiotic strains in fecal cultures from OB subjects, but in the NW group *B. bifidum* TMC3115 and *L. gasseri* BM7/10 promoted lower levels of this bacterial group when compared to the control culture (**Supplementary Table 1**).

Considering the basal differences existing on the microbial composition between fecal samples of OB and NW subjects,

TABLE 1 | Cumulative gas produced (mL) and decreases of pH values (Δ pH) after 24 h of incubation in fecal cultures from normal-weight (NW) and severely obesity (OB) individuals.

Group	Probiotic	Δ pH	Cumulative gas	*A*	μ	R^2
OB	Control	−1.54 ± 0.18	19.47^{b} ± 4.68	19.862	1.587	0.990
	B. animalis IF20/1	−1.57 ± 0.24	12.97^{a} ± 2.16	15.102	1.078	0.997
	B. bifidum TMC3108	−1.67 ± 0.11	14.22^{ab} ± 0.82	14.004	1.181	0.996
	B. bifidum TMC3115	−1.71 ± 0.17	13.69^{a} ± 2.96	13.555	1.114	0.997
	B. longum IF14/11	−1.78 ± 0.09	12.87^{a} ± 1.62	12.637	1.127	0.997
	L. gasseri BM7/10	−1.74 ± 0.17	14.40^{ab} ± 1.63	14.215	1.286	0.995
	L. rhamnosus GG	−1.70 ± 0.17	15.54^{ab} ± 2.80	15.441	1.335	0.996
NW	Control	-1.42^{a} ± 0.18	24.37 ± 12.28	24.623	2.228	0.999
	B. animalis IF20/1	-1.49^{ab} ± 0.18	22.01 ± 9.84	21.918	2.192	0.999
	B. bifidum TMC3108	-1.63^{abc} ± 0.07	20.69 ± 9.11	20.154	2.470	0.997
	B. bifidum TMC3115	-1.64^{c} ± 0.07	19.49 ± 7.20	19.108	2.248	0.998
	B. longum IF14/11	-1.64^{bc} ± 0.18	18.88 ± 8.46	18.629	2.121	0.998
	L. gasseri BM7/10	-1.63^{bc} ± 0.13	16.67 ± 6.75	16.431	1.783	0.996
	L. rhamnosus GG	-1.58^{abc} ± 0.16	22.35 ± 11.07	22.371	2.168	0.998

Kinetic parameters were determined using the modified-Gompertz equation, in which "A" represents the upper asymptote (mL) and "μ" is the rate of gas production (mL/h). The values not sharing the same superscript (a, b, or c) indicate significant differences (p-value <0.05) among probiotic strains and/or control for fecal cultures from each population group (NW or OB).

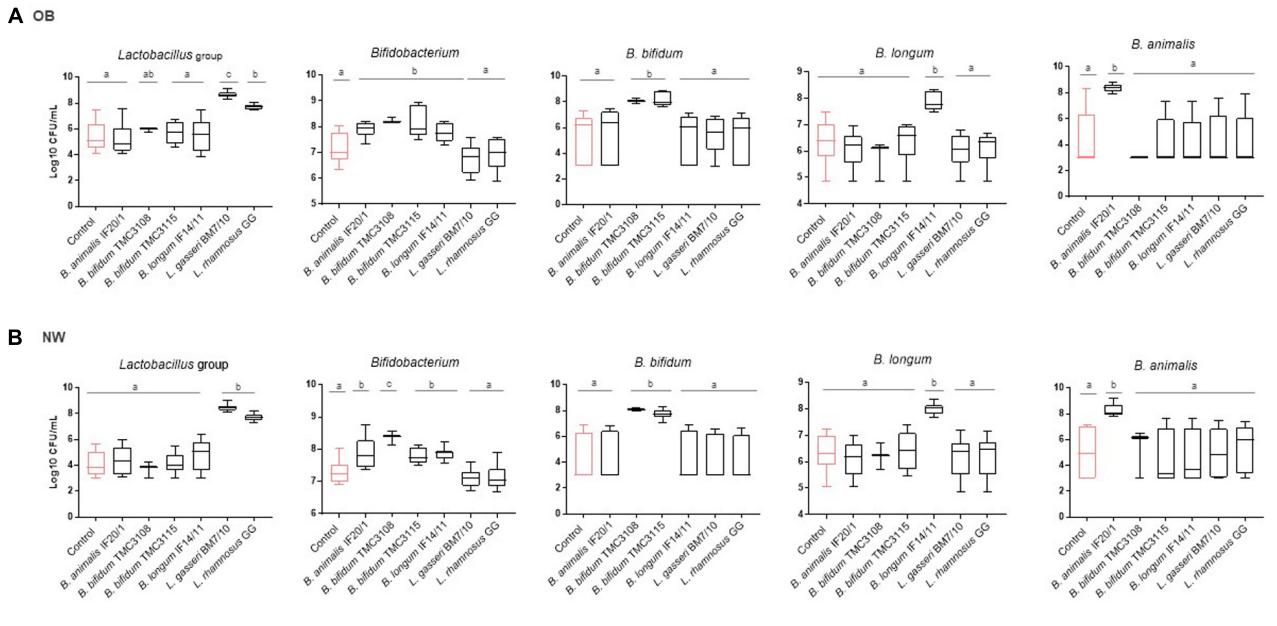

FIGURE 1 | Absolute levels (Log_{10} CFU/mL) of intestinal microbial groups determined by qPCR in fecal cultures of **(A)** OB and **(B)** NW subjects. For each condition, the box and whiskers plot represent median, interquartile range and minimum and maximum values obtained in each human group (NW or OB). Different letters above the boxes indicate significant differences (*p*-value <0.05) among probiotic strains for the microbial groups considered.

the fold change in bacterial levels were calculated for the different probiotics in fecal cultures of both groups of individuals. Interestingly, when the response to the different probiotic strains in the cultures of the two human groups studied were compared some statistically significant differences were found (**Supplementary Figure 1**). *B. animalis* subsp *lactis* IF20/1 and *B. bifidum* TMC3115 induced significantly higher increments (*p*-value <0.05) in the levels of *Bifidobacterium* in cultures of OB than in those of NW subjects. A similar trend, although not reaching statistical significance (*p*-value <0.1), was observed for *B. longum* IF14/11. Whereas *B. bifidum* TMC3108 also showed a trend (*p*-value <0.1) toward a larger reduction on the levels of *Clostridium* cluster XIVa in the OB than in NW group. Regarding the *Lactobacillus*/*Lacticaseibacillus* strains tested, *L. rhamnosus* GG led to an increase on lactobacilli levels which resulted significantly higher (*p*-value <0.05) in cultures of NW than in cultures of OB, and the same trend (*p*-value <0.1) was also observed with *L. gasseri* BM7/10. These results indicate that the increases in the corresponding groups induced by the probiotic *Lactobacillus*/ *Lacticaseibacillus* and *Bifidobacterium*

strains tested, is not limited to the increase of the administered strain. The difference in the increases induced by the same probiotic observed between OB and NW groups suggest that the probiotics may also affect the intestinal populations of lactobacilli or bifidobacteria in a way that depends on the basal microbiota, known to be different for OB and NW subjects.

When we assessed the production of SCFA no major differences were observed in the response to the different probiotics between OB and NW fecal cultures. In cultures of both groups of individuals, acetic acid was the SCFA present at higher concentration, with propionic and butyric acids being detected at lower levels (**Figure 2**). No differences among probiotic strains or between these ones with respect to the control culture were observed for propionic and butyric acid. However, differences became apparent for acetic acid, with the four *Bifidobacterium* strains tested (*B. animalis* subsp *lactis* IF20/1, *B. bifidum* TMC3108, *B. bifidum* TMC3115 and *B. longum* IF14/11) inducing the production of larger amounts of acetic acid (p-value <0.05) than the lactobacilli, or the control culture, for OB individuals. Moreover, these four strains led to the production of a higher concentration of acetic acid (p-value <0.05) than the lactobacilli also in NW subjects, with the increments from *B. bifidum* TMC3108, *B. bifidum* TMC3115 and *B. longum* IF14/11 being significantly higher (p-value <0.05) than those obtained for all the other experimental conditions in this NW group. Given that acetic acid is clearly the predominant SCFA in the samples, these observations are mirrored as well when the total level of SCFA was considered (**Figure 2**).

Effect of the Fecal Culture Supernatants and Microbiotas Added With Probiotic Strains on the Interaction With HT29 Cells

The interaction with intestinal cells monolayers was monitored in real-time using the RTCA system as a proxy for determining the impact of the probiotics on the functional response of the intestinal epithelium to the microbiota. This system allows monitoring in real time the epithelial cell monolayer structure/integrity by measuring the impedance and detecting changes in this parameter that may be due to changes in the morphology of the cells or in their attachment. We assessed first the effect of CS obtained at 24 h of incubation. The response observed with the supernatants of OB cultures displayed lower AUC values than those with cultures obtained from NW donors, which could be reflecting the significant difference found (p-value <0.05) between the control conditions from both human groups (**Figure 3A**). However, within cultures from each human group no statistically significant differences were observed among the different probiotics, with a similar response against all the strains tested (**Figure 3A**). Next, we assessed the effect of the addition of the different strains to the basal microbiota of the OB or NW individuals (**Figure 3B**). Again, differences (p-value <0.05) were observed between the basal microbiota of OB and NW subjects, with the microbiotas from OB subjects showing higher AUC values than that of NW individuals. When the different strains were compared, the *Lactobacillus*/*Lacticaseibacillus* strains displayed lower AUC values than the bifidobacteria in the NW group, whereas the results reached statistical significance (p-value <0.05) only for *L. gasseri* BM7/10 in NW subjects. Moreover, in OB subjects all bifidobacteria and *L. rhamnosus* GG strains induced significantly lower (p-value <0.05) AUC values than that obtained for the basal microbiota without probiotics added (**Figure 3B**), reaching values similar to those obtained for the microbiota of NW subjects.

DISCUSSION

Restoration of the eubiotic condition of the GIM is of great interest for the prevention of different diseases and probiotics have been among the most used tools to this end (Sanders et al., 2019). However, in spite of the several beneficial effects attributed to probiotics only some of them have been substantiated by clinical evidence (Merenstein et al., 2020). Therefore, in Europe with the sole exception of the yogurt and lactose intolerance, no other probiotic-based health claim has been approved. This is in contrast to the high amount of *in vitro* studies claiming the probiotic potential of several strains (Vinderola et al., 2017). This lack of agreement suggests that the conventional and perhaps over-simplified *in vitro* models most used until now in probiotics research (adhesion to mucus or intestinal cells, tolerance to acid and bile, pathogen inhibition or immune modulation etc.) show a poor predictive value for the *in vivo* situation where other microorganisms are present, at very large amounts, in the ecosystem. Actually, most often the strains have been tested alone, without the accompanying microbiota which in addition may be differ among population groups. This may explain why in some cases the experiments may have failed in the identification of the most suited strains for a given target population. The use of fecal culture models, or intestinal simulators, has been also common in the search for prebiotics with microbiota modulating abilities (Roberfroid et al., 2010; Shen et al., 2011; Bajury et al., 2018; Nogacka et al., 2020b). However, such models have not been so extensively used in probiotics research. Fecal culture models, similar to those applied in this study, have been used for the selection of potentially probiotic strains for GIM modulation in newborns (Arboleya et al., 2013b) or elderly (Valdés et al., 2017), among others. However, in contrast with the current work, in most of the available studies the selection was based exclusively on the effects upon the GIM composition, without taking into consideration the functionality of the microbiota. Only a few *in vitro* anaerobic bacterial co-culture systems that consider host-gut microbiota interaction have been employed for that purpose (von Martels et al., 2017), moving from the simplest ones, as the Host-Microbiota Interaction (HMI) and the Human oxygen Bacteria anaerobic (HoxBan) models, to the most sophisticated ones such as the microdevices Gut-on-a-chip and the Human Microbial crosstalk model (HuMiX). In the present work, affordable and simple *in vitro* tests for the screening and selection of probiotics in a target-population specific manner were assessed. We monitored, in real-time, the production of gas in fecal cultures and the interaction with intestinal epithelial cells of the culture supernatants or the isolated microbiotas from

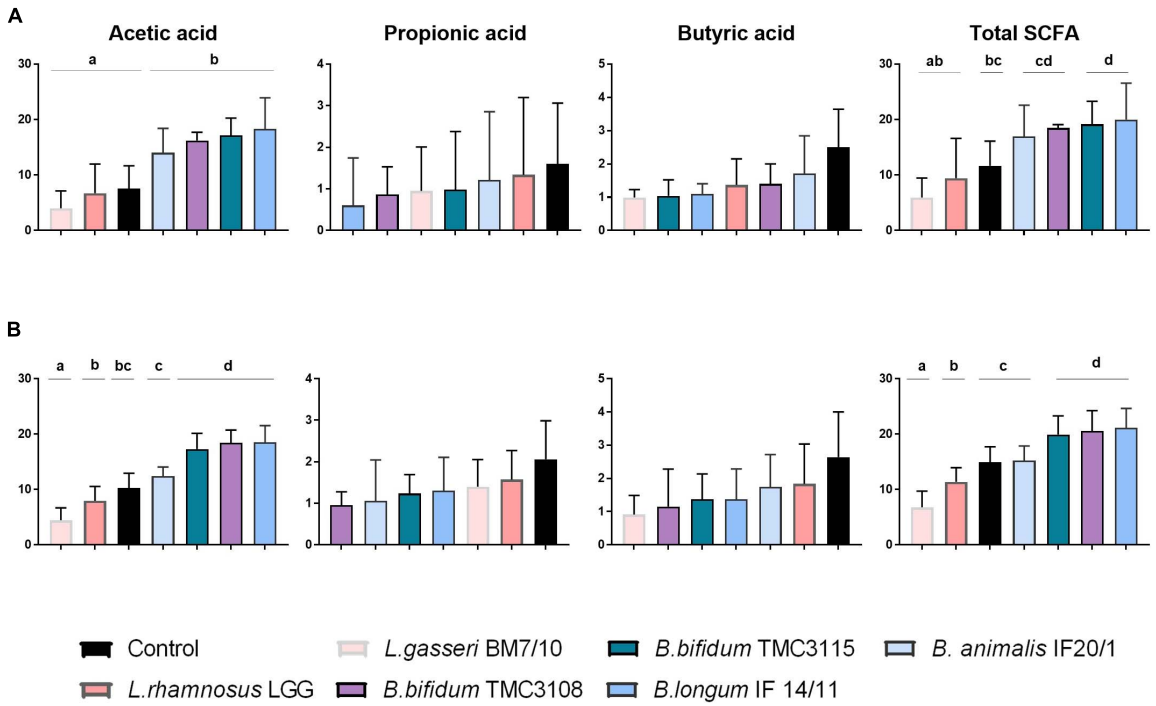

FIGURE 2 | Increments in ascending order, with respect to time 0, in the concentration (mM) of the major short-chain fatty acids (acetic, propionic, and butyric acids) after 24 h of incubation with different probiotic strains in fecal cultures from OB **(A)** and NW **(B)** groups. Differences are shown for each SCFA, columns that do not share the same letter are significantly different ($p < 0.05$).

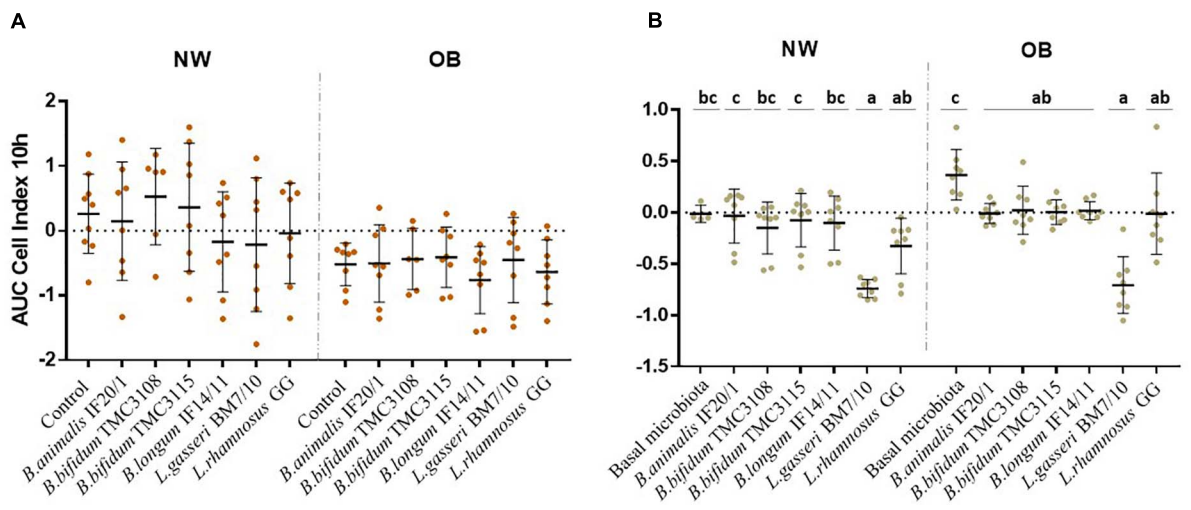

FIGURE 3 | Real-time monitoring the interaction with HT29 intestinal epithelial cells between **(A)** supernatants obtained after fecal culture with probiotic and **(B)** a mixture of a probiotic strain with the gut microbiota from NW and OB population groups. Values (media ± SD) correspond to the AUC resulting from monitoring Cell Index (CI) during 10 h. Significant differences (p-value <0.05) represent the comparison of results before and after probiotic addition in each condition.

NW and OB individuals supplemented with six *Bifidobacterium*, *Lactobacillus* and *Lacticaseibacillus* strains. Our *in vitro* models have taken into consideration not only the composition but also the functionality of the basal gut microbiota from NW and OB volunteers demonstrating their different response after probiotic administration.

Higher levels of gas production were generally achieved in fecal cultures from NW subjects than in the severely obese population, which is in good agreement with previous observations (Nogacka et al., 2020b). This result underlines the existence of functional differences between the GIM of NW and OB subjects and suggests a metabolically less active

microbiota in OB subjects. Changes in the gas production are related with differences in the composition and metabolic activity of the basal intestinal microbiota. Additionally, prebiotics are known to affect microorganisms of the intestinal microbiota such as bifidobacteria and lactobacilli that produce acetate and lactate; these compounds could be involved in cross-feeding mechanisms with gas-producing microorganisms such as *Clostridium* and sulfate-reducing bacteria (Sarbini and Rastall, 2011). We screened different probiotic strains for their ability to influence *in vitro* the GIM composition and activity. To this end, we evaluated gas production over the fecal culture in real-time using the ANKOM RF technology. Although this method had been already applied to fecal cultures (Rotbart et al., 2018; Yao et al., 2018; Nogacka et al., 2020b), this is the first time that it is applied in the evaluation of probiotics. This real-time monitoring of gas production has made possible to discriminate between probiotics according to their different ability to modulate gas production in fecal cultures of severely OB subjects, with *B. bifidum* TMC3115, *B. animalis* IF20/1 and *B. longum* IF14/11 being able to reduce the production of gas as compared with the control culture with no probiotics added. The fecal cultures of the NW individuals presented greater heterogeneity than those of OB individuals, which may partly explain why we failed to obtain statistically significant differences in the NW population.

As with regard to the response of the GIM to the addition of the different strains tested in fecal cultures, in general we did not observe any major changes in the absolute levels of the microbial groups analyzed, with significant increases only detected for the species of the added probiotics. The sole exception was the microbial group *Clostridium* XIVa in NW individuals with the strain *B. bifidum* TMC3108, which promoted higher levels of this microbial group than the other strains. In contrast, *B. bifidum* TMC3115 and *L. gasseri* BM7/10 were able to reduce the levels of this microbial group when compared to the control cultures from NW subjects. Interestingly, the *Lactobacillus*/ *Lacticaseibacillus* strains tested led to higher levels of lactobacilli in cultures from NW than in those from OB subjects, whereas the contrary occurred for bifidobacteria. These differences are likely due to the distinct basal microbiota between groups. Indeed, when comparing lactobacilli and bifidobacteria levels between both groups of individuals, at time zero, we observed that the fecal cultures from OB subjects showed significantly higher levels of lactobacilli and lower of bifidobacteria (5.14 ± 1.07 and 6.44 ± 0.37 Log_{10} CFU/mL, respectively) than those from NW individuals (3.9 ± 0.75 and 6.91 ± 0.41 Log_{10} CFU/mL, respectively). Moreover, these observations suggest that, regardless of the strain, the genus to which the probiotic strain belongs may constitute a first choice for selecting the best probiotic for microbiota modulation in a certain target population, i.e., OB or NW. These different responses observed between fecal cultures from both population groups underline previous studies reporting that the composition of the basal microbiota conditions the response to the probiotic (Arboleya et al., 2013a; Maldonado-Gómez et al., 2016; Hou et al., 2020).

Regarding SCFA, we did not observe mayor differences in the response to the different probiotics between fecal cultures of both human groups. As expected in both cases, NW and OB, acetic acid was the main SCFA followed by propionic and butyric acids. All the *Bifidobacterium* strains tested led to greater increases in the total SCFA, and of acetic acid, than the strains of lactobacilli, with *B. bifidum* TMC3108, *B. bifidum* TMC3115 and *B. longum* IF14/11 being those promoting higher values.

Finally, we studied the interaction with intestinal epithelial cells of the CS or the isolated microbiotas from NW and OB individuals supplemented with probiotics. No differences among the probiotics tested was observed when the CS were assessed, whereas GIM added with the different strains showed clear differences among them. Lactobacilli showed lower AUC values than bifidobacteria. Interestingly in OB but not in NW subjects all tested strains were able to down-regulate the HT29 cells response to the basal microbiota of these subjects. This is interesting given that the response to the basal microbiota of OB individuals was higher when compared with that of NW subjects. Therefore, the addition of the probiotic strains was able to restore this elevated response observed in the OB group bringing this functional response back to the levels observed in NW subjects.

Our results from *in vitro* models, although performed with a low number of samples, underline the need for the study and selection of probiotics in a target-population specific manner. The effects of strains in the fecal microbiota of NW individuals may be different from those that occur in OB, as it is the case of the data reported here. This complexity is often not considered in models where the strains are studied in isolation, without taking into account the mediation of the surrounding microbiota in the final effect on the host. It is, thus, necessary to select microorganisms with large functional capacity, as a previous step to carrying out human studies that entail a high economic cost. To achieve this, affordable *in vitro* study models such as those used here are necessary allowing the identification of the strains of potential interest, such as *B. bifidum* TMC3115, for its application in a specific human population group such as severely obese subjects. Nevertheless, it has to be taken into consideration that any *in vitro* screening for potential probiotic strains will require of later human clinical trials with higher number of individuals to evidence efficacy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethical Committee of CSIC and from the Regional Ethics Committee for Clinical Research of the Principality of Asturias. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CR-G, FH, GH, AE, NS, and MG conceived and designed the study. AS and CM-F recruited the volunteers and obtained the samples. AN and PR-M conducted the research. AN, CR-G, SA, NS, and MG analyzed the data. MG wrote and prepared the original draft. AN, CR-G, and NS helped with the manuscript review. All authors have read and approved the final version of the manuscript.

FUNDING

The present work was mainly financed by the project AGL2013-43770-R (receiving funds from the Ministry of Economy and Competitiveness—MINECO and the European Union FEDER funds), and partly by projects AGL2017-83653-R (AEI/FEDER, UE), and RTI2018-098288-B-I00 (MCIU/AEI/FEDER, UE) and by a contract with the company Takanashi Milk Products (Yokohama, Japan). AN was the recipient of a predoctoral contract granted by MINECO (reference BES-2014-068796). SA is currently financed by a postdoctoral Juan de la Cierva contract (Ministry of Science, Innovation and Universities, Spain, ref. IJCI-2017-32156) and NS is currently the recipient of a postdoctoral contract awarded by the Fundación para la Investigación y la Innovación Biosanitaria del Principado de Asturias (FINBA).

ACKNOWLEDGMENTS

We wish to thank the volunteers participating in the study and the Scientific-Technical Service of IPLA-CSIC for carrying out the SCFA determination.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.630572/full#supplementary-material

REFERENCES

Al-Tamimi, M. A. H. M., Palframan, R. J., Cooper, J. M., Gibson, G. R., and Rastall, R. A. (2006). In vitro fermentation of sugar beet arabinan and arabino-oligosaccharides by the human gut microflora. *J. Appl. Microbiol.* 100, 407–414. doi: 10.1111/j.1365-2672.2005.02780.x

Arboleya, S., Bahrami, B., Macfarlane, S., Gueimonde, M., Macfarlane, G. T., and de los Reyes-Gavilán, C. G. (2015). Production of immune response mediators by HT-29 intestinal cell-lines in the presence of bifidobacterium -treated infant microbiota. *Benef. Microbes* 6, 543–552. doi: 10.3920/BM2014.0111

Arboleya, S., Salazar, N., Solís, G., Fernández, N., Gueimonde, M., and de los Reyes-Gavilán, C. G. (2013a). In vitro evaluation of the impact of human background microbiota on the response to *Bifidobacterium* strains and fructo-oligosaccharides. *Br. J. Nutr.* 110, 2030–2036. doi: 10.1017/S0007114513001487

Arboleya, S., Salazar, N., Solís, G., Fernández, N., Hernández-Barranco, A. M., Cuesta, I., et al. (2013b). Assessment of intestinal microbiota modulation ability of *Bifidobacterium* strains in in vitro fecal batch cultures from preterm neonates. *Anaerobe* 19, 9–16. doi: 10.1016/j.anaerobe.2012.11.001

Arboleya, S., Saturio, S., Suárez, M., Fernández, N., Mancabelli, L., de los Reyes-Gavilán, C. G., et al. (2020). Donated human milk as a determinant factor for the gut bifidobacterial ecology in premature babies. *Microorganisms* 8, 760. doi: 10.3390/microorganisms8050760

Aron-Wisnewsky, J., Prifti, E., Belda, E., Ichou, F., Kayser, B. D., Dao, M. C., et al. (2019). Major microbiota dysbiosis in severe obesity: fate after bariatric surgery. *Gut* 68, 70–82. doi: 10.1136/gutjnl-2018-316103

Bajury, D. M., Nashri, S. M., King Jie Hung, P., and Sarbini, S. R. (2018). Evaluation of potential prebiotics: a review. *Food Rev. Int.* 34, 639–664. doi: 10.1080/87559129.2017.1373287

Cani, P. D. (2019). Severe obesity and gut microbiota: does bariatric surgery really reset the system? *Gut* 68, 5–6. doi: 10.1136/gutjnl-2018-316815

Duvallet, C., Gibbons, S. M., Gurry, T., Irizarry, R. A., and Alm, E. J. (2017). Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. *Nat. Commun.* 8, 1784. doi: 10.1038/s41467-017-01973-8

Gomes, A. C., Hoffmann, C., and Mota, J. F. (2018). The human gut microbiota: metabolism and perspective in obesity. *Gut Microbes* 9, 308–325. doi: 10.1080/19490976.2018.1465157

Grześkowiak, Ł, Grönlund, M.-M., Beckmann, C., Salminen, S., von Berg, A., and Isolauri, E. (2012). The impact of perinatal probiotic intervention on gut microbiota: double-blind placebo-controlled trials in Finland and Germany. *Anaerobe* 18, 7–13. doi: 10.1016/j.anaerobe.2011.09.006

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hou, Q., Zhao, F., Liu, W., Lv, W., Khine, W. W. T., Han, J., et al. (2020). Probiotic-directed modulation of gut microbiota is basal microbiome dependent. *Gut Microbes* 12:e1736974. doi: 10.1080/19490976.2020.1736974

Kim, K. N., Yao, Y., and Ju, S. Y. (2019). Short chain fatty acids and fecal microbiota abundance in humans with obesity: a systematic review and meta-analysis. *Nutrients* 11:2512. doi: 10.3390/nu11102512

Maldonado-Gómez, M. X., Martínez, I., Bottacini, F., O'Callaghan, A., Ventura, M., van Sinderen, D., et al. (2016). Stable engraftment of *Bifidobacterium longum* AH1206 in the human gut depends on individualized features of the resident microbiome. *Cell Host Microbe* 20, 515–526. doi: 10.1016/j.chom.2016.09.001

Merenstein, D. J., Sanders, M. E., and Tancredi, D. J. (2020). Probiotics as a Tx resource in primary care. *J. Fam. Prac.* 69, E1–E10.

Nogacka, A. M., Ruas-Madiedo, P., Gómez, E., Solís, G., Fernández, N., Suárez, M., et al. (2018). Real-time monitoring of HT29 epithelial cells as an in vitro model for assessing functional differences among intestinal microbiotas from different human population groups. *J. Microbiol. Methods* 152, 210–216. doi: 10.1016/j.mimet.2018.07.003

Nogacka, A. M., Salazar, N., Arboleya, S., Ruas-Madiedo, P., Mancabelli, L., Suarez, A., et al. (2020b). In vitro evaluation of different prebiotics on the modulation of gut microbiota composition and function in morbid obese and normal-weight subjects. *Int. J. Mol. Sci.* 21:906. doi: 10.3390/ijms21030906

Nogacka, A. M. G., de los Reyes-Gavilán, C., Martínez-Faedo, C., Ruas-Madiedo, P., Suarez, A., Mancabelli, L., et al. (2020a). Impact of extreme obesity and diet−induced weight loss on the fecal metabolome and gut microbiota. *Mol. Nutr. Food Res.* doi: 10.1002/mnfr.202000030 [Epub ahead of print].

Richards, A. L., Muehlbauer, A. L., Alazizi, A., Burns, M. B., Findley, A., Messina, F., et al. (2019). Gut microbiota has a widespread and modifiable Effect on host gene regulation. *MSystems* 4, e323–e318. doi: 10.1128/mSystems.00323-18

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C. G., and Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* 7:185. doi: 10.3389/fmicb.2016.00185

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., et al. (2010). Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* 104, S1–S63. doi: 10.1017/S0007114510003363

Rotbart, A., Moate, P. J., Yao, C. K., Ou, J. Z., and Kalantar-Zadeh, K. (2018). A novel mathematical model for the dynamic assessment of gas composition and production in closed or vented fermentation systems. *Sens. Actuators B.* 254, 354–362. doi: 10.1016/j.snb.2017.07.066

Salazar, N., Dewulf, E. M., Neyrinck, A. M., Bindels, L. B., Cani, P. D., Mahillon, J., et al. (2015). Inulin-type fructans modulate intestinal bifidobacterium species populations and decrease fecal short-chain fatty acids in obese women. *Clin. Nutr.* 34, 501–507. doi: 10.1016/j.clnu.2014.06.001

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nat. Rev. Gastroenterol. Hepatol.* 16, 605–616. doi: 10.1038/s41575-019-0173-3

Sarbini, S. R., and Rastall, R. A. (2011). Prebiotics: metabolism, structure, and function. *Funct. Food Rev.* 3, 93–106. doi: 10.2310/6180.2011.00004

Shen, Q., Tuohy, K. M., Gibson, G. R., and Ward, R. E. (2011). In vitro measurement of the impact of human milk oligosaccharides on the faecal microbiota of weaned formula-fed infants compared to a mixture of prebiotic fructooligosaccharides and galactooligosaccharides. *Lett. Appl. Microbiol.* 52, 337–343. doi: 10.1111/j.1472-765X.2011.03005.x

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem. J.* 474, 1823–1836. doi: 10.1042/BCJ20160510

Valdés, L., Gueimonde, M., and Ruas-Madiedo, P. (2015). Monitoring in real time the cytotoxic effect of *Clostridium difficile* upon the intestinal epithelial cell line HT29. *J. Microbiol. Methods* 119, 66–73. doi: 10.1016/j.mimet.2015.09.022

Valdés, L., Salazar, N., González, S., Arboleya, S., Ríos-Covián, D., Genovés, S., et al. (2017). Selection of potential probiotic bifidobacteria and prebiotics for elderly by using in vitro faecal batch cultures. *Eur. Food Res. Technol.* 243, 157–165. doi: 10.1007/s00217-016-2732-y

Vallianou, N., Stratigou, T., Christodoulatos, G. S., and Dalamaga, M. (2019). Understanding the role of the gut microbiome and microbial metabolites in obesity and obesity-associated metabolic disorders: current evidence and perspectives. *Curr. Obes. Rep.* 8, 317–332. doi: 10.1007/s13679-019-00352-2

van Baarlen, P., Troost, F., van der Meer, C., Hooiveld, G., Boekschoten, M., Brummer, R. J. M., et al. (2011). Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. *Proc. Natl. Acad. Sci. USA.* 108, 4562–4569. doi: 10.1073/pnas.1000079107

Vinderola, G., Gueimonde, M., Gomez-Gallego, C., Delfederico, L., and Salminen, S. (2017). Correlation between in vitro and in vivo assays in selection of probiotics from traditional species of bacteria. *Trends Food Sci. Technol.* 68, 83–90. doi: 10.1016/j.tifs.2017.08.005

von Martels, J. Z. H., Sadaghian Sadabad, M., Bourgonje, A. R., Blokzijl, T., Dijkstra, G., Faber, K. N., et al. (2017). The role of gut microbiota in health and disease: *in vitro* modeling of host-microbe interactions at the aerobe-anaerobe interphase of the human gut. *Anaerobe* 44, 3–12. doi: 10.1016/j.anaerobe.2017.01.001

Ware, A., and Power, N. (2017). Modelling methane production kinetics of complex poultry slaughterhouse wastes using sigmoidal growth functions. *Renew. Energy* 104, 50–59. doi: 10.1016/j.renene.2016.11.045

Williams, C. F., Walton, G. E., Jiang, L., Plummer, S., Garaiova, I., and Gibson, G. R. (2015). Comparative analysis of intestinal tract models. *Ann. Rev. Food Sci. Technol.* 6, 329–350. doi: 10.1146/annurev-food-022814-015429

Yao, C. K., Rotbart, A., Ou, J. Z., Kalantar-Zadeh, K., Muir, J. G., and Gibson, P. R. (2018). Modulation of colonic hydrogen sulfide production by diet and mesalazine utilizing a novel gas-profiling technology. *Gut Microbes* 9, 510–522. doi: 10.1080/19490976.2018.1451280

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., et al. (2020). Taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactibacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* 70, 2782–2858. doi: 10.1099/ijsem.0.004107

Conflict of Interest: FH and GH were employed by the company Takanashi Milk Products Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as potential conflict of interest. The authors declare that this study received funding from Takanashi Milk products. The funder had the following involvement with the study: study design.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 01 April 2021
doi: 10.3389/fmicb.2021.623951

Orange Juice and Yogurt Carrying Probiotic *Bacillus coagulans* GBI-30 6086: Impact of Intake on Wistar Male Rats Health Parameters and Gut Bacterial Diversity

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Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 30 October 2020
Accepted: 03 March 2021
Published: 01 April 2021

Citation:
Almada-Érix CN, Almada CN, Cabral L, Barros de Medeiros VP, Roquetto AR, Santos-Junior VA, Fontes M, Gonçalves AESS, dos Santos A, Lollo PC, Magnani M and Sant'Ana AS (2021) Orange Juice and Yogurt Carrying Probiotic Bacillus coagulans GBI-30 6086: Impact of Intake on Wistar Male Rats Health Parameters and Gut Bacterial Diversity. Front. Microbiol. 12:623951. doi: 10.3389/fmicb.2021.623951

This study aimed to investigate the impact of the food matrix (orange juice and yogurt) on the effects of the spore-forming probiotic microorganism *Bacillus coagulans* GBI-30 6086 in health parameters and gastrointestinal tract (gut) bacterial diversity in *Wistar* male rats. Rats (n = 48) were randomly distributed into six groups. The groups were the Control (which received sterile distilled water), Juice (which received orange juice), Yogurt (which received yogurt), Probiotic *Bacillus* (which received *B. coagulans* GBI-30 6086 in distilled water), Probiotic Juice (which received orange juice with *B. coagulans* GBI-30 6086), and Probiotic Yogurt (which received yogurt with *B. coagulans* GBI-30 6086). Each animal belonging to the different groups was treated for 21 days. The daily administration of probiotic juice or probiotic yogurt did not affect the rats' food or body weight. Rats fed with Probiotic Yogurt showed lower glucose and triglycerides levels ($p < 0.05$) in comparison to the control group ($p < 0.05$), while no changes in these parameters were observed in the rats fed with Probiotic Juice. Rats fed with Probiotic Yogurt showed a higher gut bacterial diversity than the control group ($p < 0.05$), and higher abundance ($p < 0.05$) of *Vibrionales*, *Enterobacteriales*, *Burkholderiales*, *Erysipelotrichales*, and *Bifidobacteriales* compared to all other groups. No changes were observed in the expression levels of antioxidant enzymes or heat shock protein 70 of rats fed with probiotic yogurt or probiotic juice. Results reveal that the consumption of yogurt containing *B. coagulans* GBI-30 6086 decreases triglycerides and glucose levels and positively impacts the gut bacterial ecology in healthy rats. These animal model findings indicate that the matrix also impacts the functionality of foods carrying spore-forming probiotics. Besides, this research indicates that yogurt is also a suitable food carrier of *Bacillus coagulans* GBI-30 6086.

Keywords: spore-forming bacteria, beneficial microbes, intestinal microbiome, functional food, fermented food

INTRODUCTION

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits on the host (Hill et al., 2014). *Bifidobacterium, Lactobacillus*, and amended genera have been the main probiotic microorganisms incorporated in food matrices. However, there is a growing interest in probiotic *Bacillus coagulans* and *B. subtilis* in foods (Cutting, 2011; Soares et al., 2019). Probiotic *Bacillus* (PB) is resistant to several unit operations used during food processing. It survives better under adverse gastric and intestinal conditions than non-spore-forming probiotics due to the spores' greater resistance (Fouad et al., 2017; Cao et al., 2020).

Health benefits related to the consumption of PB include prevention and treatment of gastrointestinal diseases (Dolin, 2009), modulation of the intestinal microbiota (Sun et al., 2011), immune modulation, and relief of lactose intolerance symptoms (Kimmel et al., 2010). Studies have reported intestinal microbiota modulation by PB in cereals-mix fermented (Ng et al., 2013) and positive effects of PB incorporated in milk on immune response (Sun et al., 2011).

For the successful application in food matrices, probiotics must survive the processing and also during storage. The food matrix must transport and deliver the cells to the gastrointestinal tract (gut; Fazilah et al., 2018). The interaction of probiotics with food components is directly linked to the carrier matrix's physicochemical and nutritional characteristics (Soares et al., 2019). Specific components of the food matrix may confer protection during the storage and when cells are exposed to several stresses such as low pH, bile acids, and digestive enzymes (Fazilah et al., 2018).

Dairy products are food matrices widely explored for the incorporation of probiotics. Even though yogurt is considered a suitable matrix for the delivery of probiotic bacteria (Rutella et al., 2016), the probiotic yogurts' stability is related to technological operations the probiotic bacteria are subjected and their intrinsic resistance to stresses (Granato et al., 2010). The oxidative stress induced by the formation of reactive oxygen species such as superoxide ion or hydrogen peroxide affects the viability of *Bifidobacterium*, *Lactobacillus*, and amended genera in yogurts. Thus, the probiotic yogurt's shelf life is limited by post-acidification during storage, which causes a loss of viability of probiotic cells due to the persistent metabolic activity of starter lactic acid bacteria (Xu et al., 2015). The addition of glucose oxidase to yogurt during processing and the use of packages with low oxygen permeability rates have been proposed as alternatives to control these tasks (Cruz et al., 2013), but they increase the costs of the final product. Therefore, the development of probiotic yogurts carrying spore-forming probiotic strains appears a promising strategy.

On the other hand, fruit juices are perceived as healthy and refreshing beverages well-accepted by consumers of all ages. These beverages have been suggested as matrices for incorporating probiotics because they are interesting for vegans and consumers interested in low cholesterol foods (Pereira and Rodrigues, 2019). However, some factors can limit the probiotics' viability in fruit juices, such as low pH, oxygen, presence of multiple antimicrobial components, and treatment systems used in processing (Pimentel et al., 2019). Otherwise, the survival of probiotics in fruit juices may be enhanced by the absence of prior fermentation (and interaction with starter cultures), relatively fast passage through the gastrointestinal, and naturally occurring juice constituents (e.g., fibers, sugars, vitamins, minerals, and phenolics; Filho et al., 2019).

Previous studies reported the survival of PB during food processing, storage, and or exposure to *in vitro* digestion when incorporated in different non-dairy matrices such as tea (Majeed et al., 2019), jelly candies (Miranda et al., 2020), dried date pastes (Marcial-Coba et al., 2019), and orange juice (Soares et al., 2019). However, no prior studies have explored PB's functionality incorporated in fruit juice and the impacts of the food matrix on the health benefits of spore-forming probiotics *in vivo*.

Therefore, the present study was performed to assess the effects of *Bacillus coagulans* GBI-30 6086, a spore-forming bacterium presenting GRAS status and claimed probiotic properties (FDA, 2016, 2017) on biochemical parameters and gut microbiota ecology of healthy rats when incorporated in yogurt and orange juice.

MATERIALS AND METHODS

Probiotic Strain

The probiotic strain *B. coagulans* GBI-30, 6086 was kindly donated by the Ganeden Biotech Inc., (Mayfield Heights, Ohio, United States) as a powder containing the spores. It is a safe strain (Endres et al., 2009; Salvetti et al., 2016) available on the market with recognized benefits to humans (Cao et al., 2020) with a potential for application in a range of foods (Almada-Érix et al., 2021). The whole-genome shotgun project was deposited in the DDBJ/EMBL/GenBank under the accession number (JPSK00000000; Orrù et al., 2014).

Food Matrix Preparation and Inoculation of *B. coagulans* GBI-30 6086

Orange juices were prepared using commercially concentrated pulp. The total soluble solids content was adjusted to 11°Brix with water, following pasteurization at 95°C for 30 s in a water bath Quimis, model 0334M-28 (Diadema, SP, Brazil). *B. coagulans* GBI-30, 6086 spores were inoculated after the thermal processing.

The yogurt production was performed according to the procedures described by Tamime and Robinsons (2007). Milk standardized to total solids (13%) was subjected to thermal treatment (90°C/5 min) in a water bath Quimis, model 0334M-28 (Diadema, SP, Brazil) and cooled down to 42°C. Then, traditional lactic culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*, CHR-Hansen, Brazil) was added at 2.5% (v/v), the following fermentation in a kiln (Marconi, model MA 032, Piracicaba, SP, Brazil) at 45°C until pH reached 4.6 and cooled to 10°C. For the preparation of the probiotic yogurt, *Bacillus coagulans* GBI-30, 6086 spores were added after fermentation. For the Probiotic *Bacillus* group, *Bacillus coagulans*

GBI-30, 6086 spores were added to sterile distilled water. In all groups, the final concentration was 10^8 spores/ml. Probiotic *Bacillus*, probiotic juice, and probiotic yogurt were prepared weekly and stored at 4°C.

The probiotic bacteria strain presented counts around 10^8 CFU/ml in juice and yogurt throughout the 21 days of refrigerated storage (data not shown). The enumeration of *Bacillus coagulans* GBI-30, 6086 in yogurt or juice comprised the application of a heat shock at 80°C/10 min, followed by pour plate in Glucose Yeast Extract Agar (BC) and incubation at 40°C/48 h under aerobiosis. Further details on the formulation of Glucose Yeast Extract Agar are available in Soares et al. (2019).

Chemical Composition of Food Matrices

After preparation, the samples were submitted to moisture, and ash contents were determined according to standard methods described by the AOAC (2012) and total lipids following the Institute Adolfo Lutz (IAL, 2005) methods in all food matrices. Total proteins were determined in juices according to the Association of Official Analytical Chemists (AOAC, 2012) and in yogurts following the IAL (2005) method. The total carbohydrates content was estimated by difference.

Experimental Design Using Wistar Rats

The Ethical Commission previously approved all experimental procedures on Animal Use (CEUA, UNICAMP, São Paulo, Brazil, protocol n° 3456-1). A total of 48 male Wistar rats at 21 days (specific pathogen-free) were obtained from the Animal Breeding Center (University of Campinas, UNICAMP, SP, Brazil), and were used in the study. The animals were kept in individual cages under a specific condition (22 ± 1°C, 12 h photoperiod; 60–70% relative humidity) with food (AIN 93 M diet, Nutivital, São Paulo) and water provided *ad libitum* (Reeves et al., 1993) for adaptation during 3 weeks. The animals were randomly distributed into six groups of eight animals as follows: (a) Control group, which received sterile distilled water; (b) Juice, which received orange juice; (c) Yogurt, which received yogurt; (d) Probiotic *Bacillus*, which received *B. coagulans* GBI-30 6086 suspended in distilled water; (e) Probiotic Juice, which received orange juice with *B. coagulans* GBI-30 6086; and (f) Probiotic Yogurt, which received yogurt with *B. coagulans* GBI-30 6086.

All animal groups received a volume of four milliliters of liquid daily administered by orogastric gavage during 21 days. The administred volume was defined considering the volume of 1 ml/100 g according to the official protocols (Andersen et al., 2004).

Weight gain was monitored weekly, and the food intake was assessed every 2 days. After the 21-days of the experiment, six milliliters of blood were collected *via* direct cardiac puncture from anesthetized with an intraperitoneal injection of 1 ml of ketamine hydrochloride (75 mg) and 1 ml of xylazine hydrochloride (5 mg) per kg of body weight. Samples were centrifuged at 3,000 × *g*, 10 min, 4°C. Animals were euthanized, and the gastrocnemius muscle and cecum fecal samples were carefully removed and stored at −80°C (Costa et al., 2019).

Assessment of Biochemical Parameters in Wistar Rats

Blood samples (4 ml) were collected *via* direct cardiac puncture and centrifuged (3,000 × *g*, 10 min, 4°C) from anesthetized animals. Serum biochemical parameters were determined using commercial kits according to the manufacturer's instructions (Labcenter®, Tocantins, Brazil): aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, high-density lipoprotein (HDL), triglycerides, uric acid, creatinine, glucose, total protein, and albumin.

Western Blot Analyses

Analyses of expression of heat shock protein 70 (HSP70) and endogenous antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were performed according to Moura et al. (2016), with minor adaptations. Gastrocnemius muscle sample (200 mg) of each animal in each experimental group was homogenized in five volumes of extracting buffer (200 mmol/L EDTA, 1 mol/L Tris-Base, 10 mmol/L orthovanadate, 2 mmol/L phenylmethanesulfonyl fluoride, 10 mmol/L sodium pyrophosphate, 0.1 mg/ml aprotinin, 100 mmol/L sodium fluoride, Triton 10%, ultrapure water) using Polytron (Pro Scientific Model Pro 200). The mixture was centrifuged at 14,000 × g for 40 min at 4°C, and the supernatant was collected. The extracts were subjected to SDS-PAGE (8%) and transferred using a semi-dry system (Bio-Rad, CA, United States) to a nitrocellulose membrane of 0.22 μm. The nitrocellulose membranes were blocked with 3% bovine serum albumin (BSA) and incubated with specific primary antibodies overnight.

To assess the protein level, the appropriate secondary antibodies were used for detection. [HSP 70 (#ADI-SPA-810, Enzo life sciences - Farmingdale, United States), SOD (#AB51254, Abcam, Cambridge United Kingdom), GPx (#AB22604, Abcam, Cambridge UK), CAT (#AB1877, Abcam, Cambridge United Kingdom)]. A molecular weight standard was used and run concurrently on each gel to determine the antibody's proper molecular weight. Immunoreactive bands were detected by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate Kit, Thermo Scientific, United States). The bands were visualized using the UNITEC instrument (model Alliance LD2), and blots were quantified using the UN SCAN IT software (Moura et al., 2016). Results were expressed as % in comparison to the control value.

Gut Microbiota Ecology Analysis

Total DNA was extracted from the cecal contents with the QIAmp DNA Stool Kit. For profiling microbiome composition, the hyper-variable region (V3–V4) of the bacterial 16S rRNA gene was amplified using the Illumina 16S Metagenomic Sequencing Library Preparation guide Illumina 16S metagenomic sequencing library preparation (Illumina Technical Note 15044223), which uses the following sequence: 338F - 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTA CGGGNGGCWGCAG -3 and 785R - 5'- GTCTCGT GGGCTCGGAGA TGTGTATAAGAGACAGGACTACHVGGG TATCTAATCC -3'. Using 300 bp paired readings and MiSeq

v3 reagents, each law's ends were overlaid to generate high-quality complete readings of the V3 and V4 regions. More than 100,000 readings per sample were generated, commonly recognized as sufficient for metagenomic research. The sequencing was performed in the Illumina Miseq equipment (Neoprospecta Consulting and Research SA, Santa Catarina state, Brazil).

Taxonomic Assignment Obtained by 16S rRNA Gene Sequencing Analysis

Initial sequences quality check was performed by the FASTQC and then quality filtering using the Trimmomatic (0.36; Bolger et al., 2014). The search for chimera was performed using the UCHIME2 (Edgar et al., 2011). The sequences were then analyzed using the QIIME (quantitative insights into microbial ecology), version 1.9.0 software (Caporaso et al., 2010). OTUs were clustered at 97% identity using the available reference approaches (UCLUST algorithm; Edgar, 2010) and identity against the Green genes bacterial 16S rRNA database (13_5 release; McDonald et al., 2012) using the RDPII classifier (Wang et al., 2007) and PyNast for aligning sequences (Caporaso et al., 2010).

For annotation analysis, all OTUs observed less than two times (i.e., singletons) were discarded. The samples' rarefaction was performed (normalization for the same number of OTUs—45,035 OTUs). The rarefied data were used for alpha diversity evaluation through the QIIME to generate rarefaction curves, Good's coverage, Chao1 richness (Chao and Bunge, 2002), and Shannon and Simpson diversity indices (Shannon, 1948; Simpson, 1949). Beta diversity was evaluated with the UniFrac (Lozupone and Knight, 2005). Feature and sample clustering were simultaneously analyzed using the heat map exploratory data analysis tool in the XLSTAT software version 2015.2 (Adinsoft, Paris, France). Annotated sequences were deposited and are available at the National Center for Biotechnology Information (NCBI[1]; BioProject PRJNA631217).

Statistical Analysis

Data of centesimal composition are presented as mean ± standard deviation (SD). Results were analyzed by ANOVA, followed by the Scott-Knott test considering $p < 0.05$, using the Sisvar software 5.6 (Lavras, MG, Brazil). All other data were presented as means and the standard error of the mean (SEM) and analyzed by ANOVA, followed by the Duncan *post-hoc* test considering a $p < 0.05$ using the statistical package for social sciences (SPSS, Chicago, IL, United States) software, version 23.0 for windows.

RESULTS

Chemical Composition of Food Matrices

The moisture, ashes, total lipids, proteins, and total carbohydrates did not differ ($p \geq 0.05$) between the juice and probiotic juice or between the yogurt and probiotic yogurt (**Supplementary Table 1**). However, contents of ashes, lipids, proteins, and estimated total carbohydrates differ ($p < 0.05$) between the yogurt and juice and between the probiotic yogurt and probiotic juice. Yogurt and probiotic yogurt showed total ashes, lipids, and proteins contents higher than the juice and probiotic juice ($p < 0.05$). Otherwise, the total estimated carbohydrates in the juice and probiotic juice were higher ($p < 0.05$) than the yogurt and probiotic yogurt.

Diet Intake, Body Weight, and Biochemical Parameters

The daily administration of juice or yogurt probiotic did not affect the food intake (568.9 g ± 23.3 and 537.6 ± 20) or the bodyweight of rats (334.0 g ± 9.0 and 330.2 ± 7.7), respectively, in comparison to the control groups ($p \geq 0.05$; **Table 1**).

The consumption of probiotic yogurt for 21 days reduced classical health parameters in rats, such as glucose (9.82%) and triglycerides (34.66%) serum levels in comparison to the control group ($p < 0.05$). The probiotic *Bacillus* group (which received the probiotic in distilled water) also showed a significant reduction in triglycerides (23.85%) serum levels when compared to the control group ($p < 0.05$). It was interesting to note that glucose and triglycerides did not change in the rats fed with probiotic juice (**Table 2**). The other measured parameters (ALT, AST, creatinine, uric acid, cholesterol, HDL, albumin, and total protein) did not change ($p \geq 0.05$) in the probiotic groups (rats that received probiotic *Bacillus* in distilled water, probiotic yogurt, or probiotic juice) when compared to the control groups (rats that received yogurt, juice, or distilled water; **Table 2**).

Protein Expression

The consumption of probiotic juice, probiotic yogurt, or *Bacillus* in distilled water did not change the expression of antioxidant enzymes (SOD, GPx, CAT) or HSP 70 in rats when compared to the control group ($p < 0.05$; **Supplementary Figure 1**).

16S rRNA Gene Sequencing-Based Structure of the Microbiota

A total of 21,466.032 reads were generated from the Next-Generation Sequencing (NGS) of amplicons corresponding to the V3–V4 region of the bacterial 16S rRNA gene. A total of 19.771.488 reads passed the sequence quality filters applied through the Trimmomatic (0.36) software, with an average value of 681.775 reads per sample after the quality filtering was obtained (**Supplementary Table 2**). The alpha-diversity and richness through the number of ace, Chao1, Good's estimated sample coverage (ESC), OTUs, and Shannon and Simpson indices were obtained for all the samples (**Table 3**).

In general, the Probiotic Yogurt group showed higher values for all alpha diversity indices than the other groups (**Table 3**). Notably, the highest values of Chao1 indices were found in the Probiotic Yogurt and Probiotic Juice groups (17518.4 and 15171.4, respectively). The six treatments' alpha diversity data (Control, Juice, Yogurt, Probiotic *Bacillus*, Juice probiotic, and Yogurt probiotic) was analyzed using Kruskal-Wallis.

[1]ncbi.nlm.nih.gov

TABLE 1 | Bodyweight and food intake of healthy *Wistar* male rats after 21 days of administration of juice, probiotic juice, yogurt, and probiotic yogurt.

Parameter (g)	Group*					
	Control	Juice	Yogurt	Probiotic *Bacillus*	Probiotic juice	Probiotic yogurt
Bodyweight	335.9 ± 8.0[a]	331.7 ± 6.2[a]	341.1 ± 6.9[a]	329.6 ± 7.8[a]	334.0 ± 9.0[a]	330.2 ± 7.7[a]
Food intake	593.6 ± 20.2[a]	597.6 ± 13.2 [a]	551.4 ± 18.2[a]	582.2 ± 24.6[a]	568.9 ± 23.3[a]	537.6 ± 20.5[a]

Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic Bacillus: received B. coagulans GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with B. coagulans GBI-30 6086; and Probiotic Yogurt: received yogurt with B. coagulans GBI-30 6086. Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by the Duncan test ($p < 0.05$).

TABLE 2 | Effect of probiotic yogurt and probiotic juice consumption during 21 days on healthy *Wistar* male rats' biochemical parameters.

Parameter	Group*					
	Control	Juice	Yogurt	Probiotic *Bacillus*	Probiotic juice	Probiotic yogurt
Glucose (mg/dL)	133.4 ± 4.8[a]	131.8 ± 2.8[ab]	131.5 ± 2.8[ab]	125.8 ± 3.4[ab]	126.9 ± 6.4[ab]	120.3 ± 1.6[b]
Triglycerides (mg/dL)	85.1 ± 10.3[a]	70.4 ± 5.2 [ab]	63.2 ± 10.7[ab]	48.6 ± 5.4[b]	64.8 ± 11.3[ab]	55.6 ± 4.7[b]
Cholesterol (mg/dL)	54.2 ± 3.1 [a]	50.5 ± 3.7 [a]	58.3 ± 2.2 [a]	48.6 ± 3.7 [a]	51.2 ± 2.5 [a]	48.6 ± 3.5 [a]
HDL (mg/dL)	40.8 ± 3.5 [a]	40.8 ± 4.4 [a]	40.0 ± 3.9 [a]	40.8 ± 4.0 [a]	40.2 ± 3.1 [a]	40.2 ± 3.9 [a]
Total protein (g/dL)	5.9 ± 0.32 [a]	5.7 ± 0.17 [a]	5.5 ± 0.16 [a]	5.5 ± 0.14 [a]	5.5 ± 0.15 [a]	5.6 ± 0.13 [a]
Albumin (g/dL)	3.7 ± 0.31 [a]	3.7 ± 0.33 [a]	4.0 ± 0.45 [a]	3.9 ± 0.15 [a]	4.1 ± 0.43 [a]	4.1 ± 0.34 [a]
ALT (U/L)	9.3 ± 1.0[ab]	7.8 ± 0.56[a]	8.8 ± 0.48[ab]	11.5 ± 1.1[b]	9.9 ± 0.92[ab]	10.6 ± 2.1[ab]
AST (U/L)	28.0 ± 2.7 [a]	28.9 ± 1.2 [a]	35.5 ± 2.7 [a]	34.0 ± 2.6 [a]	37.0 ± 3.9 [a]	36.4 ± 4.2 [a]
Creatinine (mg/dL)	0.33 ± 0.02[ab]	0.30 ± 0.03[a]	0.37 ± 0.02[ab]	0.37 ± 0.04[ab]	0.38 ± 0.02[ab]	0.42 ± 0.03[b]
Uric acid (mg/dL)	0.81 ± 0.09[ab]	0.82 ± 0.07[ab]	0.77 ± 0.07[a]	1.0 ± 0.13[ab]	1.1 ± 0.14[b]	0.99 ± 0.11[ab]

Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic Bacillus: received B. coagulans GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with B. coagulans GBI-30 6086; and Probiotic Yogurt: received yogurt with B. coagulans GBI-30 6086. Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by the Duncan test ($p < 0.05$).

TABLE 3 | Alpha-diversity metrics (ace, Chao1, Good's estimated sample coverage (ESC), OTUs, Shannon and Simpson indices) obtained for fecal samples of healthy *Wistar* male rats after 21 days of administration of probiotic yogurt and probiotic juice inferred from the sequencing of 16S V3–V4 amplicons.*

Treatments group	ace	Chao1	ESC	OTUs	Shannon	Simpson
Control	15,204.3	14,245.5	0.94	3,905.8	7.77	0.98
Juice	15,966.8	15,141.7	0.94	4,069.0	7.96	0.99
Yogurt	15,144.3	14,089.4	0.94	3,895.6	7.74	0.98
Probiotic *Bacillus*	15,197.9	14,053.6	0.94	3,983.0	7.75	0.98
Probiotic juice	16,191.7	15,171.4	0.94	4,014.6	7.84	0.98
Probiotic yogurt	18,788.5	17,518.4	0.92	4,852.2	8.29	0.99

Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic Bacillus: received B. coagulans GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with B. coagulans GBI-30 6086; and Probiotic Yogurt: received yogurt with B. coagulans GBI-30 6086.

There was a statistical difference in OTUs, Chao1, and Shannon indices among all the treatments ($p < 0.05$; **Supplementary Table 3**). The estimated sample coverage was satisfactory for 90% of the samples. The results of the analysis of the beta diversity, based on the unweighted Uni-Frac analysis, indicated that the Probiotic Yogurt samples formed a discrete group distinguished from the other five groups (Yogurt, Juice, Probiotic Juice, Probiotic *Bacillus*, and Control; **Figure 1**). When the Permanova statistical analysis was performed using the beta-diversity data, statistical differences were observed among the treatments ($p < 0.01$; **Supplementary Table 4**).

Taxonomic Assignment Obtained by 16S rRNA Gene Sequencing Analysis

The taxonomic assignment obtained by 16S rRNA gene sequencing analysis showed that the OTUs belonged to three major different bacterial classes in the six groups treatments (Yogurt; Probiotic Yogurt; Juice; Probiotic Juice; Probiotic *Bacillus*; Control): *Clostridia* (from 51.8 to 46.4%), followed by *Bacteroidia* (from 43.3 to 33.8%), and *Bacilli* (from 10.1 to 5.5%) with no statistical differences ($p \geq 0.05$). On the other hand, *Gammaproteobacteria* (4.6%) and *Betaproteobacteria* (1.1%) showed a high abundance ($p < 0.05$) in the Probiotic Yogurt treatment (**Table 4**).

At the order level, the majority of OTU in the six treatments (Yogurt, Probiotic Yogurt, Juice, Probiotic Juice, Probiotic *Bacillus*, and Control) were attributed to three significant orders: *Clostridiales* (from 46.3 to 51.8%), *Bacteroidales* (from 33.7 to 43.3%), and *Lactobacillales* (from 5.1 to 6.8%; **Figure 2**; **Table 5**). When the abundance was observed without these three major groups (i.e., *Clostridiales, Bacteroidales*, and *Lactobacillales*), most OTUs were attributed to the same orders in all groups. However, Probiotic Yogurt samples showed a higher ($p < 0.05$) abundance of *Vibrionales* (2.2%), *Enterobacteriales* (2.1%), *Burkholderiales* (1.6%), *Erysipelotrichales* (0.5%), and *Bifidobacteriales* (0.1%) when compared to the other groups (**Figure 2**; **Table 5**).

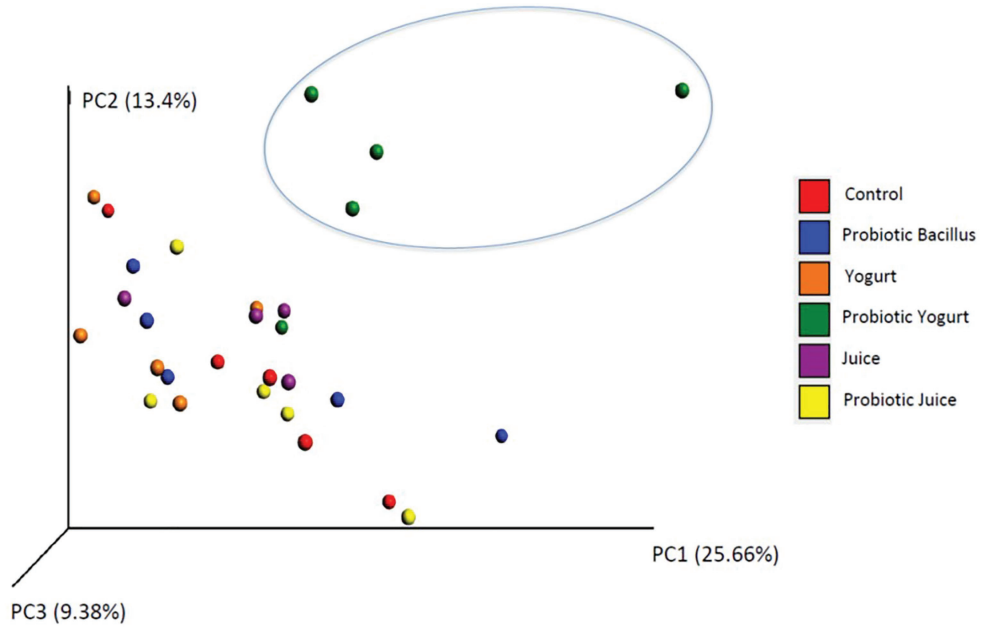

FIGURE 1 | Principal coordinate analysis of jackknifed unweighted UniFrac distances for the 16S rRNA gene sequence data. Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic *Bacillus*: received *B. coagulans* GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with *B. coagulans* GBI-30 6086; and Probiotic Yogurt: received yogurt with *B. coagulans* GBI-30 6086.

TABLE 4 | Relative abundance of bacterial classes inferred from 16S rRNA gene sequencing analysis.*

Taxonomy (%)	Control	Juice	Yogurt	Probiotic *Bacillus*	Probiotic juice	Probiotic yogurt
Clostridia	47.5 ± 9.5^{a}	49.2 ± 3.7^{a}	51.8 ± 5.0^{a}	47.3 ± 8.7^{a}	46.9 ± 6.5^{a}	46.4 ± 7.5^{a}
Bacteroidia	41.7 ± 8.9^{a}	38.9 ± 3.7^{a}	33.8 ± 5.0^{a}	41.6 ± 7.2^{a}	43.3 ± 6.2^{a}	39.3 ± 4.7^{a}
Bacilli	7.7 ± 3.5^{a}	8.0 ± 2.2^{a}	10.1 ± 2.1^{a}	7.3 ± 2.2^{a}	7.0 ± 1.0^{a}	5.5 ± 2.5^{a}
Gammaproteobacteria	0.4 ± 0.0^{b}	0.5 ± 0.2^{b}	0.3 ± 0.1^{b}	0.4 ± 0.3^{b}	0.3 ± 0.1^{b}	4.6 ± 4.0^{a}
Betaproteobacteria	0.4 ± 0.2^{b}	0.7 ± 0.3^{ab}	0.3 ± 0.2^{b}	0.3 ± 0.2^{b}	0.3 ± 0.1^{b}	1.6 ± 1.1^{a}
Erysipelotrichi	0.2 ± 0.1^{a}	0.5 ± 0.2^{a}	0.1 ± 0.0^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.5 ± 0.4^{a}
Deltaproteobacteria	0.2 ± 0.1^{a}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	0.4 ± 0.2^{a}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}
Mollicutes	0.3 ± 0.3^{a}	0.3 ± 0.2^{a}	0.9 ± 1.1^{a}	0.2 ± 0.1^{a}	0.3 ± 0.2^{a}	0.3 ± 0.2^{a}
Epsilonproteobacteria	0.3 ± 0.2^{a}	0.3 ± 0.2^{a}	0.2 ± 0.1^{a}	0.4 ± 0.4^{a}	0.4 ± 0.4^{a}	0.2 ± 0.1^{a}
*Cyanobacteria;c_*	0.6 ± 0.3^{a}	0.6 ± 0.7^{a}	0.4 ± 0.5^{a}	0.2 ± 0.1^{a}	0.3 ± 0.2^{a}	0.1 ± 0.1^{a}

**Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic Bacillus: received B. coagulans GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with B. coagulans GBI-30 6086; and Probiotic Yogurt: received yogurt with B. coagulans GBI-30 6086; Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by Tukey test ($p < 0.05$).*

A heat map analysis was performed to explore the taxonomic assignment obtained by 16S rRNA gene sequencing analysis and better visualize the similarities and differences in each bacterial affiliation among the treatments (**Figure 3**). As can be seen, the heat map presented two more prominent clusters, one composed of the Probiotic Juice and Probiotic *Bacillus* samples and the other by the Control and Juice samples. Besides, it was observed that the Probiotic Yogurt samples clustered completely separated (**Figure 3**). The microbial diversity of the samples was significantly different between each treatment. However, it was observed that the Probiotic juice and Probiotic *Bacillus* samples presented similar patterns concerning specific microbial groups: *Helicobacter, Christensenellaceae;g_, Paraprevotellaceae;g_, Helicobacteraceae;g_; Planococcaceae;g_, Clostridiales*; Other (**Figure 2**). On the other hand, the Probiotic Yogurt samples showed a high abundance of specific microbial groups than other treatments, such as *Turicibacter, Peptostreptococcaceae:g_, Lachnospira, Allobaculum, Enterobacteriaceae; Other, Prevotella; Enterobacteriaceae;g_, Vibrio, Proteus, Clostridiaceae;g_, Blautia, Phascolarctobacterium, Bifidobacterium, Erysipelotrichaceae*; Other, *Erwinia, Sutterella, Clostridium, Coprococcus, and Peptococcaceae;g_*. The Juice and Control samples presented the abundance of the *Dehalobacterium* genus.

DISCUSSION

Despite the no surprising lack of chemical variations due to *Bacillus coagulans* GBI-30, 6086 in the studied matrices since the spores are metabolically inactive cells, these results are

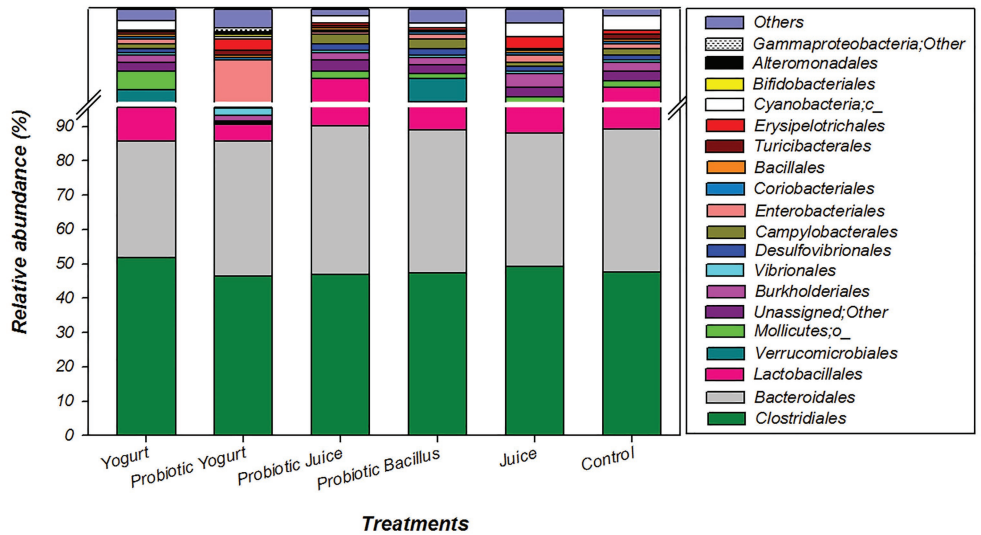

FIGURE 2 | Relative abundance of bacterial groups inferred from 16S rRNA gene amplicon sequencing analysis from samples of each experimental or control group. Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic *Bacillus*: received *B. coagulans* GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with *B. coagulans* GBI-30 6086; and Probiotic Yogurt: received yogurt with *B. coagulans* GBI-30 6086.

TABLE 5 | Relative abundance of bacterial orders inferred from 16S rRNA gene sequencing analysis.*

Taxonomy (%)	Control	Juice	Yogurt	Probiotic *Bacillus*	Probiotic juice	Probiotic yogurt
Clostridiales	47.4 ± 4.7^{a}	49.1 ± 2.1^{a}	51.8 ± 2.5^{a}	47.3 ± 4.3^{a}	46.8 ± 3.2^{a}	46.3 ± 3.7^{a}
Bacteroidales	39.0 ± 3.9^{a}	38.9 ± 2.1^{a}	33.7 ± 2.5^{a}	41.5 ± 3.6^{a}	43.3 ± 3.1^{a}	39.3 ± 2.3^{a}
Lactobacillales	7.4 ± 1.8^{a}	7.7 ± 1.2^{a}	8.9 ± 0.65^{a}	7.1 ± 1.07^{a}	6.8 ± 0.52^{a}	5.1 ± 1.1^{a}
Bacillales	0.08 ± 0.02^{a}	0.1 ± 0.00^{ab}	0.14 ± 0.02^{ab}	0.14 ± 0.02^{ab}	0.14 ± 0.02^{ab}	0.16 ± 0.009^{b}
Bifidobacteriales	0.02 ± 0.02^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.10 ± 0.05^{b}
Coriobacteriales	0.10 ± 0.00^{a}	0.12 ± 0.03^{a}	0.10 ± 0.03^{a}	0.10 ± 0.00^{a}	0.08 ± 0.02^{a}	0.14 ± 0.02^{a}
Verrucomicrobiales	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.96 ± 0.96^{a}	0.94 ± 0.91^{a}	0.02 ± 0.02^{a}	0.04 ± 0.02^{a}
Mollicutes	0.34 ± 0.15^{a}	0.32 ± 0.10^{a}	0.86 ± 0.54^{a}	0.22 ± 0.06^{a}	0.30 ± 0.10^{a}	0.28 ± 0.10^{a}
Unassigned;Other	0.38 ± 0.06^{a}	0.42 ± 0.02^{a}	0.42 ± 0.03^{a}	0.44 ± 0.04^{a}	0.48 ± 0.03^{a}	0.40 ± 0.03^{a}
Cyanobacteria	0.56 ± 0.17^{a}	0.57 ± 0.38^{a}	0.42 ± 0.25^{a}	0.22 ± 0.07^{a}	0.26 ± 0.08^{a}	0.14 ± 0.06^{a}
Burkholderiales	0.42 ± 0.09^{a}	0.65 ± 0.19^{a}	0.32 ± 0.09^{a}	0.34 ± 0.09^{a}	0.24 ± 0.02^{a}	1.62 ± 0.55^{a}
Desulfovibrionales	0.18 ± 0.03^{a}	0.27 ± 0.06^{a}	0.26 ± 0.07^{a}	0.34 ± 0.07^{a}	0.24 ± 0.05^{a}	0.22 ± 0.03^{a}
Campylobacterales	0.34 ± 0.11^{a}	0.25 ± 0.09^{a}	0.16 ± 0.06^{a}	0.38 ± 0.19^{a}	0.40 ± 0.18^{a}	0.20 ± 0.03^{a}
Enterobacteriales	0.20 ± 0.00^{a}	0.25 ± 0.08^{a}	0.20 ± 0.03^{a}	0.20 ± 0.07^{a}	0.14 ± 0.02^{a}	2.1 ± 0.89^{b}
Turicibacterales	0.20 ± 0.09^{ab}	0.10 ± 0.04^{ab}	0.06 ± 0.04^{ab}	0.02 ± 0.02^{a}	0.08 ± 0.02^{ab}	0.22 ± 0.07^{b}
Erysipelotrichales	0.18 ± 0.04^{a}	0.47 ± 0.13^{b}	0.10 ± 0.00^{a}	0.12 ± 0.04^{a}	0.12 ± 0.04^{a}	0.30 ± 0.15^{ab}
Vibrionales	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	0.08 ± 0.02^{a}	0.08 ± 0.03^{a}	0.06 ± 0.02^{a}	2.1 ± 1.0^{b}
Alteromonadales	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.06 ± 0.02^{b}

**Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic Bacillus: received B. coagulans GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with B. coagulans GBI-30 6086; and Probiotic Yogurt: received yogurt with B. coagulans GBI-30 6086; Data are expressed as means ± SEM. Different superscript letters on the same line indicate statistical differences by the Duncan test ($p < 0.05$).*

essential to clarify that the effects observed were not related to chemical changes in the matrix caused by the probiotic.

In recent years, a range of studies in animal models has reported beneficial effects of probiotics (added or not to food matrices) to the host's health, such as intestinal microbiota modulation (Marchesin et al., 2018), alleviation of inflammation (Mousavi et al., 2020) and food allergy (Maa et al., 2019), improvement of the immune system (Manuel et al., 2017), relief of symptoms caused by cardiovascular disorders (Cavalcante et al., 2019), diabetes Type 2 (Wang et al., 2020a), and colorectal cancer (Genaro et al., 2019), among others. However, few studies have focused on evaluating spore-forming probiotics effects through food consumption (Sun et al., 2011; Ng et al., 2013; Haldar and Gandhi, 2016). Therefore, the current study evaluated the effects of the probiotic spore-forming *B. coagulans* GBI-30 6086 on biochemical parameters and gut microbiota profile in healthy rats. This study also revealed the effects of the food matrices on the probiotic spore-forming *B. coagulans* GBI-30 6086 functionality.

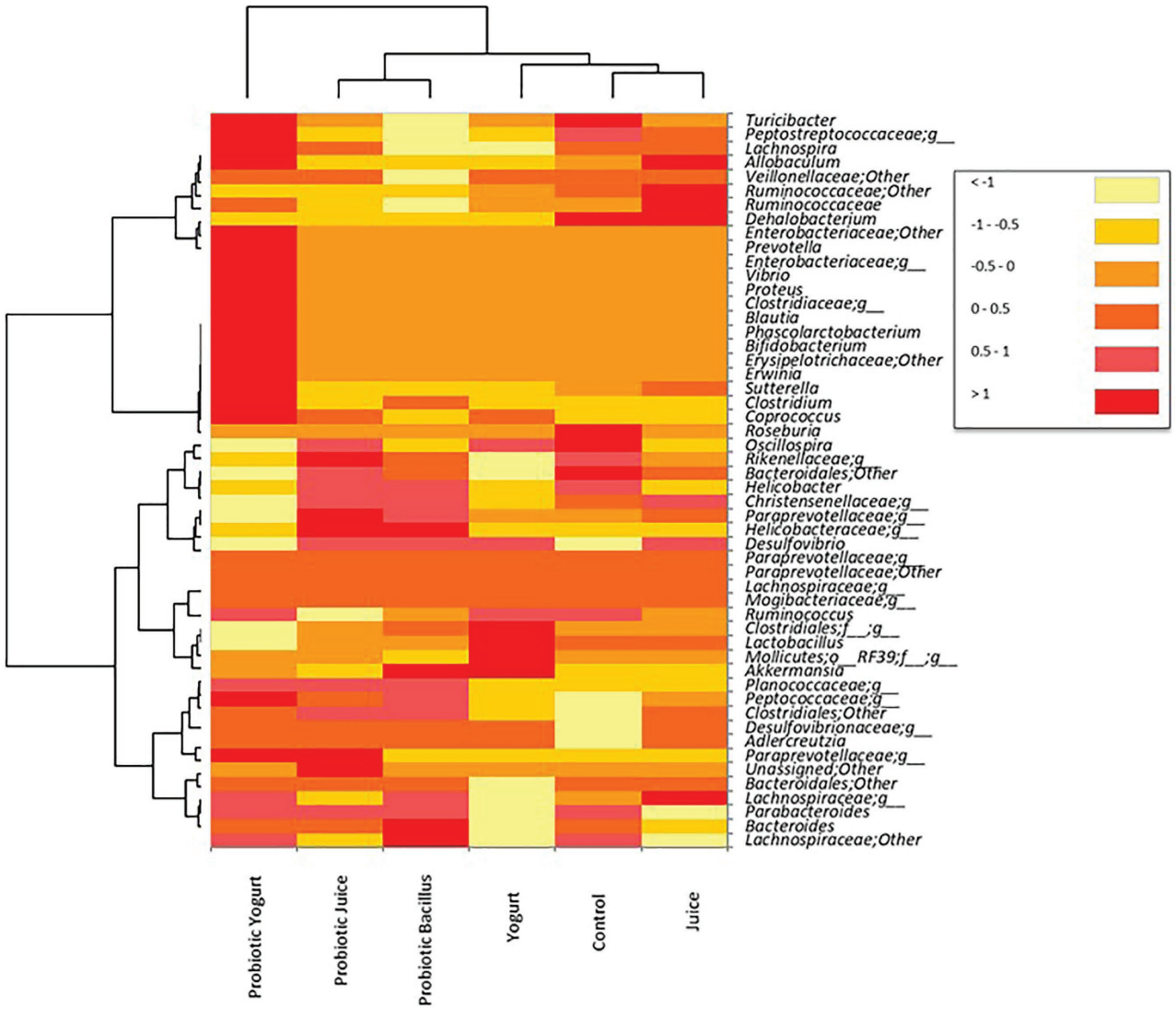

FIGURE 3 | Heatmap showing microbial taxa (mostly family level) with relative abundance obtained by 16S rRNA gene pyrosequencing analysis. Groups were as follows: Control: received distilled water; Juice: received orange juice; Yogurt: received yogurt; probiotic *Bacillus*: received *B. coagulans* GBI-30 6086 suspended in distilled water; Probiotic Juice: received orange juice with *B. coagulans* GBI-30 6086; and Probiotic Yogurt: received yogurt with *B. coagulans* GBI-30 6086. Only OTUs with abundance values above 0.1% in at least nine readings are shown.

Our results showed that the administration of the tested strain in water, juice, or yogurt for 21 days does not affect the food intake or weight gain in animals. Overall, daily ingestion of probiotic yogurt decreased serum triglycerides and glucose, while these same effects were not observed for the probiotic juice's daily ingestion. It is important to point out that the differences in the yogurt and orange juice's chemical composition can affect these biochemical parameters. Orange juice showed a higher carbohydrate content, while yogurt has a higher content of fat and proteins. The type of sugars present in each food matrix should also be considered. Orange juice stands out for its greater fructose presence, while yogurt for glucose and galactose (Ranadheera et al., 2010).

Increased triglycerides and glucose levels are risk factors associated with the development of coronary heart disease and diabetes mellitus, respectively (Karamali et al., 2016; Wang et al., 2020a). Therefore, there is a growing interest in probiotic foods that did not affect the food intake besides exerting positive effects of lipids and glucose metabolism, as observed for the probiotic yogurt in the present study. Lipids and glucose blood levels are overall classical biochemical markers elevated in animals with metabolic disorders (Roquetto et al., 2015; Costa et al., 2019). It is believed that if probiotic yogurt consumption reduced these parameters in healthy animals, they could also be attenuated when increased in the blood.

In contrast to lowering-cholesterol effects observed for probiotic yogurt (containing *L. acidophilus* e *Bifidobacterium lactis*) in hypercholesterolemic subjects (Ataie-Jafari et al., 2009), no prior studies reported effects of yogurt with PB on the serum lipid profile. In the meanwhile, the administration of non-sporulated probiotic strains (*Lactilactobacillus curvatus* HY7601 and *Lactiplantibacillus plantarum* KY1032) led to a reduction of 18% in the serum triglycerides in non-diabetic subjects with mild to moderate hypertriglyceridemia (Ahn et al., 2015), similar to the observed effects in our study by the administration of the probiotic *Bacillus* suspension.

The improvement of the glycemic and lipid parameters by probiotic strains has been primarily associated with the restoration of the gut barrier function through colonization (Wang et al., 2020a). The ability of non-spore-forming probiotic [*Lactobacillus* and amended genera strains (*Lactiplantibacillus plantarum*, *Lactobacillus helveticus*, *Lactiplantibacillus pentosus*, *Lacticaseibacillus casei*, *Limosilactobacillus mucosae*, *Lacticaseibacillus rhamnosus*, *Schleiferilactobacillus harbinensis*, and *Lentilactobacillus hilgardii*) and yeasts (*Issatchenkia orientalis*, *Candida ethanolica*, *Kluyveromyces marxianus*, and *Pichia membranifaciens*)] to reduce the blood glucose in diabetic mice have been suggested through directly glucose metabolism in the gut (Wang et al., 2020b). Inhibition of the α-glucosidase, which hydrolyzes glycosidic bonds releasing glucose, is also considered a *probiotic* (*Lacticaseibacillus rhamnosus, Lactobacillus acidophilus, Lactiplantibacillus plantarum, Bifidobacterium animalis, Bifidobacterium longun*) to regulate glucose metabolism in the blood of diabetic mice (Wang et al., 2020a). Other reports account the beneficial effects of probiotics (*Bifidobacterium, Lactobacillus*, and amended genera) on the control of glycemia and triglycerides levels to increase in hepatic natural-killer-cells, reduction of inflammatory signaling (Ma et al., 2008), up-regulation of adiponectin (Nakamura and Omaye, 2012), and increase glucagon-like peptide (GLP)-1that influence the improvement of carbohydrate metabolism (Tremaroli and Bäckhed, 2012). However, the studies have assessed these features in non-sporulated probiotics. Thus, the underlying mechanism of PB remains unclear. It can be suggested that similar mechanisms are involved in PB effects. However, experimental studies are needed to prove this relationship.

Probiotics may exert antioxidant activity through enzyme activation to protect cells against oxidative stress (Petrof et al., 2004), while heat shock proteins (HSPs) play critical roles in the regulation of both acute and chronic stresses (Zininga et al., 2018). Particularly, HSP-70 has a cytoprotective action against structural and functional damage induced by oxidative stress and inflammation. Both antioxidant enzymes and HSP help maintain homeostasis, vital for the intestinal barrier function (Arnal and Lallès, 2014). Consumption of *Bacillus coagulans* GBI-30 6086 either in distilled water or incorporated in food matrices (yogurt or juice) did not affect antioxidant enzymes and HSP expression to observed by Moura et al. (2016) in an experiment with *Lactobacillus acidophilus* LA 05 incorporated in a dairy dessert. The possible relationship between probiotics and HSPs is not yet elucidated, but it seems that low molecular weight peptides and other soluble factors secreted by probiotics in the intestinal lumen would modulate the expression of HSPs (Tao, 2005). Since HSPs are usually expressed in stress situations and act as a cellular defense, a healthy model may be related to the lack of changes in these parameters. Additionally, a limited number of probiotic strains may reduce oxidative stress (Kleniewska et al., 2016). These results are significant because they show that the amount of ingested probiotic or probiotic food did not increase the animals' stress.

Analyses of gut microbiota showed that the probiotic yogurt consumption resulted in a higher abundance and diversity of male rats' gut microbiota profile than the other samples. Lactic acid bacteria's presence, which is used as starter cultures for yogurt production, may influence these findings because they may have a synergistic effect with the probiotic *B. coagulans* modifying the microbiome composition (Ranadheera et al., 2010). Otherwise, *Clostridiales, Bacteroidales*, and *Lactobacillales* were not significantly altered among the six groups studied. Alteration in these microbial groups has been associated with chronic diseases (Ma et al., 2020). Thus, these are results that can be considered favorable for both the Probiotic juice and Probiotic Yogurt.

The ingestion of the probiotic yogurt caused an increase of specific orders such as *Bifidobacteriales* and *Bacillales*, which were not increased by the ingestion of the probiotic juice, showing a positive gut microbiota modulation by probiotic yogurt, the influence of the food carrier on these effects.

Bifidobacteriales are considered one of the main groups, including bacterial members, exhibiting probiotic health-promoting effects in humans (Zhang et al., 2016), while several groups in *Bacillales* also have remarkable health-beneficial properties (Cao et al., 2020). The increase observed in *Bacillales* by the ingestion of yogurt with *B. coagulans* GBI-30 6086 suggests that this matrix delivered the strain in the gut, where the PB germinated, grew, and multiplied as a vegetative form enabling the adhesion to the intestine and exert beneficial effects (Ghelardi et al., 2015; Haldar and Gandhi, 2016). A previous study reported that the spore germination in *Bacillus* strains with further metabolic activity in the gut is influenced by the environmental conditions (Bernardeau et al., 2017) and, as observed here, by the food matrix carrying the spores.

The gut microbiota profile changes in this study are consistent with the results of another study with *Bacillus* strains (*B. coagulans* B37 and *B. pumilus* B9) in skim milk increased lactobacilli and *Bacillus* spp. in the intestinal microbiota in rats (Haldar and Gandhi, 2016). Otherwise, Chaikham et al. (2012) observed that juice added with non-sporulated probiotics (*Lactobacillus acidophilus* LA5 or *Lacticaseibacillus casei* 01) modulated the intestinal microbiota, increasing *Bifidobacteria* and decreasing pathogenic bacteria (e.g., *Clostridia* and fecal coliforms). However, the study was performed *in vitro*, precluding a direct comparison since the environment exerts potent effects on probiotic effects.

Even though *B. coagulans* GBI-30 6086 was added to food matrices as spores, the results of this study clearly show that the food matrix is also relevant for delivering the spore-forming probiotic bacteria. Yogurt was a better carrier of *B. coagulans* GBI-30 6086 compared to orange juice, which is likely due to the yogurt's chemical composition and the presence of lactic bacteria. Yogurt has a higher fat and protein content than juice, while juice content in carbohydrates is higher (**Supplementary Table 1**). Probably these characteristics interfere with the efficacy of the matrix as carrier and maintenance of vegetative cells in the intestine through the interaction of these components with the probiotic cells, boosting its beneficial effects (Ranadheera et al., 2010).

CONCLUSION

Results obtained in the present study show that the daily consumption of yogurt containing *B. coagulans* GBI-30 6086 during 21 days decreases the glucose and triglycerides serum levels in healthy rats and positively modulated the gut microbiota by increasing *Bacillales* and *Bifidobacteriales*. These findings indicate yogurt as an efficient food carrier to deliver probiotic spore-forming bacteria and suggest that yogurt consumption containing *B. coagulans* GBI-30 6086 can be an important dietary strategy to reduce biochemical markers associated with metabolic diseases and modulate the gut microbiota ecology. These animal models' findings indicate that the food matrix impacts spore-forming probiotics' functionality and suggests yogurt as a suitable food carrier of probiotic *Bacillus*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA631217.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Commission on Animal Use (CEUA, UNICAMP, São Paulo, Brazil, protocol n° 3456-1).

AUTHOR CONTRIBUTIONS

ASS and CA-E conceived the study. ASS, CA-E, CA, AR, and PL designed the experiments. CA-E, CA, AR, VS-J, and AG conducted the experiments. CA-E, CA, AR, VS-J, LC, MF, AG, AS, PL, VB, and MM analyzed the results. CA-E, CA, LC, VB, MM, and ASS drafted the manuscript. CA-E, CA, LC, AG, PL, AS, VB, MM, and ASS revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the "*Conselho Nacional de Desenvolvimento Científico e Tecnológico*" (CNPq, Grants #302763/2014-7 and #305804/2017-0) and "*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*" (CAPES-Brazil; Finance code 001).

ACKNOWLEDGMENTS

We would like to thank the donation of the strains used in this study by Ganeden Biotech Inc., Mayfield Heights, Ohio, United States and CHR-Hansen, Brazil.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.623951/full#supplementary-material

REFERENCES

Ahn, H. Y., Kim, M., Chae, J. S., Ahn, Y. T., Sim, J. H., Choi, I. D., et al. (2015). Supplementation with two probiotic strains, *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032, reduces fasting triglycerides and enhances apolipoprotein A-V levels in non-diabetic subjects with hypertriglyceridemia. *Atherosclerosis* 241, 649–656. doi: 10.1016/j.atherosclerosis.2015.06.030

Almada-Érix, C. N., Almada, C. N., Souza Pedrosa, G. T., Santos, P., Schmiele, M., Clerici, M. T. P. S., et al. (2021). Quantifying the impact of eight-unit operations on the survival of eight bacillus strains with claimed probiotic properties. *Food Res. Int.* 142:110191. doi: 10.1016/j.foodres.2021.110191

Andersen, M. L., Dalmeida, V., Ko, G. M., Kawakami, R., Martins, P. J. F., Magalhães, L. E., et al. (2004). *Ethical and practical principles of the use of experimentation animals*. São Paulo: Cromosete.

AOAC (2012). *Official methods of analysis of a.O.a.C. 19th Edn*. Washington D.C., USA: AOAC International.

Arnal, M. E., and Lallès, J. P. (2014). Gut epithelial inducible heat-shock proteins and their modulation by diet and the microbiota. *Nutr. Rev.* 74, 181–197. doi: 10.1093/nutrit/nuv104

Ataie-Jafari, A., Larijani, B., Majd, A. H., and Tahbaz, F. (2009). Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. *Ann. Nutr. Metab.* 54, 22–27. doi: 10.1159/000203284

Bernardeau, M., Lehtinen, M. J., Forssten, S. D., and Nurminen, P. (2017). Importance of the gastrointestinal life cycle of *Bacillus* for probiotic functionality. *J. Food Sci. Technol.* 54, 2570–2584. doi: 10.1007/s13197-017-2688-3

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Cao, J., Yuc, Z., Liu, W., Zhao, J., Zhang, H., Zhaia, Q., et al. (2020). Probiotic characteristics of *Bacillus coagulans* and associated implications for human health and diseases. *J. Funct. Foods* 64, 1–11. doi: 10.1016/j.jff.2019.103643

Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., and Knight, R. (2010). Past: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Cavalcante, R. G. S., Albuquerque, T. M. R., Freire, M. O. L., Ferreira, G. A. H., Santos, L. A. C., Magnani, M., et al. (2019). The probiotic *Lactobacillus*

fermentum 296 attenuates cardiometabolic disorders in high fat diet-treated rats. *Nutr. Metab. Cardiovasc.* 29, 1408–1417. doi: 10.1016/j.numecd.2019.08.003

Chaikham, P., Apichartsrangkoon, A., Jirarattanarangsri, W., and Van de Wiele, T. (2012). Influence of encapsulated probiotics combined with pressurized longan juice on colon microflora and their metabolic activities on the exposure to simulated dynamic gastrointestinal tract. *Food Res. Int.* 49, 133–142. doi: 10.1016/j.foodres.2012.07.033

Chao, A., and Bunge, J. (2002). Estimating the number of species in a stochastic abundance model. *Biometrics* 58, 531–539. doi: 10.1111/j.0006-341X.2002.00531.x

Costa, W. K. A., Brandão, L. R., Martino, M. E., Garcia, E. F., Alves, A. F., Souza, E. L., et al. (2019). Qualification of tropical fruit-derived *Lactobacillus plantarum* strains as potential probiotics acting on blood glucose and total cholesterol levels in Wistar rats. *Food Res. Int.* 124, 109–117. doi: 10.1016/j.foodres.2018.08.035

Cruz, A. G., Castro, W. F., Faria, J. A. F., Bolini, H. M. A., Celeghini, R. M. S., Raices, R. S. L., et al. (2013). Stability of probiotic yogurt added with glucose oxidase in plastic materials with different permeability oxygen rates during the refrigerated storage. *Food Res. Int.* 51, 723–728. doi: 10.1016/j.foodres.2013.01.028

Cutting, S. M. (2011). Bacillus probiotics. *Food Microbiol.* 28, 214–220. doi: 10.1016/j.fm.2010.03.007

Dolin, B. J. (2009). Effects of a proprietary *Bacillus coagulans* preparation on symptoms of diarrhea-predominant irritable bowel syndrome. *Method Find Exp. Clin. Pharmacol.* 31, 655–659. doi: 10.1358/mf.2009.31.10.1441078

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Endres, J. R., Clewell, A., Jade, K. A., Farber, T., Hauswirth, J., and Schauss, A. G. (2009). Safety assessment of a proprietary preparation of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem. Toxicol.* 47, 1231–1238. doi: 10.1016/j.fct.2009.02.018

Fazilah, N. F., Ariffa, A. B., Khayat, M. E., Rios-Solisd, L., and Halima, M. (2018). Influence of probiotics, prebiotics, synbiotics and bioactive phytochemicals on the formulation of functional yogurt. *J. Funct. Foods* 48, 387–399. doi: 10.1016/j.jff.2018.07.039

FDA (2016). GRAS notice no. GRN 000601. Available at: https://www.fda.gov/food/gras-notice-inventory/agency-response-letter-grasnotice- no-grn-000601 (Accessed May 10, 2020).

FDA (2017). GRAS notice no. GRN 000660. Available at: https://www.fda.gov/media/104471 (Accessed May 10, 2020).

Filho, A. L. S., Freitas, H. V., Rodrigues, S., Abreu, V. K. G., Lemos, T. O., Gomes, W. F., et al. (2019). Production and stability of probiotic cocoa juice with sucralose as sugar substitute during refrigerated storage. LWT-food. *Sci. Technol.* 99, 371–378. doi: 10.1016/j.lwt.2018.10.007

Fouad, M. F. E., Namita, R., Rohini, D. G., Chetan, S., and Harsh, P. (2017). *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Front. Microbiol.* 8:1490. doi: 10.3389/fmicb.2017.01490

Genaro, S. C., Reis, L. S. L. S., and Reis, S. K. (2019). Probiotic supplementation attenuates the aggressiveness of chemically induced colorectal tumor in rats. *Life Sci.* 237, 1–7. doi: 10.1016/j.lfs.2019.116895

Ghelardi, E., Celandroni, F., Salvetti, S., Gueye, S. A., Lupetti, A., and Senesi, S. (2015). Survival and persistence of *Bacillus clausii* in the human gastrointestinal tract following oral administration as spore-based probiotic formulation. *J. Appl. Microbiol.* 119, 552–559. doi: 10.1111/jam.12848

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. A. F., and Shah, N. P. (2010). Probiotic dairy products as functional foods. *Compr. Rev. Food. Sci Food Saf.* 9, 455–470. doi: 10.1111/j.1541-4337.2010.00120.x

Haldar, L., and Gandhi, D. N. (2016). Effect of oral administration of *Bacillus coagulans* B37 and *Bacillus pumilus* B9 strains on fecal coliforms, *Lactobacillus* and *Bacillus* spp. in rat animal model. *Vet. World.* 9, 766–772. doi: 10.14202/vetworld.2016.766-772

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. *Rev. Gastroenterol.Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Instituto Adolfo Lutz (2005). Métodos físico-químicos para análise de alimentos (in portuguese). *4th Edn.* eds. Odair Zenebon, Neus Sadocco Pascuet e Paulo Tiglea. São Paulo: Brasil. 1020 p.

Karamali, M., Dadkhah, F., Sadrkhanlou, M., Jamilian, M., Ahmadi, S., Tajabadi-Ebrahimi, M., et al. (2016). Effects of probiotic supplementation on glycaemic control and lipid profiles in gestational diabetes: a randomized, double-blind, placebo-controlled trial. *Diabetes Metab.* 22, 4–11. doi: 10.1016/j.diabet.2016.04.009

Kimmel, M., Keller, D., Farmer, S., and Warrino, D. E. (2010). A controlled clinical trial to evaluate the effect of Ganeden BC (30) on immunological markers. *Methods Find. Exp. Clin. Pharmacol.* 32, 129–132. doi: 10.1358/mf.2010.32.2.1423881

Kleniewska, P., Hoffmann, A., Pniewska, E., and Pawliczak, R. (2016). The influence of probiotic *Lactobacillus casei* in combination with prebiotic inulin on the antioxidant capacity of human plasma. *Oxidative Med. Cell. Longev.* 1, 1–10. doi: 10.1155/2016/1340903

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Ma, X., Hua, J., and Li, Z. (2008). Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J. Hepatol.* 49, 821–830. doi: 10.1016/j.jhep.2008.05.025

Ma, Q., Li, Y., Wang, J., Li, P., Duan, Y., Dai, H., et al. (2020). Investigation of gut microbiome changes in type 1 diabetic mellitus rats based on high-throughput sequencing. *Biomed. Pharmacother.* 124:109873. doi: 10.1016/j.biopha.2020.109873

Maa, J., Zhang, J., Li, Q., Shi, Z., Wu, H., Zhang, H., et al. (2019). Oral administration of a mixture of probiotics protects against food allergy via induction of $CD103^+$ dendritic cells and modulates the intestinal microbiota. *J. Funct. Foods* 55, 65–77. doi: 10.1016/j.jff.2019.02.010

Majeed, M., Majeed, S., Nagabhushanam, K., Arumugama, S., Beed, K., and Ali, F. (2019). Evaluation of probiotic *Bacillus coagulans* MTCC 5856 viability after tea and coffee brewing and its growth in GIT hostile environment. *Food Res. Int.* 121, 497–505. doi: 10.1016/j.foodres.2018.12.003

Manuel, P. M., Elena, B., Carolina, M. G., and Gabriela, P. (2017). Oral probiotics supplementation can stimulate the immune system in a stress process. *Int. J. Nutr. Metab.* 8, 29–40. doi: 10.1016/j.jnim.2017.06.001

Marchesin, J. C., Celiberto, L. S., Orlando, A. B., Medeiros, A. I., Pinto, R. A., Zuanon, J. A. S., et al. (2018). A soy-based probiotic drink modulates the microbiota and reduces body weight gain in diet-induced obese mice. *J. Funct. Foods* 48, 302–313. doi: 10.1016/j.jff.2018.07.010

Marcial-Coba, M. S., Pjaca, A. S., Andersen, C. J., Knøchel, S., and Nielsen, D. S. (2019). Dried date paste as carrier of the proposed probiotic *Bacillus coagulans* BC4 and viability assessment during storage and simulated gastric passage. LWT-Food. *Sci. Technol.* 99, 197–201. doi: 10.1016/j.lwt.2018.09.052

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi: 10.1038/ismej.2011.139

Miranda, J. S., Costa, B. V., Oliveira, I. V., Lima, D. C. N., Martins, E. M. F., Júnior, B. R. C. L., et al. (2020). Probiotic jelly candies enriched with native Atlantic Forest fruits and *Bacillus coagulans* GBI-30 6086. LWT-Food. *Sci. Technol.* 126, 1–6. doi: 10.1016/j.lwt.2020.109275

Moura, C. S., Lollo, P. C. B., Morato, P. N., Esmerino, E. A., Margalho, L. P., Santos-Junior, V. A., et al. (2016). Assessment of antioxidant activity, lipid profile, general biochemical and immune system responses of Wistar rats fed with dairy dessert containing *Lactobacillus acidophilus* La-5. *Food Res. Int.* 90, 275–280. doi: 10.1016/j.foodres.2016.10.042

Mousavi, N. S., Saboori, S., and Asbaghi, O. (2020). Effect of daily probiotic yogurt consumption on inflammation: a systematic review and meta-analysis of randomized controlled clinical trials. *Obs. Med.* 18, 1–8. doi: 10.1016/j.obmed.2020.100221

Nakamura, Y. K., and Omaye, S. T. (2012). Metabolic diseases and pro- and prebiotics: mechanistic insights. *Nutr. Metab.* 9, 1–9. doi: 10.1186/1743-7075-9-60

Ng, S. C., Lam, E. F., Lam, T. T., Chan, Y., Law, W., Tse, P. C., et al. (2013). Effect of probiotic bacteria on the intestinal microbiota in irritable bowel syndrome. *J. Gastroenterol. Hepatol.* 28, 1624–1623. doi: 10.1111/jgh.12306

Orrù, L., Salvetti, L., Cattivelli, L., Lamontanara, A., Michelotti, V., Capozzi, V., et al. (2014). Draft genome sequence of *Bacillus coagulans* GBI-30, 6086, a widely used spore-forming probiotic strain. *Genome Announc.* 2:e01080–14. doi: 10.1128/genomeA.01080-14

Pereira, A. L. F., and Rodrigues, S. (2019) in *Turning fruit juice into probiotic beverages. In fruit juices extraction, composition, quality and analysis. 1st Edn*. eds. G. Rajauria and B. K. Tiwar (Dublin, Ireland: Academic Press), 279–287.

Petrof, E. O., Kojima, K., Ropeleski, M. J., Musch, M. W., Tao, Y., De Simone, C., et al. (2004). Probiotics inhibit nuclear factor-kb and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterology* 127, 1474–1487. doi: 10.1053/j.gastro.2004.09.001

Pimentel, C. T., Klososki, S. J., Rosset, M., Barão, C. E., and Marcolino, V. A. (2019). "Fruit juices as probiotic foods" in *Sports and energy drinks*. *Vol. 10*. eds. A. M. Grumezescu and A. M. Holban (Duxford, United Kingdom: Woodhead Publishing), 483–513.

Ranadheera, R. D. C. S., Baines, S. K., and Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Res. Int.* 43, 1–7. doi: 10.1016/j.foodres.2009.09.009

Reeves, P. G., Nielsen, F. H., and Fayes, G. C. J. (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* 123, 1939–1951. doi: 10.1093/jn/123.11.1939

Roquetto, A. R., Monteiro, N. E. S., Moura, C. S., Toreti, V. C., Pace, F., Santos, A., et al. (2015). Green propolis modulates gut microbiota, reduces endotoxemia and expression of TLR4 pathway in mice fed a high-fat diet. *Food Res. Int.* 76, 796–803. doi: 10.1016/j.foodres.2015.07.026

Rutella, G. S., Tagliazucchi, D., and Solieri, L. (2016). Survival and bioactivities of selected probiotic lactobacilli in yogurt fermentation and cold storage: new insights for developing a bifunctional dairy food. *Food Microbiol.* 60, 54–61. doi: 10.1016/j.fm.2016.06.017

Salvetti, E., Orrù, L., Capozzi, V., Martina, A., Lamontanara, A., Keller, D., et al. (2016). Integrate genome-based assessment of safety for probiotic strains: *Bacillus coagulans* GBI-30, 6086 as a case study. *Appl. Microbiol. Biotechnol.* 100, 4595–4605. doi: 10.1007/s00253-016-7416-9

Shannon, C. E. (1948). A mathematical theory of communication. *Bell Syst. Tech. J.* 27, 379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

Simpson, E. H. (1949). Measurement of diversity. *Nature* 163:688. doi: 10.1038/163688a0

Soares, M. B., Martinez, R. C. R., Pereira, E. P. R., Balthazar, C. F., Cruz, A. G., Ranadheera, C. S., et al. (2019). The resistance of *Bacillus, Bifidobacterium*, and *Lactobacillus* strains with claimed probiotic properties in different food matrices exposed to simulated gastrointestinal tract conditions. *Food Res. Int.* 125, 1–11. doi: 10.1016/j.foodres.2019.108542

Sun, Y. Z., Yang, H. L., Ma, R. L., Song, K., and Lin, W. Y. (2011). Molecular analysis of autochthonous microbiota along the digestive tract of juvenile grouper *Epinephelus coioides* following probiotic *Bacillus pumilus* administration. *J. Appl. Microbiol.* 110, 1093–1103. doi: 10.1111/j.1365-2672.2011.04967.x

Tamime, A. Y., and Robinson, R. K. (2007). *Tamime and Robinson's yoghurt: Science and technology*. Washington: Woodhead Publishing.

Tao, Y. (2005). Soluble factors from *Lactobacillus* GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *AJP. Cell. Physiol.* 290, 1018–1030. doi: 10.1152/ajpcell.00131.2005

Tremaroli, V., and Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature* 489, 242–249. doi: 10.1038/nature11552

Wang, Y., Dilidaxi, D., Wu, Y., Sailike, J., Sun, X., and Nabi, X. (2020b). Composite probiotics alleviate type 2 diabetes by regulating intestinal microbiota and inducing GLP-1 secretion in DB/DB mice. *Biomed. Pharmacother.* 125, 1–11. doi: 10.1016/j.biopha.2020.109914

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wang, L., Shang, Q., Guo, W., Wub, X., Wu, L., Wu, L., et al. (2020a). Evaluation of the hypoglycemic effect of probiotics via directly consuming glucose in intestines of STZ-induced diabetic mice and glucose water-induced diabetic mice. *J. Funct. Foods* 64, 1–9. doi: 10.1016/j.jff.2019.103614

Xu, Z., Li, S., Gong, G., Liu, Z., Wu, Z., and Ma, C. (2015). Influence of different acidifying strains of *Lactobacillus delbrueckii* subsp. bulgaricus on the quality of yoghurt. *Food Sci. Technol. Res.* 21, 263–269. doi: 10.3136/fstr.21.263

Zhang, G., Gao, B., Adeolu, M., Khadka, B., and Gupta, R. S. (2016). Phylogenomic analyses and comparative studies on genomes of the *Bifidobacteriales*: identification of molecular signatures specific for the order *Bifidobacteriales* and its different subclades. *Front. Microbiol.* 7:978. doi: 10.3389/fmicb.2016.00978

Zininga, T., Ramatsui, L., and Shonhai, A. (2018). Heat shock proteins as immunomodulators. *Molecules* 23, 1–17. doi: 10.3390/molecules23112846

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 April 2021
doi: 10.3389/fmicb.2021.630841

Preventive Effects of *Bacillus licheniformis* on Heat Stroke in Rats by Sustaining Intestinal Barrier Function and Modulating Gut Microbiota

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OPEN ACCESS

Edited by:
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Reviewed by:
Wei Chen,
Hefei University of Technology, China
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 18 November 2020
Accepted: 22 February 2021
Published: 06 April 2021

Citation:
Li L, Wang M, Chen J, Xu Z, Wang S, Xia X, Liu D, Wang S, Xie C, Wu J, Li J, Zhang J, Wang M, Zhu J, Ling C and Xu S (2021) Preventive Effects of Bacillus licheniformis on Heat Stroke in Rats by Sustaining Intestinal Barrier Function and Modulating Gut Microbiota. Front. Microbiol. 12:630841. doi: 10.3389/fmicb.2021.630841

Heat stroke (HS) models in rats are associated with severe intestinal injury, which is often considered as the key event at the onset of HS. Probiotics can regulate the gut microbiota by inhibiting the colonization of harmful bacteria and promoting the proliferation of beneficial bacteria. Here, we investigated the preventive effects of a probiotic *Bacillus licheniformis* strain (BL, CMCC 63516) on HS rats as well as its effects on intestinal barrier function and gut microbiota. All rats were randomly divided into four groups: control (Con) + PBS (pre-administration with 1 ml PBS twice a day for 7 days, without HS induction), Con + BL group (pre-administration with 1 ml 1×10^8 CFU/ml BL twice a day for 7 days, without HS induction), HS + PBS (PBS, with HS induction), and HS + BL (BL, with HS induction). Before the study, the BL strain was identified by genomic DNA analysis. Experimental HS was induced by placing rats in a hot and humid chamber for 60 min until meeting the diagnostic criterion of HS onset. Body weight, core body temperature, survival rate, biochemical markers, inflammatory cytokines, and histopathology were investigated to evaluate the preventive effects of BL on HS. D-Lactate, I-FABP, endotoxin, and tight-junction proteins were investigated, and the fluorescein isothiocyanate-dextran (FD-4) test administered, to assess the degree of intestinal injury and integrity. Gut microbiota of rats in each group were analyzed by 16S rRNA sequencing. The results showed that pre-administration with BL significantly attenuated hyperthermia, reduced HS-induced death, alleviated multiple-organ injury, and decreased the levels of serum inflammatory cytokines. Furthermore, BL sustained the intestinal barrier integrity of HS rats by alleviating intestinal injury and improving tight junctions. We also found that BL significantly increased the ratios of two probiotic bacteria, *Lactobacillus* and *Lactococcus*. In addition, *Romboutsia*, a candidate biomarker for HS diagnosis, was unexpectedly detected. In summary, BL pre-administration for 7 days has preventative effects on HS that may be mediated by sustaining intestinal barrier function and modulating gut microbiota.

Keywords: heat stroke, intestinal barrier, *Bacillus licheniformis*, probiotics, 16S rRNA, gut microbiota

INTRODUCTION

Heat stroke (HS) is a life-threatening disease characterized by extreme hyperthermia (usually core body temperature (Tc) > 40.5°C), central nervous system dysfunction, systemic inflammatory response syndrome (SIRS), and multiple-organ injury dysfunction syndrome (MODS) (Epstein and Yanovich, 2019). As estimated by climate models, heat waves are becoming more intense, more frequent, and longer lasting in the 21st century, resulting in the rapid increase of mortality risk of heat-related illnesses, of which HS is the most hazardous (Meehl and Tebaldi, 2004; Guo et al., 2017). Rapid cooling methods that help immediately lower the body temperature are the major emergency treatments to save patients. However, when people develop HS in areas where cooling is difficult to obtain, the (Tc) cannot be controlled at the early stage. Without effective first-aid measures, Tc can rise continually and lead to a cascade of events that includes SIRS, disseminated intravascular coagulation (DIC), MODS, and even death. At this stage, even if multiple adjuvant treatments and intensive care therapy are adopted, the case-fatality rate of HS patients is difficult to reduce and the occurrence of sequelae might be inevitable. Considering the lack of specific and effective drugs for HS, prevention is critical rather than treatment after onset (Epstein and Yanovich, 2019).

The gastrointestinal tract (GI), which needs functional intestinal barrier integrity and gut microbiota to perform properly, seems to be more sensitive to heat stress compared with other visceral organs, such as the liver, kidney, and lungs. Growing evidence indicates that intestinal injury caused by HS-induced visceral ischemia plays a key role in the pathogenesis and pathophysiology of HS (Lian et al., 2020; Ogden et al., 2020). Intestinal injury comprised of enterocyte death and tight-junction disintegration results in intestinal barrier dysfunction that triggers gut-derived endotoxemia, which activates subsequent systemic inflammatory responses and multiple-organ injury (Ye et al., 2019). Therefore, the intestines can be considered as the main route of HS progression, and sustaining the intestinal barrier might help prevent HS onset and subsequent pathology.

Probiotics are live microorganisms that, when administered in proper amounts, confer a health benefit on the host. Supplementation with probiotics can inhibit the colonization of pathogenic bacteria, improve gut barrier function, and improve gut flora (Hill et al., 2014). Recently, several studies have suggested that dietary probiotics can alleviate the adverse effects of stress. Notably, some *Bacillus* probiotic strains are able to fight against heat stress in poultry and in laboratory studies (Deng et al., 2012; Lei et al., 2013; Song et al., 2014). *Bacillus licheniformis* (BL) is a Gram-positive, spore-forming bacteria that has been widely applied in the livestock industry, and live BL powder (CMCC63516) is used as a treatment for gastrointestinal diseases and as a probiotic in China (Li et al., 2019). Since BL has probiotic benefits by enhancing intestinal barrier function and modulating gut flora, and because the intestine plays a key role in HS, we hypothesized that BL might ameliorate HS onset and alleviate HS progression by advanced short-term dietary application. To the best of our knowledge, there have been no studies on the preventive effects of BL against HS in rat models, and the influence of BL on gut microbiota has not been clarified. Thus, in the current study, we determined whether BL has preventive effects on HS rats and evaluated the influence of BL on the intestinal barrier and gut microbiota.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley (SD) rats, weighing 250–300 g, were purchased from Sippr B&K Laboratory Animal Ltd. (Shanghai, China). All rats were raised in the Specific Pathogen Free Animal Experiment Center of the Navy Medical University in China. The rats (6/cage) were maintained at an ambient temperature (Ta) of 22 ± 1°C and relative humidity (RH) of 50 ± 5% with a 12-h day/night cycle. Standard pellet rat chow and distilled tap water were provided *ad libitum*. All experimental procedures were approved by the Institutional Animal Ethics Committee of the Navy Medical University according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Animal Grouping and Probiotic Administration

A total of 172 rats evaluated as suitable for research were randomly allocated into four groups by using a computerized randomization procedure. Rats in each group were processed according to uniform standards and then allocated to each experiment. The four groups were as follows: control + phosphate-buffered saline group (Con + PBS, $n = 34$); control + BL group (Con + BL, $n = 34$); HS + PBS group (HS + PBS, $n = 52$); and HS + BL group (Con + BL, $n = 52$). The whole research animal model process occurred in two stages that included intragastric administration and heat stress exposure (**Figure 1A**). In the first stage, rats were administered intragastrically with 1 ml PBS or 1×10^8 colony-forming units (CFU) of BL (CMCC63516, Northeast Pharmaceutical Group, Shenyang No. 1 Pharmaceutical Co. Ltd., China, Lot S10950019) suspended in 1 ml PBS twice a day at fixed time points for 7 days (Li et al., 2019). Rats were weighed every day before gavage in case of stress-induced weight fluctuation. In the second stage, rats in the HS groups were exposed to heat stress, and rats in the Con groups were placed in a conventional environment without food and water.

Heat Stroke Protocol and Core Body Temperature Measurements

Tc (±0.1°C) was monitored continuously at 5-min intervals in conscious, free-moving rats implanted with a temperature-monitoring capsule (SV223 capsule thermometer, Shenzhen Flamingo Technology Co., Ltd.). Briefly, each rat was anesthetized with isoflurane, and then an activated temperature-monitoring capsule, which was around 1.5 cm in length, 0.5 cm in diameter, and 2 g in weight, was surgically implanted intra-abdominally via a small incision with aseptic techniques. The capsule recorded Tc at 5-min intervals and sent the

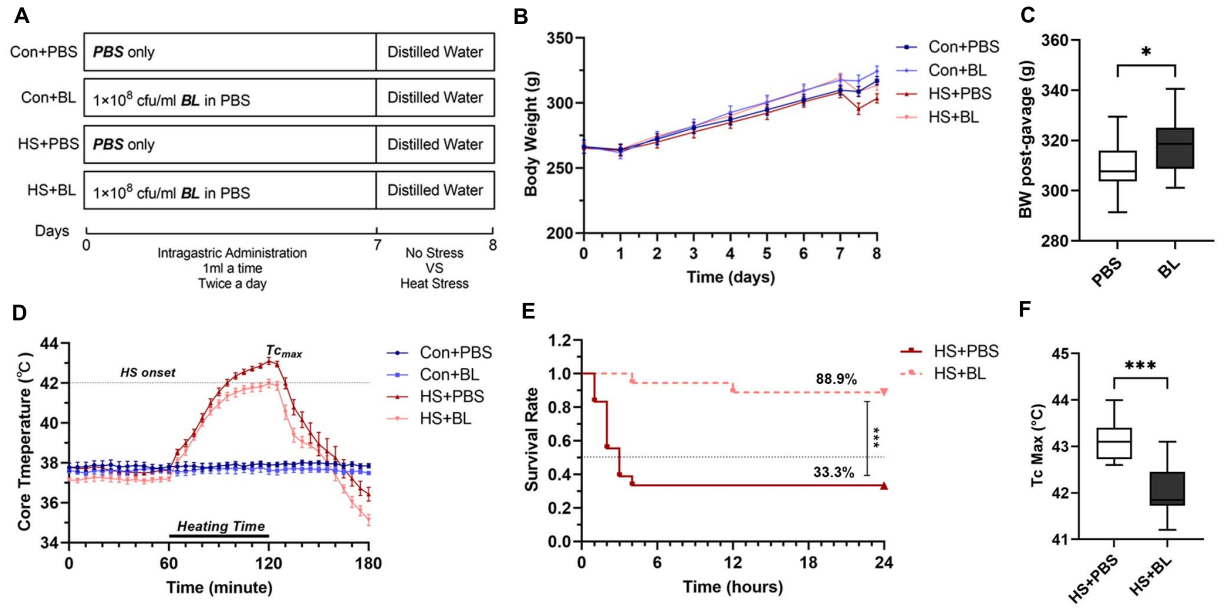

FIGURE 1 | Experimental design and effects of *Bacillus licheniformis* (BL) on weight, core body temperature (Tc), and survival rate of rats with heat stroke (HS). **(A)** The BL group was administered 1×10^8 CFU BL each twice a day for 7 days. After gavage, the HS model was induced by placing rats in an artificial climate chamber with Ta maintained at 40°C ± 1 and RH at 65% until the Tc of rats was beyond 42°C for approximately 60 min. **(B)** Body weight (BW) changes recorded every day are expressed from the starting gavage day. HS experiments were conducted just after the gavage stage, and pre-heating and post-heating BW were measured at day 7.5. **(C)** BW of post-gavage in the PBS groups was compared with BW in the BL groups. **(D)** Core body temperature (Tc, °C) was plotted for every 5 min from 1 h before heating to 1 h after heating. **(E)** Survival was monitored for 24 h after HS onset. **(F)** The maximum Tc in HS + PBS was compared with Tc Max in HS + BL. Data of Con + PBS (dark blue line with square), Con + BL (light blue line with circle), HS + PBS (dark red line with upright triangle), and HS + BL (light red line with inverted triangle) are plotted in **(B,D,E)**. Data in **(B,D)** are presented as the mean ± SEM of eight rats per group. Data in **(C,F)** are presented as a box-and-whisker plot of eight rats per group. Eighteen rats in each HS group were used for the survival study. $^*P < 0.05$, $^{***}P < 0.001$.

data to a cell phone by Bluetooth transmission. Due to the tiny size of the capsule and strong wound healing ability of rats, the physical habits of rats implanted with capsules were observed. To be included in the experiment, rats were implanted with capsules 1 week ahead of the study and their physical condition was confirmed as healthy with no abnormal behaviors (Helwig et al., 2012).

An artificial climate chamber (LTH-575N-01, Shanghai Drawell Scientific Instrument Co., Ltd.) where Ta was controlled at 40 ± 1°C and RH at 65 ± 5% to create an environment with high temperature and humidity was used to give rats heat stress (Xia et al., 2017). Rats in the HS groups were placed in the chamber at least 2 h a day during the first gavage stage to avoid stress-induced hyperthermia. To induce HS, rats were placed in a hot and humid chamber maintained at stable Ta and RH without food and water during the whole stage. Once rats were exposed to heat stress, Tc was monitored at 5-min intervals. The timepoint of stable hyperthermia occurrence (Tc > 42.7°C) was taken as the primary criterion of HS onset (Geng et al., 2015) and was allowed to continue for around 60 min with heating according to previous experience under our experimental environment. Then, rats meeting the criterion were transferred to regular housing with food and water access *ad libitum*. Body weights of rats in all groups were measured before and after the HS induction stage. Rats that died during or after this stage were recorded, and additional experiments were conducted to meet the sample size requirement. Overall, 36 rats in the HS + PBS and HS + BL groups each were used to observe and record survival within 24 h after the onset of HS.

Sample Collection

Samples including serum, plasma, organ tissues, and feces were collected at 3 h after the onset of HS from rats anesthetized with isoflurane. Blood samples were collected from the abdominal aorta and separated by centrifugation at 3,000 rpm for 10 min at 4°C, then sub-packed and stored at −80°C for later examinations. Samples of liver, kidney, lung, and colon segments beside the ileocecal valve were harvested immediately after the blood collection and fixed in 4% paraformaldehyde for histopathological examination. Samples of colon specimens were cut into tiny parts that were fixed in 0.25% glutaraldehyde at 4°C for transmission electron microscopy. Fecal samples were quickly collected from colons from anesthetized rats and placed into liquid nitrogen to ensure their freshness for microbiological detection.

Serum Biochemical Markers and Inflammatory Cytokine Analysis

Serum biochemical markers can represent the level of internal-organ injury. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea (BU), creatine kinase

(CK), and creatinine (CREA) were determined by a HITACHI 7080 automated analyzer (Tokyo, Japan). Serum inflammatory cytokines can represent the inflammatory level, so we detected the concentrations of IL-6, IL-1β, and TNF-α using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) kit [Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China] and a SpectraMax M2e Microplate Spectrophotometer (Bio-Rad, Berkeley, CA, United States) according to the protocol provided (Xia et al., 2017).

Histological Examination and Scoring

All the organ samples fixed in paraformaldehyde were dehydrated through a graded alcohol series and embedded in paraffin wax, sectioned into 10-mm sections, stained with hematoxylin and eosin (H&E), and examined under a light microscope (Leica DM 2000, Wetzlar, Germany). Selected H&E slides were scanned, and images were taken using a Panoramic MIDI Slide scanner (3DHISTECH, Hungary). Slide images were visualized by CaseViewer software (3DHISTECH). Then, histological profiles of organs in each group were analyzed with pathological injury scores. Typical pathological changes such as inflammation, necrosis, degeneration, hyperplasia, and fibrosis were described, and representative characteristics were chosen to construct an overall rating. Liver injury scoring was performed based on necrosis, inflammation, and vacuolar degeneration; kidney injury was based on congestion and inflammation; lung injury was based on alveolar wall thickening, congestion, and inflammation; and intestinal injury was based on necrosis, inflammation, and hemorrhage. The lesion degree of each kind of tissue was divided into five stages: no lesions or very few lesions, 0; mild or small lesions, 1; moderate lesions, 2; severe or multiple lesions, 3; and extremely severe or numerous lesions, 4; we then summarized each score for organs in each group rat for histopathological comparison.

D-Lactate, I-FABP, and Endotoxin Detection

Plasma D-Lactate, intestinal fatty acid-binding protein (I-FABP), and endotoxin levels were, respectively, detected by a D-Lactate Colorimetric Assay Kit (BioVision, Milpitas, CA, United States), a Rat I-FABP ELISA Kit (F12794, YANJING Biological Co., Ltd., Shanghai, China), and a limulus amebocyte lysate (LAL) test kit (EC64405S, Xiamen Bioendo Technology Co., Ltd., Xiamen, China) according to the manufacturers' instructions. The three detection kits all used 98-well plates with methods similar to the ELISA kit. Plasma samples and detection agents in each kit were mixed according to the provided instructions and measured at O.D. 450 and 545 nm in a microplate spectrophotometer. Standard curves were plotted, and then concentrations of each sample were calculated.

FITC-Dextran 4-kD Detection

Intestinal barrier permeability was assessed using 4 kDa fluorescein isothiocyanate (FITC)-dextran (FD4, Sigma-Aldrich, St. Louis, MO, United States). In brief, a 200-mg/ml solution of FD4 was prepared with PBS and stored in a black Eppendorf tube. Then, the FD4 solution was orally administered to rats in each group at 200 mg/kg body weight, and rats were held in an upright position for 30 s to avoid regurgitation before the HS stage. Then, plasma samples were collected according to the study plan after the HS intervention and added to black 96-well microplates. Concentrations of FD4 were detected using a microplate spectrophotometer (excitation wavelength, 485 nm; emission wavelength, 530 nm). Concentrations of FD4 were calculated by preparing a standard curve with a serial dilution of FD4.

Immunofluorescence Staining

Paraffin-embedded sections of the colon samples were also used for immunofluorescence staining. Samples were also obtained through deparaffinizing and rehydrating, antigen retrieval, circling, blocking endogenous peroxidase, blocking with serum, primary antibody (ZO-1, occludin, and E-cadherin) incubation, corresponding secondary antibody with HRP incubation, addition of TSA-FITC solution, microwave treatment, second primary antibody incubation, spontaneous fluorescence quenching, DAPI counterstaining of nuclei, mounting, microscopy detection, and image collection by fluorescent microscopy (NIKON ECLIPSE TI-SR, Tokyo, Japan). The image acquisition system (NIKON DS-U3) and microscope settings were maintained throughout the process.

Transmission Electron Microscopy

Colon samples were quickly harvested and cut into small fresh tissues within 1–3 min for transmission electron microscopy (TEM). Colon tissue sizes were no more than 1 mm and were placed into fixative for TEM immediately. Colon tissues were kept in the dark and post-fixed with 1% OsO_4 in 0.1 M PBS (pH 7.4) for 2 h at room temperature. After removal of OsO_4, the tissues were rinsed in 0.1 M PBS (pH 7.4) three times for 15 min each. Then, samples were dehydrated with ethanol at room temperature, embedded in Epon-812 resin, and finally polymerized for more than 48 h at 65°C. Ultrathin sections (60–80 nm) were cut and stained with 2% uranyl acetate and 2.6% lead citrate (Xia et al., 2017). The sections were observed, and images were captured under a Hitachi-7000 electron microscope (Hitachi, Naka, Japan).

Identification of *Bacillus licheniformis*

Bacillus licheniformis, which was stored as a spore powder, was provided by Northeast Pharmaceutical Group, Shenyang No. 1 Pharmaceutical Co. Ltd., China. The species-level identification of the *B. licheniformis* strain was conducted by genomic DNA analysis (Joseph et al., 2013; Selvarajan and Mohanasrinivasan, 2015). Total DNA of BL samples was extracted using a DNA purification kit (TIANamp Genomic DNA Kit, Tiangen Biotech CO., Ltd., China) according to the manufacturer's instructions. PCR amplification of the 16s rDNA fragment was used for DNA amplification for 16S rRNA sequencing. The sequences obtained by 16S rRNA sequencing were subjected to a BLAST search[1] of the NCBI database.

[1] https://pubmed.ncbi.nlm.nih.gov/

Phylogenetic tree analysis was conducted on the NCBI website, and blast tree view was produced using BLAST pairwise alignments. Then, precisely designed amplified polymorphic DNA primers according to the genome sequence provided by the company (F 5′-GGTCGTATGCCTTCACCAGAT-3′ and R 5′-CGCTTTTTGCTCGGAAATGAT-3′) were used for polymerase chain reaction (PCR) amplification. An 807-bp PCR-amplified product was detected by agarose gel electrophoresis and analyzed to determine whether a sample was the BL strain (CMCC63516). Then, the amplified gene segment was subjected to a BLAST search in the NCBI database for blast tree view to confirm the species again.

Fecal Microbiota Composition Analysis

Microbial community genomic DNA was extracted from 24 frozen fecal samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States). The DNA concentration and purification were checked with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, United States). The V3–V4 region of the bacterial 16S ribosomal RNA (rRNA) gene was amplified by PCR with primer pairs (338F 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′) on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corporation, United States) following the manufacturer's protocols (Wang et al., 2020).

Amplicons were then purified by gel extraction with an AxyPrep DNA GelExtraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using a Quantus™ Fluorometer (Promega, United States). Purified amplicons were pooled in equimolar amounts and subject to paired-end sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) according to the standard protocols.

The raw 16S rRNA gene sequencing data were demultiplexed, quality-filtered by Trimmomatic, and merged by Fast Length Adjustment of Short reads (FLASH, v 1.2.11). Then, sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff using UPARSE (version 7.1[2]), and chimeric sequences were identified and removed using UCHIME. The taxonomy of the acquired OTUs was analyzed by the RDP Classifier Bayesian algorithm[3] against the SILVA (SSU138) 16S rRNA database with a confidence threshold of 0.7. Then, we subsampled each sample to an equal sequencing depth and clustered them for subsequent microbial bioinformatic analysis. The raw reads have been uploaded to the Sequence Read Archive (SRA) database (Accession: PRJNA674334 ID: 674334).

The 16Sr RNA sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology platform (QIIME) with i-Sanger platform[4] provided by Majorbio BioTech Co., Ltd. (Shanghai, China). Briefly, community diversity was evaluated using alpha diversity indexes that included species richness indices (Ace and Sobs) and species diversity indices (Shannon). Analysis of differences in beta-diversity as revealed by principal component analysis (PCA), principal coordinate analysis (PCoA), and non-metric multidimensional scaling analysis (NMDS) was conducted based on the OTU level from the weighted UniFrac distances. Differences between groups were tested by ANOSIM/Adonis, PERMANOVA, and partial least square discriminant analysis (PLS-DA). The Wilcoxon rank-sum test or Mann–Whitney *U* test was used to compare two targeted groups, and the Kruskal–Wallis *H* test was used to compare multiple groups at the phylum and genus levels. To investigate the effects of BL and HS on the gut bacterial communities in two groups, Student's *t*-test was performed. A collinearity diagram was constructed by Circos software[5] to visualize the corresponding abundance relationship between samples of each group and bacterial communities at the phylum and genus levels. Linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe), which is an algorithm for biomarker discovery that identifies taxa characterizing the differences between two metadata classes, was used to determine differentially abundant features consistent with biologically meaningful categories among these groups. Species composition analyses were conducted based on the results of taxonomic analysis and are shown as bar graphs combined with statistical analysis.

Statistical Analysis

All experimental data are presented as means ± standard error. All statistical analyses were performed with GraphPad Prism (Version 8.3.0, GraphPad Software, La Jolla, CA, United States), except the data of microbiota using multivariate and advanced statistical analysis, which is described above. Results from multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple-comparison test. Results from two unpaired groups were compared using the two-tailed Student's *t*-test. Survival was analyzed with the log-rank test. Histological scores were analyzed by the Mann–Whitney rank-sum test. A two-tailed *P*-value of 0.05 or less was considered to be statistically significant.

RESULTS

BL Pre-administration Attenuated Hyperthermia and Reduced HS-Induced Death

After the oral administration of BL, body weights (BW) of rats in the BL groups were heavier than in the PBS groups (**Figure 1B**). Combining the data of the PBS groups and BL groups, the BW post-gavage between the groups were found to be significantly different (**Figure 1C**; 309.0 ± 11.11 vs. 318.5 ± 10.77, $P = 0.0201$, 16 rats/group). Then, the HS rat model, which is widely used for research of HS, was used to evaluate the effects of BL on BW and Tc. The BW pre-gavage (Day 1), post-gavage (Day 7), and post-heating (Day 7.5) are shown in **Table 1**. Due to HS-induced fluid loss, the post-heating BW decreased around 10 g

[2]http://drive5.com/uparse/

[3]http://rdp.cme.msu.edu/

[4]http://www.i-sanger.com/

[5]http://circos.ca/software/download/circos/

TABLE 1 | Experimental data and characteristics of rats subjected to BL and HS.

	Con		HS	
	PBS (n = 8 ∼ 10)	**BL (n = 8 ∼ 10)**	**PBS (n = 8 ∼ 10)**	**BL (n = 8 ∼ 10)**
BW pre-gavage, g	266.4 ± 14.43	266.4 ± 13.81	265.3 ± 11.63	265.4 ± 11.09
BW post-gavage, g	309.8 ± 11.01	317.5 ± 12.19	308.2 ± 11.91	319.4 ± 9.91
BW post-heating, g	–	–	285.6 ± 12.48	308.7 ± 11.33
BW lose-heating, g	–	–	12.6 ± 2.38	10.7 ± 2.65
Tc max, °C	–	–	43.1 ± 0.46	42.0 ± 0.58$^{\#}$
Time of HS onset, min	–	–	96.9 ± 6.51	113.3 ± 11.09$^{\#}$
ALT, U/L	25.2 ± 8.59	24.5 ± 6.47*	127.9 ± 36.17*	79.8 ± 15.6$^{\#}$
AST, U/L	81.8 ± 10.99	80.3 ± 11.44*	322.6 ± 60.27*	146.9 ± 34.07$^{\#}$
BU, mol/L	4.8 ± 2.26	5.3 ± 0.98*	19.5 ± 5.50*	14.2 ± 2.90$^{\#}$
CREA, μmol/L	6.7 ± 1.77	8.2 ± 1.44*	58.2 ± 18.17*	27.5 ± 7.054$^{\#}$
CK, U/L	291.3 ± 172.90	462.2 ± 116.10*	1631 ± 1002.00*	808.9 ± 336.60$^{\#}$
TNF-α, pg/ml	1.5 ± 0.31	1.1 ± 0.66*	3.3 ± 0.65*	1.8 ± 0.41$^{\#}$
IL-1β, pg/ml	5.9 ± 4.39	8.6 ± 4.54*	34.7 ± 6.69*	28.1 ± 3.94$^{\#}$
IL-6, pg/ml	4.0 ± 2.92	3.2 ± 1.92*	40.7 ± 10.87*	20.8 ± 4.41$^{\#}$
D-Lactate, μmol/mL	0.2 ± 0.03	0.2 ± 0.041*	0.7 ± 0.13*	0.3 ± 0.03$^{\#}$
I-FABP, pg/mL	216.3 ± 80.17	212.7 ± 63.04*	754.7 ± 266.2*	368.9 ± 117.3$^{\#}$
Endotoxin, EU/mL	0.1 ± 0.01	0.1 ± 0.01*	0.8 ± 0.13*	0.6 ± 0.13$^{\#}$
FITC-4kD, μmol/mL	0.1 ± 0.03	0.1 ± 0.04*	1.8 ± 0.86*	0.9 ± 0.43$^{\#}$

*Data are shown as means ± SD; sample sizes are indicated in (n); BW, body weight; Tc max, mean value and SD of the maximum Tc during the heating period. Time of HS onset, the time from the beginning of heating to Tc reaching the standard of HS onset or to the end of the heating period in the BL group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BU, blood urea; CK, creatine kinase; CREA, creatinine; I-FABP, intestinal fatty acid-binding protein; FITC-4kD, 4-kDa fluorescein isothiocyanate. * vs. Con + PBS; # vs. HS + PBS.*

compared with the post-gavage BW but was not significant. Tc curves were plotted from 1 h before heating to 1 h after heating. All rats in the HS + PBS group (n = 8) met the HS onset standard (Tc > 42°C), while only two rats in the HS + BL group (n = 8) met the standard. The mean Tc of the HS + PBS group was higher than that of the HS + BL group as shown in **Figure 1D**, and the maximum Tc of rats in the BL groups was higher than in the PBS groups with significant difference (43.14 ± 0.46 vs. 42.01 ± 0.58, P = 0.008, n = 8 rats/group) as shown in **Table 1** and **Figure 1F**. Furthermore, we found that the survival rate 24 h after HS onset was 33.3 and 88.9%, respectively, for the HS + PBS and HS + BL groups as shown in **Table 1**. The survival time and survival rate of the HS + BL group were notably improved by BL pre-administration compared with the HS + PBS group (log rank, P = 0.0003, 18 rats/group; **Figure 1E**).

BL Pre-administration Attenuated Multiple-Organ Injury and Decreased Levels of Serum Inflammatory Cytokines

Multiple-organ injury in rats of each group was evaluated by checking biochemical markers that represent each related organ's function and histopathological examination with H&E staining. Serum biochemical markers of liver function (ALT and AST), kidney function (BU and CREA), and skeletal and/or cardiac muscle function (CK) were chosen to evaluate typical organ injury. Typical serum inflammatory cytokines TNF-α, IL-1β, and IL-6 were selected to evaluate the inflammatory response. No statistically significant difference in these indexes was found between the Con + PBS and Con + BL groups, while levels of serum biochemical markers and inflammatory cytokines were notably increased in the HS groups compared with the Con groups with a significant difference as shown in **Figures 2**, **3**. Consistent with the serum biochemical markers, livers from the HS + PBS group showed massive hepatocyte necrosis, hyperchromatic or cataclysmic nuclei, enhanced eosinophils, round vacuoles in the cytoplasm, and granulocyte infiltration around the local bile duct (**Figure 3C**, arrows). Typical kidney injuries were observed in the cortex of HS rats as characterized by edema, renal vesicle stenosis, granulocyte infiltration in the glomeruli, eosinophilic material in renal tubules, and congestion in the blood vessels (**Figure 3G**, arrows). Furthermore, massive alveolar wall thickening, narrowed alveolae, many monocytes scattered with granulocytes, and severe congestion in vessels were found in the lungs of HS rats (**Figure 3K**, arrows). Meanwhile, intestinal (colon) injuries were seen with extensive epithelial necrosis in mucosa marked with hyperchromatic or cataclysmic nuclei, subepithelial edema, congestion in most blood vessels, and destruction of villi structures (**Figure 3O**, arrows). However, BL pre-administration attenuated HS-induced multiple-organ injury, which was also reflected by biochemical markers (**Figure 2**) and histopathological images (**Figure 3**). All of these mitigative effects were statistically significant, except for the histopathological scores of lungs. Since BL pre-administration resulted in obvious preventive effects of organ injury, systematic inflammatory responses were checked by measuring the concentrations of inflammatory cytokines. Encouragingly, significant decreases of TNF-α, IL-1β, and IL-6 in the HS + BL group were found compared with the HS + PBS group. Altogether, this evidence

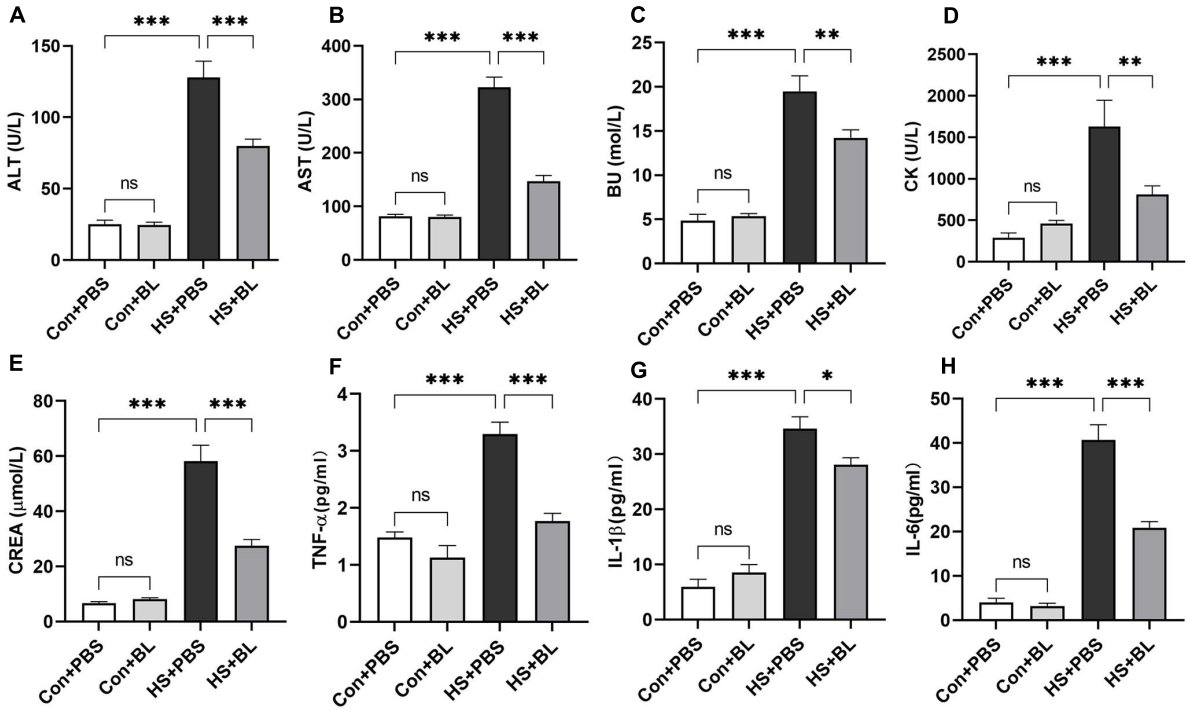

FIGURE 2 | BL pre-administration decreased levels of serum biochemical markers and inflammatory cytokines. Rats were pre-administered with 1×10^8 CFU twice a day (HS + BL group) for 7 days or PBS (HS + PBS group), and blood samples were collected 3 h after HS onset and at the same time for the control group rats. Serum biochemical marker levels of ALT **(A)**, AST **(B)**, BU **(C)**, CK **(D)**, and CREA **(E)** and inflammatory cytokines TNF-α **(F)**, IL-1β **(G)**, and IL-6 **(H)** are presented as means ± SEM, $n = 10$ per group. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BU, blood urea; CK, creatine kinase; CREA, creatinine.

indicated that BL pre-administration attenuates multiple-organ injury and significantly decreases the levels of serum inflammatory cytokines.

BL Pre-administration Attenuated Intestinal Injury and Enhanced Intestinal Barrier Function

As intestinal injury plays a key role in the progress of HS and might be the target organ protected by BL, typical biomarkers D-Lactate and I-FABP were chosen to evaluate the degree of intestinal injury. In addition, intestinal barrier function is highly associated with gastrointestinal permeability as determined by the level of plasma endotoxin and the FD4 test. Endotoxin escaping from the injured gut barrier might activate systematic inflammatory responses, resulting in multiple-organ injury. Significantly high levels of plasma D-lactate and I-FABP were detected in HS + PBS, while the levels of the two biomarkers were relatively low in HS + BL (**Figures 4A,B**). The initial degree of intestinal injury was thus lower in the HS + BL group. In addition, plasma endotoxin concentrations in the HS + BL group were lower than in the HS + PBS group with significant difference, which indicated that pre-administration of BL decreased intestinal barrier permeability (**Figure 4C**). Therefore, FD4 solution was pre-administered to rats for further investigation of intestinal permeability differences among groups. Once intestinal barrier dysfunction and permeability decrease occur, FD4 passes through the "leaky" gut into the blood and can be detected in the plasma. Quite high levels of FD4 were detected in the plasma of the HS + PBS group compared with Con + PBS, but relatively low FD4 levels were detected in the HS + BL group compared with HS + BL (**Figure 4D**). In addition, there was no significant difference of the above indexes between Con + PBS and Con + BL groups, which indicates that guts with or without BL both were healthy and "unleaky." Representative proteins of tight junctions (TJs; ZO-1, occludin, and E-cadherin), which represent the permeability and function of the intestinal barrier, had a markedly lowered expression in the intestines of rats subjected to heat as shown in **Figure 4E**. Interestingly, the expressions of TJ markers were slightly increased in the rat in the Con + BL group, though the changes were subtle. However, the expressions of TJ markers were notably higher in the HS + BL group than in the HS + PBS group, which indicated that BL partly counteracted the HS-induced intestinal damage. To further explore the protective effects of BL on the gut, TEM was conducted to observe the ultrastructure of the intestinal alternations among groups. Consistent with the other results, we observed that BL pre-administration did not damage the intestinal barrier as represented by TJ structures and alleviated HS-induced intestinal injury. In brief, in the Con + PBS (**Figure 4F**) and Con + BL (**Figure 4G**) groups, intestine samples had similarly

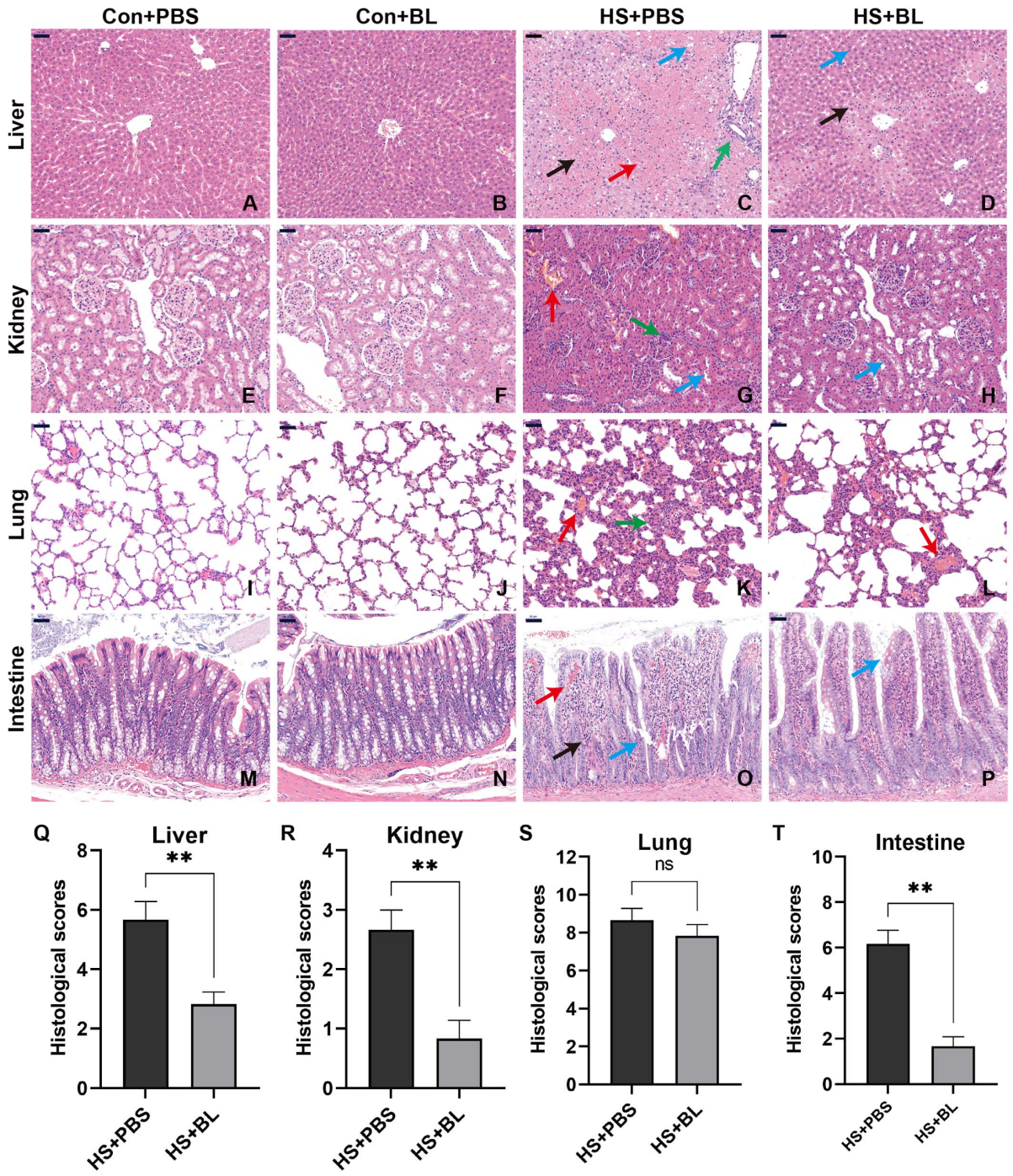

FIGURE 3 | BL pre-administration attenuated multiple-organ injury in HS rats. Organ samples were harvested at 3 h after HS and stained with H&E. Representative histopathological images are shown of liver **(A–D)**, kidney **(E–H)**, lung **(I–L)**, and intestine **(M–P)** from Con + PBS, Con + BL, HS + PBS, and HS + BL (left to right) groups, magnification 200×. Scale bar = 50 μm. Arrows indicate typical pathological changes including necrosis, inflammation, vacuolar degeneration, congestion, hemorrhage, and alveolar wall thickening. Histological scores of liver **(Q)**, kidney **(R)**, lung **(S)**, and intestine **(T)** were counted and plotted. Values are presented as means ± SEM; $n = 6$ per group. $^{ns}P > 0.05$, $^{**}P < 0.01$. Black arrows indicate necrosis; green arrows, inflammation; red arrows, congestion or hemorrhage; blue arrows, organ-specific alternations, such as villi destruction or hepatocyte vacuolar degeneration.

normal cell structures, mitochondria, and regularly aligned microvilli on the membranes, and clearly visible TJ structures between enterocytes, while in the HS + PBS (**Figure 4H**) group, intestine samples showed widespread damage to microvilli and TJ structure disruption as well as cell death. As expected, BL pre-administration attenuated these injuries and preserved

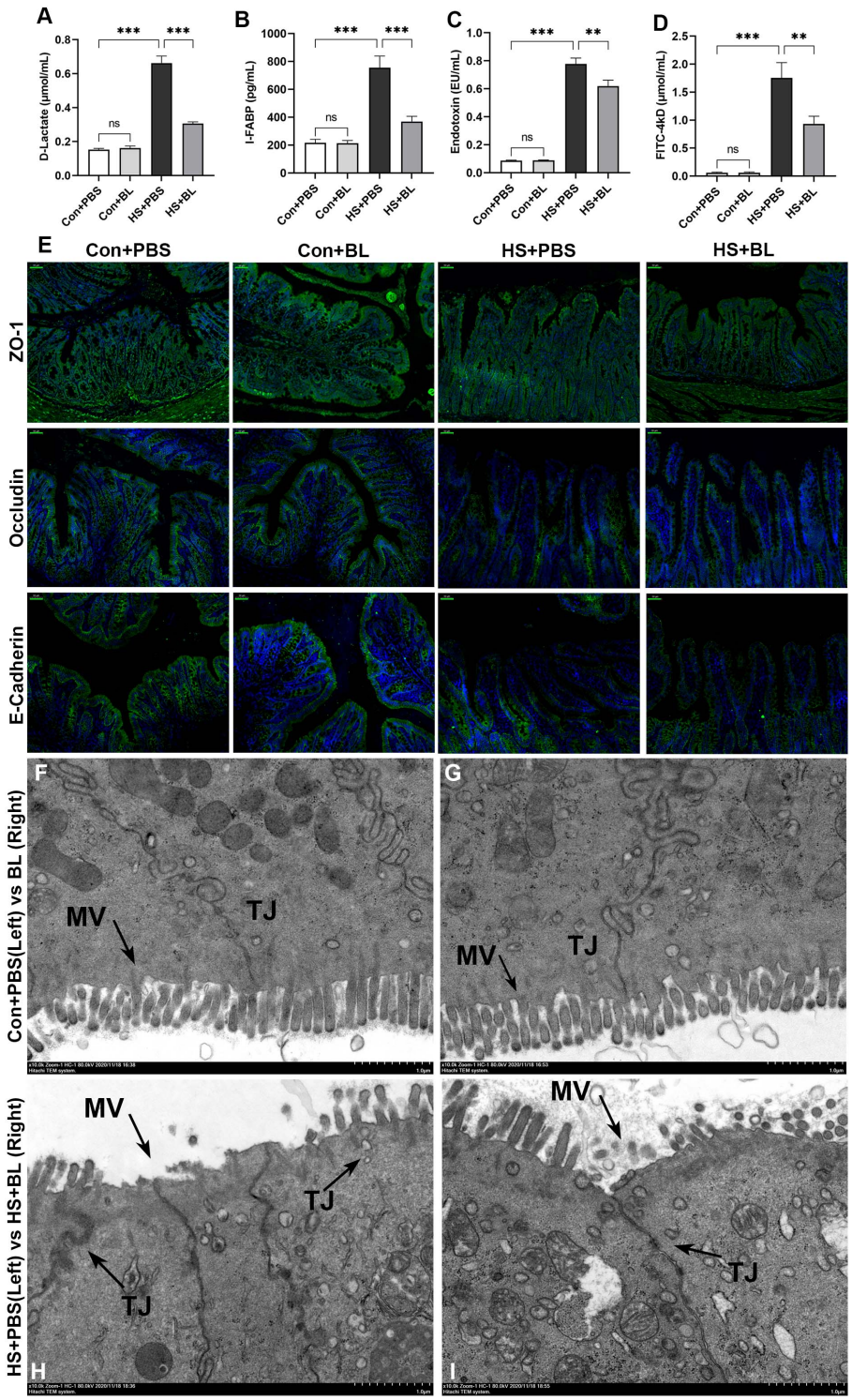

FIGURE 4 | BL pre-administration attenuated intestinal injury and enhanced intestinal barrier function. Plasma D-Lactate **(A)**, I-FABP **(B)**, endotoxin **(C)**, and FD4 **(D)** were detected at 3 h after the HS onset in the Con + PBS, Con + BL, HS + PBS, and HS + BL groups. Concentrations are presented as means ± SEM; $n = 10$ per group. $^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(E)** Representative images of intestinal sections of rats from Con + PBS, Con + BL, HS + PBS, and HS + BL groups. Cell nuclei were stained using DAPI (blue), and ZO-1, occludin, and E-cadherin proteins were stained with corresponding antibodies (green). Scale bar = 50 μm, $n = 6$ per group. **(F–I)** Representative ultrastructural transmission electron photomicrographs of intestinal samples from Con + PBS **(F)**, Con + BL **(G)**, HS + PBS **(H)**, and HS + BL **(I)** show the morphology and sizes of cell nuclei, membrane microvilli (MV, arrows), and tight junctions (TJ, arrows). Scale bar = 1 μm. $n = 3$ per group.

intact TJ structures (**Figure 4I**). Taken together, the present data suggests that BL pre-administration alleviated intestinal injury and improved gut barrier function during and after HS onset.

BL Pre-administration and HS Induction Modulated the Gut Microbiota Structure of Rats

The gut microbiota from rat fecal samples in the four groups were investigated by analysis of the V3–V4 regions of 16S rRNA gene sequences to determine the composition of the gut bacterial community. A total of 835,056 sequences were obtained from 24 fecal samples from the four groups through size filtering, quality control, and chimera removal. Moreover, 831 operational taxonomic units (OTUs) including 280 species, 181 genera, 86 families, 52 orders, 20 classes, and 14 phyla of gut microbes from 24 samples were identified and annotated for subsequent analyses. The refraction curve of each sample from each group at the OTU level indicated that the sequencing data of these samples was sufficient to reflect the overall structure of the gut microbiota (**Supplementary Figures 2C,D**). A Venn diagram at the OTU level showed that, although the four groups shared 566 identical OTUs, each group still contained some unique bacteria (**Figure 5A**), and 15, 20, 15, and 12 unique OTUs were present in the Con + PBS, Con + BL, HS + PBS, and HS + BL groups, respectively. The richness of the microbial communities indicated by the ACE index (**Figure 5B**) and Sobs index (**Figure 5C**), and the diversity of microbial communities indicated by the Shannon index (**Figure 5D**), were reduced significantly at the OTU level by BL pre-administration and HS induction. Furthermore, significant differences (ANOSIM $R = 0.283$, $p = 0.001$) in β-diversity based on the weighted UniFrac distances among the four groups were determined and displayed by PCoA and NMDS at the OTU level (**Figures 5E,F**). The Con + PBS group was clearly separate from the Con + BL and HS + PBS groups, which indicated that BL pre-administration and HS induction altered the gut microbiota. Though the HS + BL group was not clearly separated from the Con + BL and HS + PBS groups, a certain degree of difference was observed among these groups. Moreover, samples from the Con + PBS, Con + BL, and HS + PBS groups were more clustered compared with the HS + BL group.

The gut microbiota community structure was illustrated by Circos and histograms (**Figures 6A–C,E**) at the phylum and genus levels, respectively. Then, multiple-group comparisons were conducted by the Kruskal–Wallis H test at the phylum and genus levels. A total of four phyla and the top 15 genera with significant differences were identified and are shown among the four groups (**Figures 6D,F**). Firmicutes, Bacteroidota, Desulfobacterota, Patescibacteria, and Actinobacteria were the predominant phyla in each group with relative abundances of >1%. *Lactobacillus*, *Monoglobus*, *Romboutsia*, and *Desulfovibrio* combined with 24 other genera were present in abundance in each group at the genus level. Though multiple-group comparisons identified that the average relative abundance in the microbial community composition for each group was notably different, partially distinguished microbiota compositions might be ignored or even not detected. Thus, eight pairwise comparisons of gut microbiota compositions were conducted to detect significant differences between groups subjected to BL or HS at the phylum and genus levels. The mean relative abundances of the five most abundant phyla and genera (non-ranked or unclassified types were excluded) are shown by an extended error bar plot (**Figures 7A,B**) and the significantly different phyla and genera are marked accordingly. In summary, following 7 days of BL pre-administration, BL-treated rats showed an increase in Firmicutes and a decrease in Bacteroidota at the phylum level. Correspondingly, the percent of *Lactobacillus*, which is a probiotic bacterium, was notably increased in BL-treated rats at the genus level. However, after HS induction, HS-treated rats had the same trend for Firmicutes and Bacteroidota on the phylum level with significant difference. Furthermore, the percent of *Romboutsia*, which is distinct and unreported in HS studies previously, was found to be increased in HS rats compared with Con rats. Interestingly, no significant difference was found between the Con + BL and HS + BL groups, which seemed to indicate that gut microbiota from BL-treated rats were not disturbed much by HS induction. To further determine the kinds of specific bacterial taxa in each group, the LEfSe analysis method, which uses LDA coupled with effect size analysis, was applied. Due to the number of differentially abundant phyla and genera, only taxa having p-values of <0.05 and LDA >3.5 are shown in **Figures 7C,D,F**, though **Figure 7E** shows LDA >2.0 due to the subtle difference in this comparison. The results of LEfSe analysis were basically consistent with the pairwise comparisons mentioned above and could identify multilevel species differences. In particular, the increase of *Bacillus* in BL rats, which is the genus of BL, confirms that BL colonized the guts of rats. Correspondingly, no differential species were determined for taxa of LDA >3.5 and only a tiny number of species were found when the LDA >2.0 between the Con + BL and HS + BL groups. These results collectively indicate that BL pre-administration and HS induction each modulated the gut microbiota of rats, and BL helped make the gut microbiota more probiotic and more heat-tolerable as characterized by the notable increase of *Lactobacillus* and the tiny alternations of gut microbiota of rats in the HS + BL group compared with the Con + BL group.

DISCUSSION

As the duration, intensity, and frequency of heat waves resulting from global warming increase, the incidence and mortality of HS are rapidly growing (Perkins-Kirkpatrick and Gibson, 2017). Prevention of HS is highly preferable to treatment. Therefore, alleviating intestinal injury and sustaining the intestinal barrier are conducive to the prevention of HS onset and its subsequent pathological cycle. BL pre-administration is a convenient and safe choice to prevent heat stress (Deng et al., 2012; Shing et al., 2014). However, the preventive effects of BL and its underlying mechanisms have yet to be determined. In this study, we demonstrated that BL pre-administration for 7 days exerted preventive effects on HS in rats, including attenuating

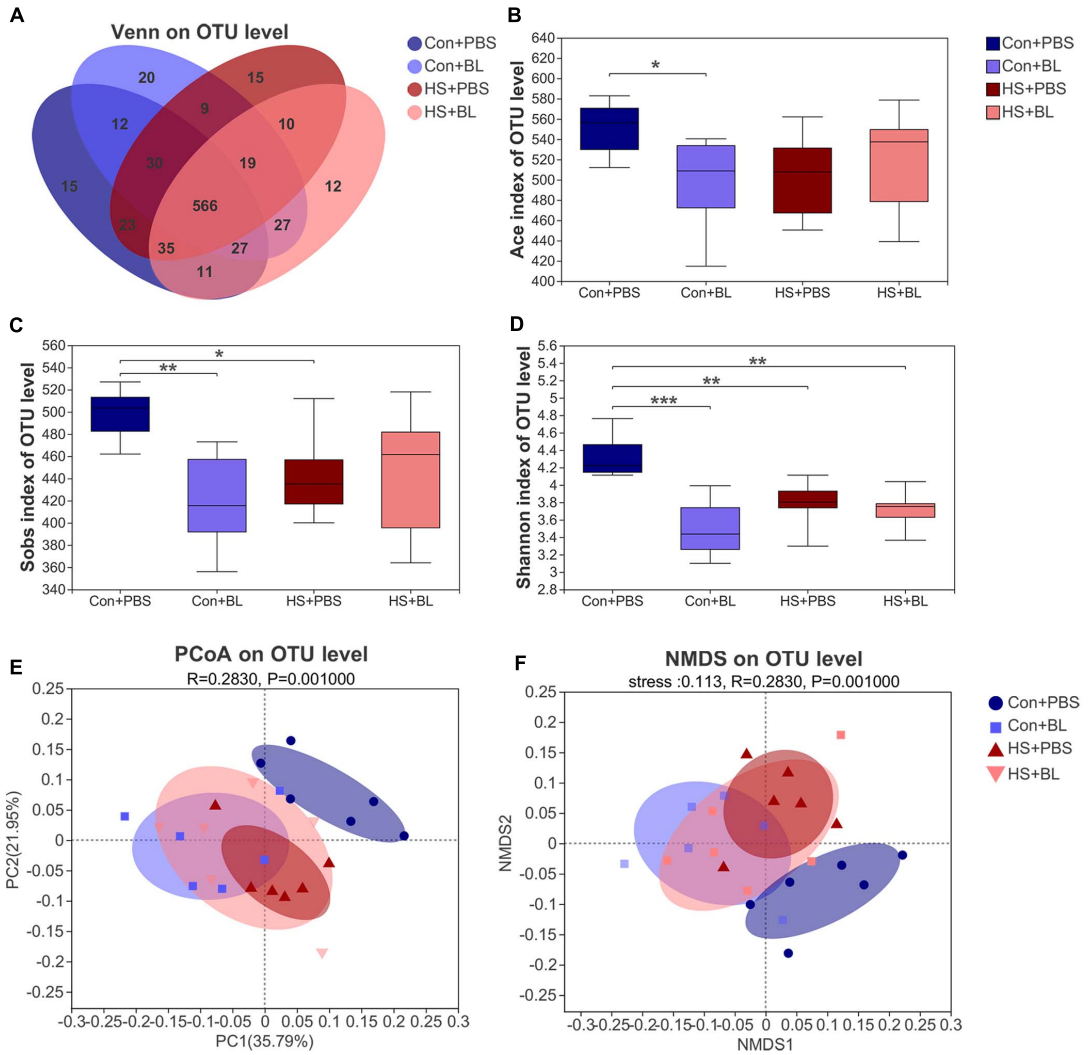

FIGURE 5 | BL pre-administration and HS induction modulated the overall structure of gut microbiota of rats according to analysis of α-diversity and β-diversity. **(A)** Venn diagram of the operational taxonomic unit (OTU) distribution shows unique and shared OTUs between different experimental groups ($n = 4$). The Ace **(B)**, Sobs **(C)**, and Shannon **(D)** indexes of α-diversity analysis at the OTU level were calculated and compared among four groups. **(E,F)** β-Diversity was calculated by multivariate statistical analysis including principal coordinate analysis (PCoA), non-metric multidimensional scaling analysis (NMDS), and analysis of similarities (ANOSIM) at the OTU level based on weighted unifrac analysis, indicating significantly different gut microbiota structures among the four groups (ANOSIM, $R = 0.283$, $P = 0.001$). Data **(B–D)** are presented as a box-and-whisker plot of six rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

hyperthermia, reducing HS-induced death, decreasing systematic inflammatory responses, and minimizing multiple-organ injury, by sustaining intestinal barrier function and modulating gut microbiota under HS.

Heat stroke is a life-threatening disease with severe outcomes if patients cannot be rapidly recognized and effectively treated. Although simple preventive and first-aid measures such as avoiding intense physical work in hot environments, conducting heat acclimation in advance, and medical rapid cooling are recommended (Liu et al., 2020), once HS occurs in a hot environment, rapid progression and limited medical treatment methods often lead to poor outcomes. The present understanding of the pathophysiology of HS indicates the need for mechanism-based intervention measures. In brief, heat exposure (from the environment) and/or exercise (from the body) can cause heat stress, which activates the thermoregulatory response. When excessive heat cannot be dissipated from the body and body temperature homeostasis cannot be maintained, the core body temperature rises continuously and stimulates multiple reflexive adjustments (Epstein and Roberts, 2011). Primarily, heat stress can produce direct thermal injury via heat cytotoxicity, acute-phase response, heat-shock response, and vascular endothelium damage. Secondly, to promote heat dissipation and reduce heat production, skin blood flow is increased to facilitate heat loss and visceral blood flow is decreased to compensate. Prolonged ischemia of visceral organs causes oxidative/nitrosative stress, and the gut, which needs functioning barrier integrity and microbiota to perform, is highly

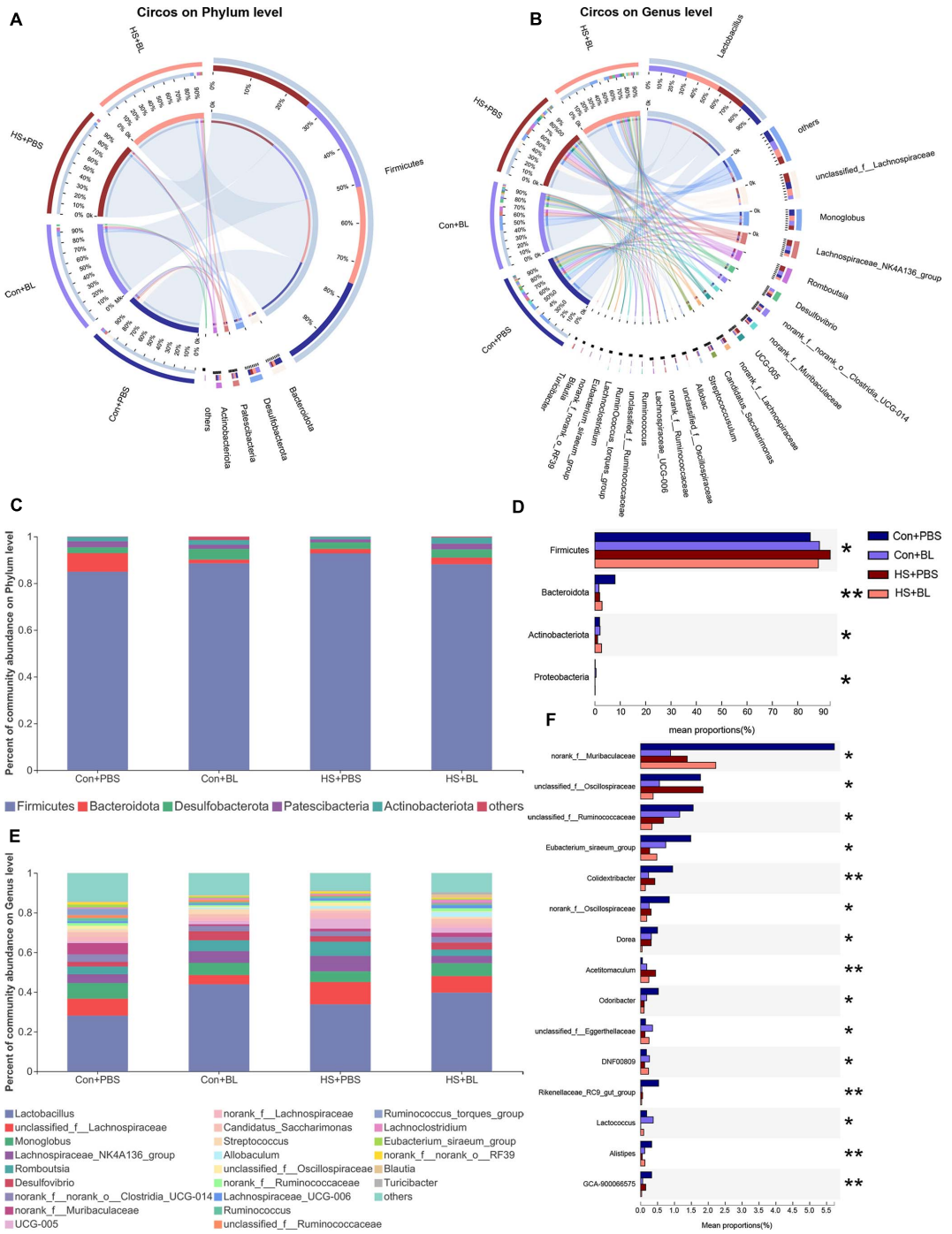

FIGURE 6 | Alterations in the composition of gut microbiota in rats among each group. **(A,B)** Distribution of the microbial community for each group at the phylum and genus levels. The data were visualized by Circos software, and the width of the bars from each phylum or genus represents the relative abundance of that phylum or genus in this group. **(C,D)** Average relative abundances of microbial community composition for each group are shown by bar plots for the phylum level **(C)**, and a total of four phyla were found **(D)** with significant difference among the four groups. **(E,F)** Average relative abundances of microbial community composition for each group are shown by bar plots for the genus level **(E)**, and the top 15 abundant genera **(F)** with significant differences are shown among the four groups. Data are shown as the mean by bar plot analysis. $n = 6$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

sensitive to heat stress and ischemia, so intestinal injury is found in the early stage of HS (Lian et al., 2020). As intestinal injury combined with a dysfunctional barrier and gastrointestinal microbial translocation from the "leaky" gut contributes to the pathophysiology of HS as characterized by SIRS and MODS, the etiology of intestinal barrier dysfunction following heat stress

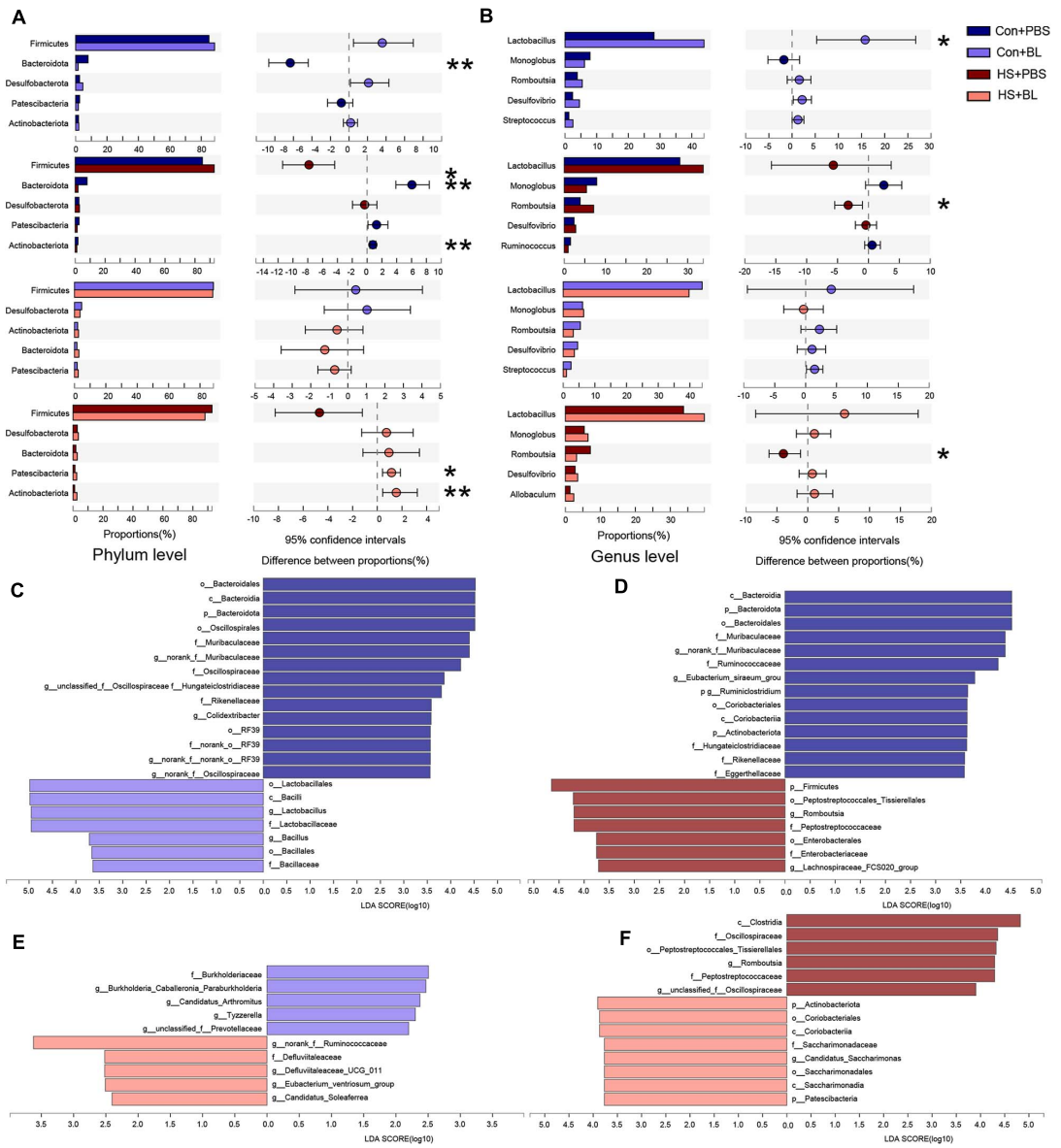

FIGURE 7 | Pairwise comparisons of gut microbiota compositions between each group. **(A,B)** Mean relative abundances of the five most abundant phyla and genera (non-ranked or unclassified types were excluded) are shown by extended error bar plot and were compared between two groups at the phylum and genus levels. **(C–F)** Gut microbiota comparisons from phylum to genus between two groups by linear discriminant analysis effect size (LEfSe) analysis. Linear discriminant analysis (LDA) scores for the differentially abundant bacterial taxa between two groups were calculated by LEfSe to assess the effect size of each differentially abundant taxon. Only taxa having a *p*-value < 0.05 and LDA > 3.5 are shown in **(C,D,F)**, except **(E)** uses LDA > 2.0 due to the subtle difference in this comparison. Only taxa meeting an LDA significance threshold of 2 were included and shown. Enriched taxa in each group are indicated in red or blue. $n = 6$ in each group. $^*P < 0.05$, $^{**}P < 0.01$.

has become a focus of contemporary HS research (Hu et al., 2019). Therefore, countermeasures to maintain intestinal barrier integrity following heat stress are promising.

Probiotics are live microorganisms that confer a health benefit mainly through gut microbiota modulation, increased turnover of enterocytes, and competitive exclusion of pathogens (Liu et al., 2020). Each probiotic strain may generate species-level effects and strain-specific effects that include gut barrier reinforcement, nutrient production, and immunological effects. Recently, the administration of probiotics to sustain intestinal barrier integrity has been reported in several human studies, consistent with many *in vitro* and *in vivo* experiments (Hemert et al., 2013). Furthermore, several studies of poultry production have found that dietary supplementation of probiotics can alleviate heat stress-induced damage (Bron et al., 2012). A probiotics mixture containing *B. licheniformis*, *Bacillus subtilis*, and *Lactobacillus plantarum* has been found to increase growth performance, modulate intestinal microbiota, ameliorate jejunal morphology,

and decrease the intestinal permeability of broilers subjected to heat stress (Song et al., 2014). Short-term administration of *B. subtilis* has been shown to prevent heat stress-induced adverse effects based on a simple rat model subjected to 45°C for 25 min (Sorokulova et al., 2016). Since some bacterial strains can modulate gut microbiota, sustain intestinal barrier integrity, and even fight against heat stress, combined with the advantages of safety, convenience, and accessibility, probiotics are a promising choice for the prevention and protection against HS. *B. licheniformis* (CMCC63516), a Gram-positive, spore-forming bacteria, was isolated from the vagina of a healthy woman in 1986 and has been used as an over-the-counter treatment for gut problems in China for more than 30 years (Li et al., 2019). It has been demonstrated that BL administration can counteract colon inflammation and gut dysbiosis caused by colitis in a mouse model, normalize the ileum microbiota of chickens with necrotic enteritis, and improve growth performance in broilers (Song et al., 2014; Xu et al., 2018). In brief, BL can sustain gut barrier function, modulate gut microbiota, counteract inflammatory responses caused by gut problems, and even improve growth. Moreover, BL, like other bacilli, can tolerate extreme environments and colonize in the gut easily and stably. In view of these advantages, we examined whether BL pre-administration could help prevent HS in rats. Since BL powder was obtained commercially, the probiotic benefits are probably from strain CMCC63516. Not all BL strains are non-pathogenic, and we performed genomic DNA analysis of the BL samples (Joseph et al., 2013; Selvarajan and Mohanasrinivasan, 2015). Sequencing of 16S rRNA and phylogenetics accompanied by BLAST searches of the NCBI database demonstrated that BL samples were of the phylum Firmicutes, class Bacilli, order Bacillales, family Bacillaceae, and genus *Bacillus*, showing more than 96% identity with several *B. licheniformis* strains (**Supplementary Figure 1**). An 807-bp PCR product, amplified with primers for the genome sequence provided by the company for identification, was detected by agarose gel electrophoresis. The amplified sequences separated by gel electrophoresis and sequenced were subjected to BLAST searches in the NCBI database using blast tree view to confirm the species, which provided similar results (**Supplementary Figure 1D**). After identification of BL, intragastric administration with 1×10^8 CFU in 1 ml PBS twice a day at fixed timepoints for 7 days was used according to the literature and preliminary experimental results.

We established a classic HS rat model according to previous studies and made some improvements. We provided a high-temperature and high-humidity environment by building an artificial climate chamber, as we found that the long heating period in some studies might be attributed to the ambient environment without reaching the target parameters according to our experiments. In previous studies, many researchers have measured rectal temperature by inserting a thermometer into the rectum and monitoring Tc by timed measurements. However, the inserted length of the thermometer, operative skills, and stress-induced hyperthermia caused by temperature measurement can affect the Tc of rats significantly (Robertson et al., 2020). Therefore, in this study, an activated temperature monitoring capsule, which can record Tc in conscious and free-moving rats at 5-min intervals and transmit data, was surgically implanted intra-abdominally. We found that all adult male SD rats met the criterion (Tc > 42.7°C) during a 60-min heating period in the hot and humid chamber. So a 60-min heating protocol for HS induction was used in this study. When rats were exposed to heat stress, Tc rose rapidly at the beginning. When Tc was beyond 40°C, it escalated slowly but continuously. At the end of the heating period, the rats in the HS + PBS group all met the HS onset criterion, while only two rats in the HS + BL group met the standard. The maximum Tc in the HS + PBS group reached 43.1°C ± 0.46, which was significantly higher than in the HS + BL group (**Table 1**). Therefore, BL pre-administration attenuated HS-induced hyperthermia. Since uncontrolled hyperthermia in HS was the main cause of death, and this kind of hyperthermia was reduced by BL, the survival rates between HS + PBS and HS + BL groups were compared using a log-rank test. We found that the survival time and survival rate of the HS + BL group were notably improved by BL pre-administration. The present research indicates that systematic inflammatory response and multiple-organ injury are important driving factors of hyperthermia and death. We detected significant increases of biochemical markers of organ injury and serum inflammatory cytokines with visible histopathological injuries in the liver, kidney, lungs, and intestines of HS rats (**Figures 2**, **3**), which is consistent with the pathophysiology of HS (Epstein and Roberts, 2011). This further validated our HS model. We found that BL pre-administration attenuated multiple-organ injury and decreased the levels of serum inflammatory cytokines significantly. Moreover, no evidence was found that BL pre-administration caused damage or inflammation. Taken together, our results preliminarily demonstrated that BL pre-administration prevents HS onset, alleviates HS-induced damage, and increases survival.

As mentioned above, the gut is considered the "motor" of SIRS, MODS, and other pathophysiological alternations in HS (Ogden et al., 2020). Visceral blood flow reduction caused by HS-induced blood redistribution can lead to intestinal ischemia and injury followed by intestinal barrier dysfunction and hyperpermeability. D-Lactate, which is normally maintained at a concentration of only about 0.01 mM, may be elevated in the plasma under various gastrointestinal conditions such as ischemia, short bowel syndrome, appendicitis, and Crohn's disease, making it useful as a biomarker (Levitt and Levitt, 2020). I-FABP, which is solely expressed in the intestine and is released extracellularly after intestinal epithelial injury, can be a suitable early biomarker of intestinal injuries (Voth et al., 2017). Therefore, D-Lactate and I-FABP were chosen to evaluate HS-induced intestinal injury in the early stage. In this study, BL pre-administration showed no harm to the gut, while the two intestinal injury biomarkers were elevated in plasma with a significant difference in HS rats, consistent with H&E staining. As expected, BL pre-administration significantly decreased the levels of D-Lactate and I-FABP, which indicated that intestinal injury was attenuated. Intestinal injury can lead to intestinal barrier dysfunction, which generates a "leaky" gut permitting microorganisms and microbial products (e.g.,

endotoxin, flagellin, and bacterial DNA) to pass through. Of concern, lipopolysaccharides (LPS), also known as endotoxins, are the major component of the outer membranes of Gram-negative bacteria that are a large part of the gut microbiota. LPS can bind pathogen-associated molecular patterns to toll-like receptors (TLR), and TLR activation can initiate the production of many pro-inflammatory cytokines if too much LPS passes through the gut to the blood without effective detoxification (Liu et al., 2012; Guo et al., 2013; Rathinam et al., 2019). Therefore, the FD4 test and endotoxin detection were used to assess intestinal barrier permeability (Xia et al., 2017). Lower FD4 and endotoxin in the HS + BL group were detected than in the HS + PBS group, indicating that gut barrier integrity was sustained successfully by BL.

Tight junctions are dynamic structures with complex architecture that are composed of transmembrane barrier proteins (e.g., claudins, junctional adhesion molecules, occludin, and tricellulin) and cytoplasmic scaffolding proteins (e.g., the ZO family, cingulin, and afadin) (Dokladny et al., 2016). These proteins are directly connected with the intracellular cytoskeleton and linked to regulatory proteins. An injured intestine results in a "leaky" gut with increased intestinal permeability and decreased expression of TJ proteins as well as disrupted TJ structures. A dysfunctional intestinal barrier and disrupted TJ structures have been reported in many studies, which can be examined by immunofluorescence of TJ proteins and TEM of TJ structures (Uerlings et al., 2018; Hu et al., 2019; Koch et al., 2019). In the present study, similar alterations were detected in HS rats, represented as lower expression of TJ proteins ZO-1, occludin, and E-cadherin (**Figure 4E**), and as disrupted TJ structures, enterocytes, and microvilli in TEM images (**Figures 4F–I**). BL pre-administration significantly counteracted these effects and maintained the integrity of the intestinal barrier. Altogether, these results demonstrated that BL pre-administration can alleviate HS-induced intestinal injury and sustain intestinal integrity and function.

Previous studies have demonstrated that the spores of *Bacillus* can germinate, colonize, and act as a probiotic in the gut (Li et al., 2019). Our results indicated that BL pre-administration influenced the richness and diversity of gut microbiota compared with PBS as illustrated in **Figure 5**. However, we found that our BL pre-administration protocol downregulated richness and diversity in contrast to previous studies. We estimated that BL administered for 7 days might inhibit some harmful bacteria more effectively than promote desirable bacteria. According to PCoA and NMDS analysis (**Figure 5**), gut microbiota in the Con + PBS group were clearly distinguished from those in the Con + BL group, which demonstrated that BL pre-administration for 7 days modulated gut microbiota successfully. Surprisingly, gut microbiota in the Con + PBS group were distinguished from HS + PBS, which indicated that heat stress altered the gut microbiota. Despite many researchers having applied probiotics or prebiotics in poultry to help livestock fight heat stress, few have focused on the impact of heat stress on gut microbiota based on an HS model (Armstrong et al., 2018). In this study, the structures of gut microbiota in the HS + PBS and HS + BL groups could not be distinguished clearly, which indicates that the microbiota of BL rats can stay stable even with heat stress. The results from PCA and PLS-DA analyses imply a similar conclusion (**Supplementary Figure 2**).

Firmicutes and Bacteroidota account for more than 90% of the gut microbiota, and there was a significant increase in the ratio of Firmicutes in BL rats with a notable decreased ratio of Bacteroidota. BL belongs to the phylum Firmicutes, and this increase could be attributed to both the colonization of BL in the gut and the elevation of *Lactobacillus*. In fact, from the LEfSe analysis (**Figure 7C**), the proportion of the genus *Bacillus* was notably higher in BL rats than in PBS rats. This clearly demonstrated that BL colonized the guts of rats. BL, which is a Gram-positive and aerobic bacterium, can cause oxygen deprivation and promote the growth of anaerobic bacteria. *Lactobacillus*, which is a genus of Gram-positive, aerotolerant anaerobes or microaerophilic bacteria that belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae, is a probiotic used for centuries (Zhang et al., 2018). Moreover, as shown in **Figure 6F**, *Lactococcus*, a genus of lactic acid bacteria, was also elevated significantly in BL rats and is one of the most important bacteria in the gut (Song et al., 2017). Studies have shown that *Lactobacillus* functions by maintaining the bacterial community in the gut, facilitating the absorption of nutrients and improving body immunity, and can even inhibit the inflammatory response in the gut by enhancing innate and adaptive immunity and defending against intestinal pathogens (Mu et al., 2018; Wang et al., 2018). *Lactococcus* species can produce antimicrobial peptides that have potent antibacterial effects against many Gram-negative bacteria, including *Staphylococcus*, *Listeria*, and *Clostridium* (Field et al., 2015). Altogether, the above results suggest that BL pre-administration might not increase the richness or diversity of gut microbiota but can significantly increase two kinds of important probiotics. Although several studies have investigated the impact of heat stress on gut microbiota, they have mainly focused on poultry production or agricultural animals such as hens, broilers, and pigs. There has been limited research based on rat models investigating HS and gut microbiota alterations.

Patients with HS who are treated in intensive care units may still die, and sepsis in the end-stage happens frequently in many case reports (Graber et al., 1971). Some doctors refer to HS as a sepsis-like disease. As bacteria and endotoxins originating from the gut are key exacerbating factors, we hypothesized that the high morbidity of HS might be related to specific pathogenic gut bacteria. We thus compared gut microbiota between Con + PBS and HS + PBS groups, and the results demonstrated that microbiota between the two groups were notably different (**Figure 5**). An analysis of their compositions showed that *Romboutsia ilealis* was significantly elevated in HS + PBS rats. We also compared the gut microbiota of Con + BL and HS + BL groups, but no significant difference was detected at the phylum or genus levels, and LEfSe analysis did not find any meaningfully different bacteria with LDA > 2.0. These results indicated that the gut microbiota of rats with BL pre-administration maintained stable compositions of gut bacteria after HS induction. Conversely, *Romboutsia* was also significantly elevated in the HS + BL group compared with the HS + BL

group. Therefore, we speculated that *Romboutsia* might play a special role in the gut. *R. ilealis*, which is a Gram-positive obligately anaerobic bacterium and the dominant member of the ileal microbiota, is a strain that can utilize carbohydrates via different and partially redundant pathways and has not been proven harmful so far (Gerritsen et al., 2017, 2018). Limited research has demonstrated that *R. ilealis* is at a higher proportion in the gut microbiota of patients with neurodevelopmental disorders, type 2 diabetes, or dementia (Araos et al., 2018; Kang et al., 2019; Frazier and Leone, 2020). However, some researchers have classified *R. ilealis* as a probiotic listed with *Lactobacillus*, which implies that the elevation of *Lactobacillus* and *Romboutsia* may represent a kind of beneficial alteration of the gut (Wu et al., 2020). Interestingly, we observed a slight increase of the ratio of *Lactobacillus* in HS + PBS rats, though it was not significant. Some researchers have speculated that probiotics can promote the elevation of *Romboutsia*, but our results in BL rats do not support this hypothesis. Healthier rats in the HS + BL group did not show an increase in *Romboutsia*, in contrast to the rats in the HS + PBS group. Therefore, we speculate that the increased ratio of *Romboutsia* during the early stage of HS may represent an important signal indicating HS onset or rapid progression, and it may be used as a biomarker for early diagnosis. Any relationship between *Romboutsia* and subsequent pathology still needs further exploration and verification. Furthermore, in the present study, 16S rRNA sequencing allowed us to study alterations of the composition of the gut microbiota in each group and the potential beneficial effects. However, changes in the metabolites of gut flora should be investigated. Therefore, in future research, we will use two detection methods to uncover the mechanism of the preventive effects. First, untargeted metabolomics detection based on liquid chromatography–mass spectrometry will be conducted to compare metabolomics profiles of gut flora to discover how beneficial bacteria perform. Second, targeted metabolomics detection based on gas chromatography–mass spectrometry will be conducted to find alterations in the levels of short-chain fatty acids (SCFAs), as SCFAs are typical metabolites of gut bacteria that can sustain intestinal integrity and rehabilitate gut function (Chang et al., 2020). We believe that more explicit and distinct mechanistic explanations of this preventive effect will be clarified with the results of our planned experiments. If some potential significant metabolites can be detected, our team will verify their protective effects based on an intestinal epithelial cell barrier model of heat stress.

CONCLUSION

In the present study, BL pre-administration for 7 days showed preventive effects against HS by attenuating hyperthermia, increasing survival rate, and alleviating systematic immune responses and multiple-organ injury. The preventive effects of BL may be mediated by sustaining intestinal barrier integrity and modulating gut microbiota. We demonstrated that BL modulates gut microbiota by upregulating probiotic bacteria strains and inhibiting harmful bacteria. Moreover, *Romboutsia*, a candidate biomarker for HS, was detected. Overall, our results demonstrated that 7 days of BL pre-administration prevented HS onset and alleviate its progression. Further studies of the use of probiotics for the prevention of HS are warranted.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committee of the Navy Medical University.

AUTHOR CONTRIBUTIONS

SX, CL, and JBZ conceived the idea and designed the research. LL, MW, JC, ZX, SKW, XX, SW, CX, JW, JL, and JQZ performed the research and analyzed the data. LL, MW, and JC wrote the manuscript. DL, MTW, JBZ, CL, and SX have taken part in the revision of the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This study was financially supported by Shanghai Military Civilian Integration Industry Development Project (Grant No. 2019-jmrh1-kj52), Shanghai Shenkang City Hospital Emerging Frontier Technology Joint Research Project (Grant No. SHDC12017106), National Natural Science Foundation of China (Grant No. 81703627), and National Key Research and Development Project of China (Grant. 2019YFC0312604).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.630841/full#supplementary-material

Supplementary Figure 1 | Species level identification of BL by genomic DNA analysis. **(A)** The obtained 16S rRNA sequences were subjected to BLAST searches in the NCBI database, and top 10 BLAST hits are shown. **(B)** BLAST hits on the query sequence for BL. **(C)** A BLAST tree view was produced using BLAST pairwise alignments for 16S rRNA of BL (using both neighbor-joining and maximum-likelihood algorithms). **(D)** Electrophoresis of BL samples after PCR amplification with special primers for identification, and BLAST pairwise alignments for the complete sequence of BL. Bar, 0.009 changes per nucleotide position.

Supplementary Figure 2 | Species level identification of BL by 16S r RNA sequencing. **(A,B)** Pan and Core OTU analysis of samples from each group for evaluating whether the sample size for this sequencing is sufficient according to whether the Pan/Core species curve is flat. **(C,D)** The refraction curve of each sample from each group on OTU level indicated that the sequencing data of these samples was sufficient to reflect the overall structure of gut microbiota. **(E)** Analysis of differences in beta-diversity revealed by PCA. **(F–H)** Differences

between groups were tested by ANOSIM/Adonis from weighted unifrac distances at the phylum and genus levels and by PLS-DA. Data in **(G,H)** are presented as a box-and-whiskers plot of six rats per group.

Supplementary Figure 3 | Comparisons of gut microbiota compositions among the four groups. **(A)** Gut microbiota comparisons from phylum to genus among the four groups were analyzed by LEfSe. LDA scores for the bacterial taxa differentially abundant between two groups were calculated by LEfSe to assess the effect size of each differentially abundant taxon. Only taxa having a p-value < 0.05 and LDA > 3.5 are shown in the figure. **(B,C)** Average relative abundances of microbial community compositions for each group are shown by bar plots at the phylum and genus levels. The top 15 abundant phyla and genera are shown in the figure. Data are shown as the mean by bar plot analysis. $n = 6$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Supplementary Figure 4 | Summary of HS pathophysiological alterations that lead to a cascade of events including SIRS, DIC, MODS, and even death. Intestinal injury caused by HS-induced visceral ischemia plays a key role in HS pathogenesis and pathophysiology. Intestinal injury, which is comprised of enterocyte death and tight junction disintegration, results in intestinal barrier dysfunction and triggers gut-derived endotoxemia, which activates subsequent systemic inflammatory response and multiple organ injury.

REFERENCES

Araos, R., Andreatos, N., Ugalde, J., Mitchell, S., Mylonakis, E., D'Agata, E. M. C., et al. (2018). Fecal microbiome among nursing home residents with advanced dementia and clostridium difficile. *Digestive Dis. Sci.* 63, 1525–1531. doi: 10.1007/s10620-018-5030-7

Armstrong, L. E., Lee, E. C., and Armstrong, E. M. (2018). Interactions of gut microbiota, endotoxemia, immune function, and diet in exertional heatstroke. *J. Sports Med.* 2018, 1–33. doi: 10.1155/2018/5724575

Bron, P. A., van Baarlen, P., and Kleerebezem, M. (2012). Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nat. Rev. Microbiol.* 10, 66–78. doi: 10.1038/nrmicro2690

Chang, Y., Chen, Y., Zhou, Q., Wang, C., Chen, L., Di, W., et al. (2020). Short-chain fatty acids accompanying changes in the gut microbiome contribute to the development of hypertension in patients with preeclampsia. *Clin. Sci.* 134, 289–302. doi: 10.1042/CS20191253

Deng, W., Dong, X. F., Tong, J. M., and Zhang, Q. (2012). The probiotic *Bacillus* licheniformis ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poult. Sci.* 91, 575–582. doi: 10.3382/ps.2010-01293

Dokladny, K., Zuhl, M. N., and Moseley, P. L. (2016). Intestinal epithelial barrier function and tight junction proteins with heat and exercise. *J. Appl. Physiol.* 120, 692–701.

Epstein, Y., and Roberts, W. O. (2011). The pathopysiology of heat stroke: an integrative view of the final common pathway. *Scand J. Med. Sci. Sports* 21, 742–748. doi: 10.1111/j.1600-0838.2011.01333.x

Epstein, Y., and Yanovich, R. (2019). Heatstroke. *New Eng. J. Med.* 380, 2449–2459.

Field, D., Gaudin, N., Lyons, F., O'Connor, P. M., Cotter, P. D., Hill, C., et al. (2015). A bioengineered nisin derivative to control biofilms of *Staphylococcus* pseudintermedius. *Plos One* 10:e119684. doi: 10.1371/journal.pone.0119684

Frazier, K., and Leone, V. A. (2020). Microbiota can't keep time in type 2 diabetes. *Cell Host Microbe* 28, 157–159. doi: 10.1016/j.chom.2020.07.015

Geng, Y., Ma, Q., Liu, Y. N., Peng, N., Yuan, F. F., Li, X. G., et al. (2015). Heatstroke induces liver injury via IL-1β and HMGB1-induced pyroptosis. *J. Hepatol.* 63, 622–633.

Gerritsen, J., Hornung, B., Renckens, B., van Hijum, S. A. F. T., Martins Dos Santos, V. A. P., Rijkers, G. T., et al. (2017). Genomic and functional analysis of romboutsia ilealis CRIBT reveals adaptation to the small intestine. *Peer J.* 5:e3698. doi: 10.7717/peerj.3698

Gerritsen, J., Umanets, A., Staneva, I., Hornung, B., Ritari, J., Paulin, L., et al. (2018). Romboutsia hominis sp. Nov., The first human gut-derived representative of the genus Romboutsia, isolated from ileostoma effluent. *Int. J. Syst. Evol. Microbiol.* 68, 3479–3486. doi: 10.1099/ijsem.0.003012

Graber, C. D., Reinhold, R. B., Breman, J. G., Harley, R. A., and Hennigar, G. R. (1971). Fatal heat stroke. Circulating endotoxin and gram-negative sepsis as complications. *Jama* 216:1195.

Guo, S., Al-Sadi, R., Said, H. M., and Ma, T. Y. (2013). Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. *Am. J. Pathol.* 182, 375–387. doi: 10.1016/j.ajpath.2012.10.014

Guo, Y., Gasparrini, A., Armstrong, B. G., Tawatsupa, B., Tobias, A., Lavigne, E., et al. (2017). Heat wave and mortality: a multicountry, multicommunity study. *Environ. Health Perspec.* 125:87006. doi: 10.1289/EHP1026

Helwig, B. G., Ward, J. A., Blaha, M. D., and Leon, L. R. (2012). Effect of intraperitoneal radiotelemetry instrumentation on voluntary wheel running and surgical recovery in mice. *J. Am. Assoc. Lab. Anim. Sci.* 51, 600–608.

Hemert, S. V., Verwer, J., and Schütz, B. (2013). Clinical studies evaluating effects of probiotics on parameters of intestinal barrier function. *Adv. Microbiol.* 03, 212–221. doi: 10.4236/aim.2013.32032

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hu, J., Kang, H., Liu, C., Hu, P., Yang, M., Zhou, F., et al. (2019). Regulatory t cells could improve intestinal barrier dysfunction in heatstroke. *Inflammation* 42, 1228–1238. doi: 10.1007/s10753-019-00983-6

Joseph, B., Dhas, B., Hena, V., and Raj, J. (2013). Bacteriocin from *Bacillus* subtilis as a novel drug against diabetic foot ulcer bacterial pathogens. *Asian Pacific J. Tropical Biomed.* 3, 942–946. doi: 10.1016/S2221-1691(13)60183-5

Kang, Y., Feng, D., Law, H. K., Qu, W., Wu, Y., Zhu, G. H., et al. (2019). Compositional alterations of gut microbiota in children with primary nephrotic syndrome after initial therapy. *BMC Nephrol.* 20:434. doi: 10.1186/s12882-019-1615-4

Koch, F., Thom, U., Albrecht, E., Weikard, R., Nolte, W., Kuhla, B., et al. (2019). Heat stress directly impairs gut integrity and recruits distinct immune cell populations into the bovine intestine. *Proc. Natl. Acad. Sci.* 116, 10333–10338. doi: 10.1073/pnas.1820130116

Lei, K., Li, Y. L., Yu, D. Y., Rajput, I. R., and Li, W. F. (2013). Influence of dietary inclusion of *Bacillus* licheniformis on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. *Poult Sci.* 92, 2389–2395. doi: 10.3382/ps.2012-02686

Levitt, M. D., and Levitt, D. G. (2020). Quantitative evaluation of D-Lactate pathophysiology: New insights into the mechanisms involved and the many areas in need of further investigation. *Clin. Exp. Gastroenterol.* 13, 321–337. doi: 10.2147/CEG.S260600

Li, Y., Liu, M., Zhou, J., Hou, B., Su, X., Liu, Z., et al. (2019). *Bacillus* licheniformis zhengchangsheng(R) attenuates DSS-induced colitis and modulates the gut microbiota in mice. *Benef Microbes* 10, 543–553. doi: 10.3920/BM2018.0122

Lian, P., Braber, S., Garssen, J., Wichers, H. J., Folkerts, G., Fink-Gremmels, J., et al. (2020). Beyond heat stress: intestinal integrity disruption and mechanism-based intervention strategies. *Nutrients* 12:734. doi: 10.3390/nu12030734

Liu, S., Song, J., Mao, H., Zhao, J., and Song, Q. (2020). Expert consensus on the diagnosis and treatment of heat stroke in China. *Mil. Med. Res.* 7:1. doi: 10.1186/s40779-019-0229-2

Liu, X., Li, H., Lu, A., Zhong, Y., Hou, X., Wang, N., et al. (2012). Reduction of intestinal mucosal immune function in heat-stressed rats and bacterial translocation. *Int. J. Hyperthermia* 28, 756–765. doi: 10.3109/02656736.2012.729173

Meehl, G. A., and Tebaldi, C. (2004). More intense, more frequent, and longer lasting heat waves in the 21st century. *Science* 305, 994–997. doi: 10.1126/science.1098704

Mu, Q., Tavella, V. J., and Luo, X. M. (2018). Role of *Lactobacillus* reuteri in human health and diseases. *Front. Microbiol.* 9:757. doi: 10.3389/fmicb.2018.00757

Ogden, H. B., Child, R. B., Fallowfield, J. L., Delves, S. K., Westwood, C. S., Layden, J. D., et al. (2020). The gastrointestinal exertional heat stroke paradigm: pathophysiology, assessment, severity, aetiology and nutritional countermeasures. *Nutrients* 12:537. doi: 10.3390/nu12020537

Perkins-Kirkpatrick, S. E., and Gibson, P. B. (2017). Changes in regional heatwave characteristics as a function of increasing global temperature. *Sci. Rep.* 7, 12212–12256. doi: 10.1038/s41598-017-12520-2

Rathinam, V. A. K., Zhao, Y., and Shao, F. (2019). Innate immunity to intracellular LPS. *Nat. Immunol.* 20, 527–533. doi: 10.1038/s41590-019-0368-3

Robertson, J. K., Mastromonaco, G., and Burness, G. (2020). Evidence that stress-induced changes in surface temperature serve a thermoregulatory function. *J. Exp. Biol.* 223:b213421. doi: 10.1242/jeb.213421

Selvarajan, E., and Mohanasrinivasan, V. (2015). Kinetic studies on exploring lactose hydrolysis potential of β galactosidase extracted from *Lactobacillus* plantarum HF571129. *J. Food Sci. Technol.* 52, 6206–6217. doi: 10.1007/s13197-015-1729-z

Shing, C. M., Peake, J. M., Lim, C. L., Briskey, D., Walsh, N. P., Fortes, M. B., et al. (2014). Effects of probiotics supplementation on gastrointestinal permeability, inflammation and exercise performance in the heat. *Eur. J. Appl. Physiol.* 114, 93–103. doi: 10.1007/s00421-013-2748-y

Song, A. A., In, L. L. A., Lim, S. H. E., and Rahim, R. A. (2017). A review on *Lactococcus lactis*: from food to factory. *Microbial. Cell. Fact.* 16:55. doi: 10.1186/s12934-017-0669-x

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., et al. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poult. Sci.* 93, 581–588. doi: 10.3382/ps.2013-03455

Sorokulova, I., Globa, L., Pustovyy, O., and Vodyanoy, V. (2016). Prevention of heat stress adverse effects in rats by *Bacillus* subtilis strain. *J. Vis. Exp.* 113:e54122. doi: 10.3791/54122

Uerlings, J., Song, Z. G., Hu, X. Y., Wang, S. K., Lin, H., Buyse, J., et al. (2018). Heat exposure affects jejunal tight junction remodeling independently of adenosine monophosphate-activated protein kinase in 9-day-old broiler chicks. *Poult. Sci.* 97, 3681–3690. doi: 10.3382/ps/pey229

Voth, M., Duchene, M., Auner, B., Lustenberger, T., Relja, B., Marzi, I., et al. (2017). I-FABP is a novel marker for the detection of intestinal injury in severely injured trauma patients. *World J. Surg.* 41, 3120–3127. doi: 10.1007/s00268-017-4124-2

Wang, J., Ji, H., Wang, S., Liu, H., Zhang, W., Zhang, D., et al. (2018). Probiotic lactobacillus plantarum promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Front. Microbiol.* 9:1953. doi: 10.3389/fmicb.2018.01953

Wang, L., Tang, L., Feng, Y., Zhao, S., Han, M., Zhang, C., et al. (2020). A purified membrane protein from Akkermansia muciniphila or the pasteurised bacterium blunts colitis associated tumourigenesis by modulation of CD8+ T cells in mice. *Gut* 69, 1988–1997. doi: 10.1136/gutjnl-2019-320105

Wu, Y., Zhang, X., Han, D., Ye, H., Tao, S., Pi, Y., et al. (2020). Short administration of combined prebiotics improved microbial colonization, gut barrier, and growth performance of neonatal piglets. *ACS Omega* 5, 20506–20516. doi: 10.1021/acsomega.0c02667

Xia, Z. N., Zong, Y., Zhang, Z. T., Chen, J. K., Ma, X. J., Liu, Y. G., et al. (2017). Dexmedetomidine protects against multi-organ dysfunction induced by heatstroke via sustaining the intestinal integrity. *Shock* 48, 260–269. doi: 10.1097/SHK.0000000000000826

Xu, S., Lin, Y., Zeng, D., Zhou, M., Zeng, Y., Wang, H., et al. (2018). *Bacillus* licheniformis normalize the ileum microbiota of chickens infected with necrotic enteritis. *Sci. Rep.* 8:1744. doi: 10.1038/s41598-018-20059-z

Ye, N., Yu, T., Guo, H., and Li, J. (2019). Intestinal injury in heat stroke. *J. Emerg. Med.* 57, 791–797. doi: 10.1016/j.jemermed.2019.08.033

Zhang, Z., Lv, J., Pan, L., and Zhang, Y. (2018). Roles and applications of probiotic *Lactobacillus* strains. *Appl. Microbiol. Biotechnol.* 102, 8135–8143. doi: 10.1007/s00253-018-9217-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 29 April 2021
doi: 10.3389/fmicb.2021.659465

Regional Diets Targeting Gut Microbial Dynamics to Support Prolonged Healthspan

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In the last 150 years, we have seen a significant increase in average life expectancy, associated with a shift from infectious to non-communicable diseases. The rising incidence of these diseases, for which age is often the largest risk factor, highlights the need for contemporary societies to improve healthy ageing for their growing silver generations. As ageing is an inevitable, non-reversing and highly individualised process, we need to better understand how non-genetic factors like diet choices and commensal gut microbes can modulate the biology of ageing. In this review, we discuss how geographical and ethnic variations influence habitual dietary patterns, nutrient structure, and gut microbial profiles with potential impact on the human healthspan. Several gut microbial genera have been associated with healthy elderly populations but are highly variable across populations. It seems unlikely that a universal pro-longevity gut microbiome exists. Rather, the optimal microbiome appears to be conditional on the microbial functionality acting on regional- and ethnicity-specific trends driven by cultural food context. We also highlight dietary and microbial factors that have been observed to elicit individual and clustered biological responses. Finally, we identify next generation avenues to modify otherwise fixed host functions and the individual ageing trajectory by manipulating the malleable gut microbiome with regionally adapted, personalised food intervention regimens targeted at prolonging human healthspan.

Keywords: gut microbiome, gut microbiota, healthy ageing, prolonged healthspan, personalised diet, bioactive compounds, phytonutrients

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *27 January 2021*
Accepted: *29 March 2021*
Published: *29 April 2021*

Citation:
Low DY, Hejndorf S, Tharmabalan RT, Poppema S and Pettersson S (2021) Regional Diets Targeting Gut Microbial Dynamics to Support Prolonged Healthspan. Front. Microbiol. 12:659465. doi: 10.3389/fmicb.2021.659465

AGEING IS A VARIABLE AND PERSONALISED PROCESS

While ageing is an inherent genetically determined biological process, the chronological outcome of an individual's lifespan is extraordinarily variable. Remarkably, so-called "blue zones" have been identified around the world where the proportion of centenarians (i.e., people who are 100 years old or more) is significantly higher than in neighbouring communities (Buettner and Skemp, 2016), but there are no validated biological explanations for these age-privileged cultural hotspots. At present, the global centenarian population is estimated to be between 500,000 and 600,000 (Robine and Cubaynes, 2017).

Ageing is associated with life-long accumulation of molecular damage that results in cellular deterioration and impairment in organ functionality and crosstalk, ultimately leading to collapse of body physiology and death (Melzer et al., 2020). The cellular and molecular damage that drive the ageing process may arise from genetic, environmental, and lifestyle factors. The heritability of lifespan is estimated in genome-wide association studies (GWAS) and twin studies to be ∼25% (Herskind et al., 1996), implying that up to 75% of our lifespan is determined by environmental and/or lifestyle factors.

However, a long chronological life may not be equivalent to a long healthspan (i.e., number of years spent in good health). While some estimates of genetic contribution to healthspan have been proposed (Ruby et al., 2018), we are in the early stages of understanding the factors that determine the onset and occurrence of age-related non-communicable diseases (e.g., cancers, neurodegeneration, osteoporosis, fractures, and cardiovascular diseases). Current estimates of heritability of non-communicable diseases average 40% but vary greatly from <10% in Parkinson's disease to >60% in osteoporosis (Steves et al., 2012). This suggests that modifiable environmental and lifestyle factors (e.g., diet, gut microbiota; **Figure 1**) represent an avenue to prolong healthspan.

In recent decades, we have seen remarkable progress in understanding how molecular pathways are linked to the ageing process, leading to the conceptualisation of nine molecular "Hallmarks of Ageing" (López-Otín et al., 2013), and spurring research into modifiable or druggable molecular targets within each hallmark (Campisi et al., 2019). Despite sharing common hallmarks, the ageing process is still highly individualised as evident by the increased variability in organ function and inter-organ crosstalk (Steves et al., 2012). For example, Ahadi et al. (2020) characterised the variability of ageing with 184 individual molecular markers, which can be grouped into four partially overlapping domains of ageing or ageotypes: immunological, metabolic, hepatic, and renal. They observed that individuals tended to age asymmetrically, displaying different rates of ageing between the domains (Ahadi et al., 2020). Hence, to achieve a comprehensive and clinically applicable understanding of ageing, insights into common molecular principles of ageing must be accompanied by a decipherment of variability and individuality of the ageing process as well as underlying and modifiable factors behind this variability (**Figure 2**). The imminent need to address how environmental and lifestyle factors drive the individuality of the ageing process is therefore highly warranted and certainly within the vision of the twenty-first century of precision medicine.

FIGURE 1 | An individual's healthspan is determined by inter-organ crosstalk of genetic, and modifiable environmental and lifestyle events that may be illustrated as a characteristic fingerprint, which influences body physiology, metabolism, and excretion.

DYNAMIC GUT MICROBIOME AS AN ACCESSIBLE TARGET FOR HEALTHSPAN INTERVENTIONS

Increasingly, gut microbes and their plethora of genes and secreted molecules are being recognised as an integral part of the holobiont (**Figure 3**), where the human host and prokaryotic microbial counterparts coexist to mutually benefit and function as a meta-organism (Byndloss and Bäumler, 2018). While the human genome is fixed at birth, the gut microbiome is dynamic, characterised by a rapid cellular turnover (average of 5 days, in the human host) (Sender and Milo, 2021) and can be considered superior in its plasticity to transcriptionally respond to dietary interventions as compared to host eukaryotic cells (**Figure 3**). This highlights the potential to target the gut microbiota to alleviate, for example, accelerated ageing by dietary interventions. The observation that gut microbes display considerable changes in composition (and by extension, their functions as a community) in many age-associated non-communicable diseases (Byndloss and Bäumler, 2018) allows for prospective dietary interventions to be applied in situations where impairments in microbe-microbe functions and/or dysfunctional microbe-host interactions are driving the disease. It is tempting to speculate that the spectrum of symptoms associated with accelerated ageing may in part be reversed or mitigated due to the dynamic nature of the gut microbiome.

Accumulating evidence points to diet as a central determinant of gut microbial composition. A study into South East Asian populations of various ethnic origins showed that diet overruled ethnicity, lifestyle, and environmental factors in determining the gut microbial configuration (Khine et al., 2019), confirming an Israeli cohort study, which found no significant association between genetic ancestry and microbial composition (Rothschild et al., 2018). In contrast, the Dutch HELIUS study identified ethnicity as a strong determinant of microbial composition [main operational taxonomic units (OTU) characterised as *Prevotella* in Moroccans, Turks, and Ghanaians, *Bacteroides* in African Surinamese and South-Asian Surinamese, and *Clostridales* in the Dutch] (Deschasaux et al., 2018). However, the same study also showed that there was a strong correlation between ethnicity and dietary pattern, suggesting that diet might account for inter-ethnic differences (Deschasaux et al., 2018). Additionally, ethnicity comprises other aspects such as cultural habits, socioeconomic status, health care, antibiotics use, and early-life environment, which may all contribute to shape the gut

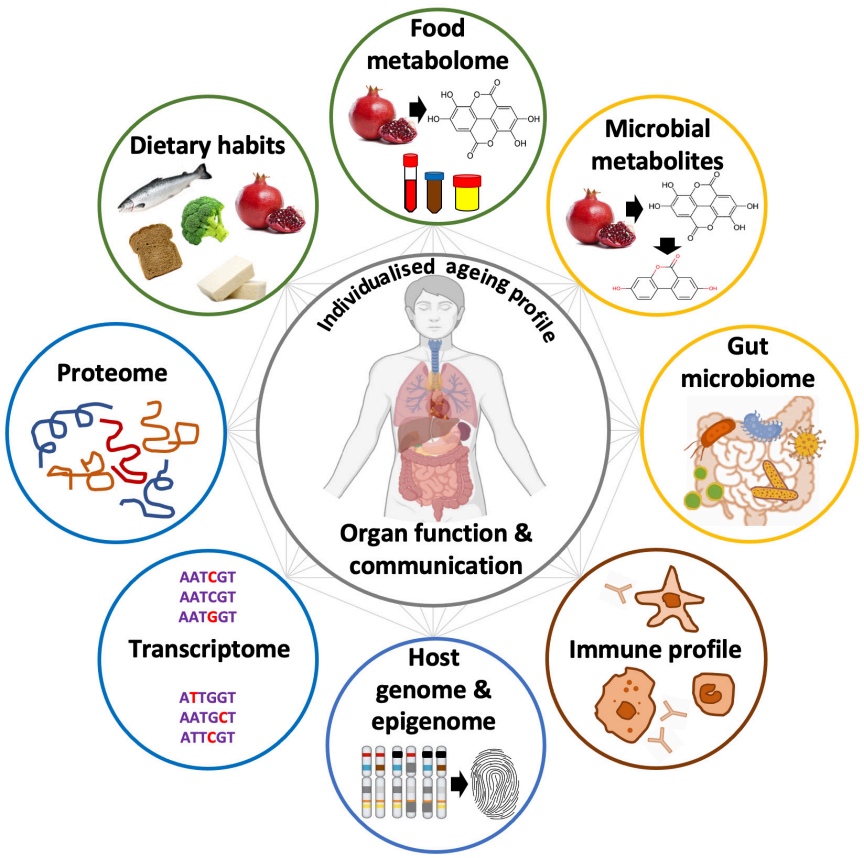

FIGURE 2 | Life-long accumulation of damage to body functions and organ systems leads to a discrepancy between chronological and biological ageing. Further, these individual nodes and inter-organ crosstalk may contribute to variability of an individual's ageing process.

microbiome in a specific fashion by determining nutrient availability and xenobiotic exposure. Furthermore, differences in early-life colonisation trajectories by maternal microbes may shape organ-to-organ communication, immune system development, maturation of epithelial linings, and tuning of brain development and function.

THE DIET-MICROBIOME AXIS IS IMPLICATED IN HEALTHY AGEING

Dietary pattern and gut microbial composition correlate with longevity and markers of healthy ageing. Simultaneously, the dynamic interaction between diet and microbes is interwoven: diet regulates the phylogenetic structure and biological activity of the gut microbes, whereas the microbes, in turn, regulate the presence and/or bioactivity of food molecules through their metabolism and biotransformation (Rowland et al., 2018). Hence, understanding the relationship between diet, gut microbes, diet-gut microbe interactions, and host organ function is of utmost importance if we aim to intervene in the individualised ageing process to improve health and healthspan in the ageing population through personalised nutritional interventions.

Gut Microbiome and Longevity

Centenarians, who have escaped or survived lethal diseases earlier in life, may be considered a spontaneous model of healthy ageing. The gut microbial composition of centenarians has consistently been reported to differ in phylogenetic composition from that of younger people. Interestingly, within centenarian populations, species have been reported to display regional characteristics, further supporting that environmental and/or lifestyle factors including the diet, shape microbial composition (**Figure 4**). For example, in an Italian cohort, the centenarian microbiome was found to be dominated by the same two microbial families as in the other age groups (<75 years old) of the population, namely *Veillonellaceae* and *Ruminococcaceae* (*Firmicutes* phylum), but was specifically enriched in the genera *Akkermansia*, *Bifidobacterium*, and *Christensenella* (Biagi et al., 2016). In contrast, the Chinese Hainan Centenarian Cohort was dominated by *Bacteroides* (*Bacteroidetes* phylum) and *Escherichia* (*Proteobacteria* phylum) (Luan et al., 2020). Long-term elderly care residents in the Irish ELDERMET Cohort also had a gut microbiome dominated by *Bacteroidetes* (Claesson et al., 2012). Importantly, although the aggregate faecal microbiome in ELDERMET was dominated by *Bacteroidetes*, the residents showed extraordinary inter-individual variation with 3–92%

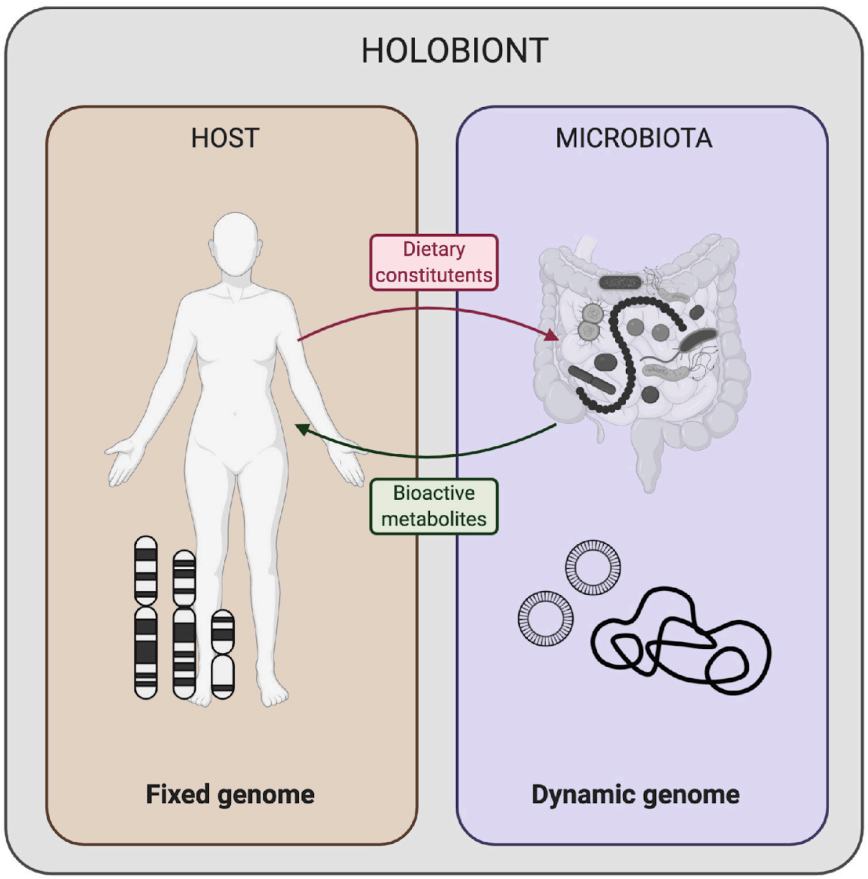

FIGURE 3 | The holobiont genome comprise of the human host genome (fixed at conception with limited accumulation of somatic mutations throughout life) and dynamic microbiome (amenable to change following dietary variations). Dietary intake and habits of the human host can determine the genomic configuration and transcriptional activity of the commensal gut microbiota that break down ingested foods to produce bioactive metabolites with a wide range of effects on the human host. Illustration was created with BioRender.com.

Bacteroidetes and 7–94% *Firmicutes*, hinting at a long-term effect of their dietary habits (Claesson et al., 2012).

The results of the Italian study are also in contrast to those of another Chinese Centenarian Cohort from the Guangxi region, who harboured significantly higher abundance of the genera *Escherichia* and *Roseburia*, and reduced abundance of *Akkermansia*, *Lactobacillus*, *Faecalibacterium*, *Parabacteroides*, *Butyricimonas*, *Coprococcus*, *Megamonas*, *Mitsuokella*, and *Sutterella* (Fang et al., 2015). A Korean centenarian study found trends similar to both Italian and Guangxi Chinese centenarians, with higher abundance of *Akkermansia and Christensenella*, and *Escherichia*, respectively. They also displayed increased abundance of *Clostridium* and *Collinsella*, and reduced abundance of *Faecalibacterium* and *Prevotella* compared to the general population (Bong-Soo et al., 2019).

At present, we do not have a good understanding to explain these geographical variations in the centenarian gut microbial composition or to unequivocally answer if there are certain microbial species globally associated with longevity. In the reviewed studies (**Supplementary Table 1**), some microbial genera associated with healthy elderly populations include *Roseburia*, *Escherichia*, *Akkermansia*, *Christensenella*, *Bifidobacterium*, and *Clostridium*, but they are all highly variable across populations. Based on these cross-sectional observations, it seems unlikely that a universal pro-longevity gut microbiome exists. Rather, the optimal microbiome for healthspan appears to be conditional on the microbial functionality acting on regional- and ethnicity-specific trends driven by cultural food context (**Supplementary Table 1**). Furthermore, because lifespan is influenced by exposures throughout life and the microbiome tends to change over time, the gut microbiome snapshot of centenarians might not capture all the microbial factors contributing to their long life leading up to the time of measurement. For example, Wilmanski et al. (2020) recently reported that the "uniqueness" of the gut microbiome, which correlates with health parameters, starts significantly increasing from the age window of 40–50 years and with each subsequent decade. Asserting that the centenarian microbiome represents a pro-longevity microbial configuration would require individually tracked longitudinal studies of pre-centenarian cohorts.

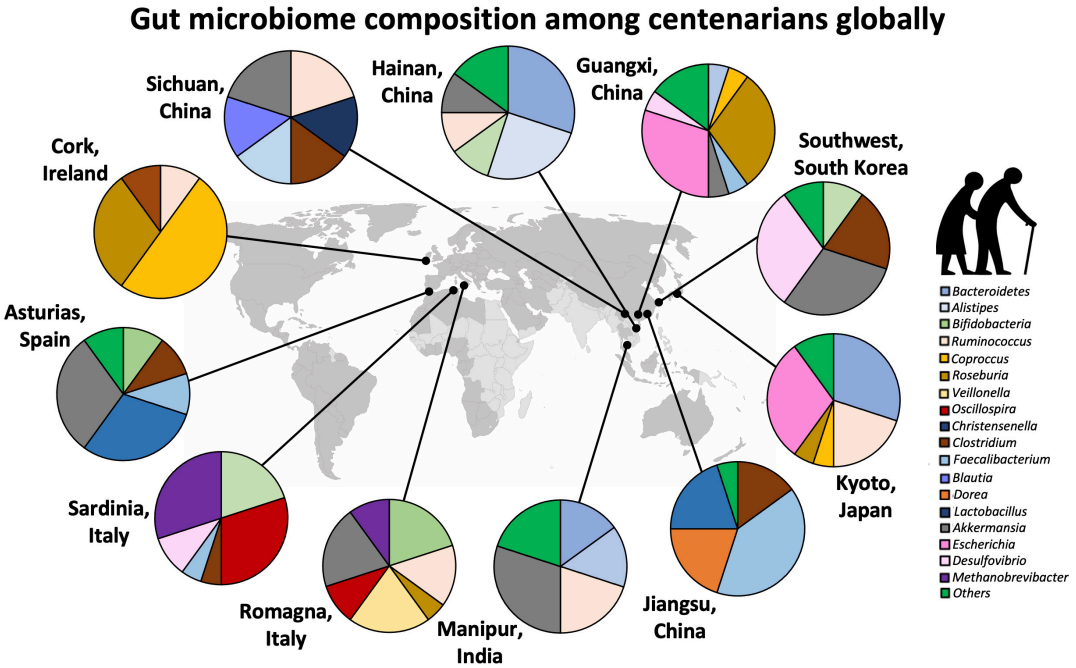

FIGURE 4 | Centenarians harbour distinct gut microbial species and community structures unique to the region, suggesting that microbial functional and biochemical properties are pivotal to healthy ageing, rather than the phylogenetic composition. Data adapted from Claesson et al. (2012), Fang et al. (2015), Biagi et al. (2016), Kong et al. (2016), Bian et al. (2017); Bong-Soo et al. (2019), Naito et al. (2019), Salazar et al. (2019), Tuikhar et al. (2019), Wu et al. (2019), and Luan et al. (2020).

Additionally, the phylogenetic composition of the gut microbiome might not accurately represent the functional output as species across genera are often able to perform similar metabolic processes. It is of interest for future studies to complement phylogenetic analysis with metabolomics and proteomics of the host blood and faecal material. For example, the abundance of microbial tryptophan synthase (TrpB) and tryptophanase (TnaA) responsible for synthesising tryptophan and indole, respectively, was reduced in a Spanish cohort of elderly (65–85 years old) compared to infants (2–5 years old) and young adults (24–45 years old) (Ruiz-Ruiz et al., 2020). Homologues of TnaA were previously identified in >85 species in a variety of genera including *Escherichia*, *Shigella*, *Porphyromonas*, *Clostridium*, *Enterobacter*, etc. (Lee and Lee, 2009).

Diet and Longevity

In the Western world, the Mediterranean Diet (MedDiet) is widely regarded as a "healthy diet" and adherence to this diet or diets with similar macronutrients content has been associated with longevity and reduced risks of age-related non-communicable diseases in culturally diverse populations (Roman et al., 2008). There is no consensus on the exact MedDiet structure, but it is characterised by high consumption of plant-based foods rich in whole grains, vegetables, fruits, nuts, legumes, and olive oil, followed by moderate consumption of fish, dairy products, and low consumption of red meat.

However, not all longevity regions exhibit adherence to the MedDiet structure. For instance, centenarians from Hainan, a region recognised by the International Expert Committee of Population Aging and Longevity as a World Longevity Island for its highest percentage of centenarians (18.75/100,000) in China, scored only 7.7 (±1.9) out of 18 on the MEDI-LITE adherence score, significantly lower than their Italian counterparts (Luan et al., 2020). This suggests that although the MedDiet pattern seems beneficial across genetic groups and cultures, some regions may benefit from developing dietary guidelines better suited to their geographical and cultural climates. Additional studies evaluating the effects of nutritional intervention on diseases, ageing, and longevity must consider geographical variation.

REGIONAL DIETARY PATTERNS AND ASSOCIATED GUT MICROBIOME TRENDS

Dietary patterns, stemming from variations in culture, beliefs, and availability of food across seasons, are evidently different regionally and an important determinant driving gut microbial diversity and richness. Generally, shifting from a low-fat, high-fibre ("healthy") diet to a high-fat, high-sugar, high-protein, low-fibre ("unhealthy") diet leads to decreased α-diversity (i.e., intra-individual microbial richness), increased β-diversity (i.e., inter-individual microbial diversity), and decreased abundance of species (e.g., *Prevotella* and *Treponema*) (De Filippo et al., 2010). Furthermore, food additives commonly used in ultra-processed foods (e.g., emulsifiers, artificial sweeteners, salt) and

Maillard reaction products (e.g., pyrazines and furans that are formed during thermal processing) were recently demonstrated to decrease α-diversity in the *Milieu Intérieur* study, while increasing the *Firmicutes:Bacteroidetes* (*F:B*) ratio (Partula et al., 2019), which has been previously associated with obesity and cardiovascular diseases.

In Asia, populations typically consume a high-starch diet based on rice or noodle, different to the Western diet rich in animal meat and processed foods or to the Nordic diet rich in whole grain cereals, fatty fish, berries and root vegetables. A carbohydrate-rich diet was shown to select for the beneficial *Bifidobacterium* genus in Indian and Chinese living in Singapore (Jain et al., 2018), a small island state (5.8 million population, land area 726 km^2) in the Straits of Malacca containing diverse ethnic populations. High *Bifidobacterium* abundance was similarly observed in Asian children living in China, Taiwan, Japan, Indonesia and Thailand (Nakayama et al., 2015), and Japanese adults (Nishijima et al., 2016) as expected based on this genus' higher expression of glycoside hydrolases for degrading starch relative to other intestinal microbes. Other genera, significantly more represented in Singaporean Indians compared to Singaporean Chinese were *Bacteroidetes* (4-times higher), *Prevotella* (21-times higher), *Megasphaera*, *Catenibacterium*, *Lactobacillus*, *Mitsuokella*, *Carnobacterium*, and *Lachnospira* (Fukkoshi et al., 2015), pointing to a gut microbe determining role of dietary components characteristic of Indian plant-based diet- heavily spiced and curried foods, ghee, lentils and coconut milk. For example, basmati rice, commonly used in Indian cuisine, has a lower glycaemic index and higher amylose-amylopectin ratio compared to other medium- or long-grain rice (Kaur et al., 2014). In contrast, the Singaporean Chinese diet is characterised by noodles, white bread, animal protein and fat, seafood, soy-based products, and heavily sweetened beverages.

Lee et al. (2017) found that globally, populations with *Prevotella*-containing gut microbiota, e.g., Indonesian, Thai, Korean, and African, share a similarity in reduced meat consumption compared to populations harbouring a higher proportion of *Bacteroides* and *Bifidobacterium*. Likewise, significant associations were detected between vegetable-based diets and increased abundance of *Prevotella* and fibre-degrading *Firmicutes* in an Italian cohort as well as increased levels of faecal short chain fatty acids (SCFA) (De Filippis et al., 2016). Interestingly, a recent paper showed that the gut microbiome of vegetarians and vegans had developed selective responses to plant-based diets rich in slowly digestible and complex carbohydrates, including increased cell motility (e.g., flagellin) to physically access nutrients, increased catalytic activities for carbohydrate and food proteins as well as the synthesis/release of bioactive compounds (De Angelis et al., 2020). In a separate study, the gut microbial composition of people with a high MedDiet adherence was found to be phylogenetically diverse, including broadly anaerobic fermenters, and more niche- and subject-specific biochemical specialists as well as major dietary fibre metabolisers (e.g., *Faecalibacterium prausnitzii*, *Eubacterium eligens*, and *Bacteroides cellulosilyticus*) (Wang et al., 2020). Their gut microbiomes were enriched for bacterial metabolism of plant-derived polysaccharide degradation, SCFA production, and secondary bile acid biosynthesis (Wang et al., 2020).

The gut microbiota of Southern Chinese population in two urbanised Malaysian (32 million population, land area 330,000 km^2) regions, Penang City (west coast) and Kelantan City (east coast), was abundant in *Bifidobacterium* and *Collinsella*, both positively correlating with refined sugar-enriched foods (Khine et al., 2019). Additionally, *Collinsella* was positively correlated with fruits and curried foods but negatively correlated with Southeast Asian vegetables, while *Bacteroides*, *Fecalibacterium and Bifidobacterium* were negatively correlated with caffeinated drinks, curried and oily foods (Khine et al., 2019). The same study did not find significant differences in microbial composition between ethnicities living in the same location. This is not surprising as Malaysian cities are populated by Malay and Chinese ethnicities, and most residents are thus exposed to and share a fusion of Malay and Chinese food cultures, e.g., Peranakan (or Nyonya) food- a blend of Chinese ingredients with distinct Malay spices or cooking methods. These dishes are pungent and often use coconut milk and galangal. Peranakans have settled mainly in Penang or Malacca with slight variations to their cuisine (Chung, 2019). Nevertheless, preferred foods are a personal choice and may explain the segregation of microbial composition between clusters within a population or city.

Some Malaysian regions are populated by forest-dwelling hunter-gatherer tribes, also known as the Orang Asli. In a study comparing the effects of ethnicity and socioeconomic status on gut microbiota profiles, pre-adolescents of Orang Asli were observed to possess richer microbial diversity compared to urbanised Malay and Chinese cohorts (Chong et al., 2015). *Ruminococcaceae* and a *Spirochaetes*-related 16S rRNA gene signature were enriched in the former gut microbial profiles, which have been previously associated with the breakdown of fibre-rich food (Chong et al., 2015). The *F:B* ratio in Orang Asli was 6 times lower than other Malaysian and Myanmarese populations (Chua et al., 2019), and can be linked to their primarily plant-based diet, reflecting their horticultural and hunting lifestyle. It is worth noting that the oral microbial composition and functional profiles between Orang Asli sub-groups differ depending on location and gender (Yeo et al., 2019). Extensive gender-specific variation in their gut microbial composition can be observed, likely due to food taboos, where women and children are restricted against the intake of certain foods (e.g., game animals, jackfruit, coconut, and "ikan kelah") while men are allowed to consume a wider variety of wild and game animals.

Another example of regional dietary influence is the higher prevalence of porphyranase- and agarase-encoding genes in the gut microbiome of Japanese and Chinese populations than in North American and European populations (Hehemann et al., 2010; Pudlo et al., 2020). These genes encode proteins that break down complex glycosidic linkages of porphyrapolysaccharides. They were originally reported to have transferred from *Zobellia galactanivorans* derived from Nori red algae (a significant portion of the Japanese

diet) to *Bacteroides plebeius* while passing through the gastrointestinal tract (Hehemann et al., 2010). *B. plebeius* was found amongst the top five species in the Singapore elderly BAMMBE cohort; its presence did not correlate with the low consumption of sushi in this cohort but likely reflects the higher level functionality of this genera of bacteria, which is responsible for producing Carbohydrate Active Enzymes (CAZymes) involved in synthesis, recognition, or metabolism of complex carbohydrates (i.e., oligosaccharides, polysaccharides, glycoconjugates). Interestingly, high levels of genes encoding for plant-degrading enzymes were found in the modern Hadza hunter-gatherer tribes of Tanzania, whose diet consists mainly of plant-based fibre-rich and unprocessed foods, whereas genes encoding enzymes targeted towards animal and mucin degradation were enriched in Americans (De Filippo et al., 2010; Fragiadakis et al., 2019), aligning with industrialisation and urbanisation. Alike the Orang Asli, *Spirochaetaceae* was similarly found in hunter-gatherer and agrarian populations, as was *Prevotellaceae*, *Succinovibrionaceae*, and *Paraprevotellaceae* (Fragiadakis et al., 2019).

Enriched levels of *B. plebeius* were also detected in a Korean sub-population (Tuikhar et al., 2019), where Nori is frequently consumed. A similarity between the diets of Korean (52 million population, land area 100,200 km^2) and Japanese (127 million population, land area 378,000 km^2) is the heavy consumption of fermented foods such as fermented vegetables (e.g., kimchi, natto, and tsukemono), soybean paste, fish products, red pepper paste, medicinal herbs, and sesame or perilla oil (Kim et al., 2016), originally developed to cover for shortfalls in food during winter. The intakes of fermented legumes, vegetables, and potatoes were found to be positively associated with higher α-diversity and *F:B* ratio in the Korean NAS-IARC cohort (Noh et al., 2021). A metagenomic analysis of kimchi revealed dominating members of *Leuconostoc*, *Lactobacillus*, and *Weissella* (Jung et al., 2011), with the latter recently isolated and identified in traditional Indian fermented foods (Månberger et al., 2020). *Leuconostoc* species were also previously found in participants of the Dutch Lifelines (DEEP) cohort who specifically consumed a fermented milk product, buttermilk (Zhernakova et al., 2016). However, it is not known how the proportion of these genera are distributed in the gut microbiota of other regional populations. Fermented foods (i.e., yoghurt) are a known source of probiotics, which are thought to bring about gastrointestinal health benefits such as increased microbial diversity and enriched resident microbiota when consumed in adequate amounts (Marco et al., 2017). Kefir, a fermented milk beverage made from colonies of lactose-fermenting yeast, and lactic- and acetic acid-producing bacteria, is gaining popularity in recent years, and has been suggested to reduce lactose malabsorption and promote *Helicobacter pylori* eradication (Dimidi et al., 2019). However, robust clinical evidence from randomised controlled human trials confirming the effects of various fermented foods on gastrointestinal health are limited.

These studies, collectively, indicate local and global variations in the human gut microbiome, which are attributed to dietary cultures and choices. A common nutritional feature of healthy or longevity diets seems to be the frequent consumption of a minimally processed plant-based diet rich in complex carbohydrates, fruits, vegetables, soy-bean based foods, nuts, and seafood, which emphasises a "healthy fat" profile (i.e., higher in unsaturated fats and omega-3, and lower in saturated fat). The healthy fat being a likely mechanism for reducing inflammation, optimising cholesterol and other risk factors (Nishijima et al., 2016), and when combined with the lower caloric density of plant-rich diets and concomitant high intake of bioactive phytonutrients, jointly reduce risk for chronic age-related diseases and promote healthy ageing and longevity.

MAPPING THE BI-DIRECTIONAL INTERACTIONS BETWEEN DIETARY COMPONENTS AND GUT MICROBIOTA TO UNDERSTAND VARIABILITY IN INTER-INDIVIDUAL RESPONSE

Studies of how diet affects healthspan are commonly limited to macronutrients (e.g., sugar, fat, vitamins) and structural components (e.g., dietary fibres). However, these nutritional components represent a small fraction of >26,000 phytonutrients described in foods[1,2] such as polyphenols, terpenoids, alkaloids, and other plant secondary metabolites. Some of these compounds have been shown to nourish the gut microbiome, which in turn, metabolises these precursors into smaller molecular weight compounds, of which many serve a regulatory role. For instance, olive oil, a well-known ingredient in the MedDiet, has more than 200 unique compounds listed in specialised food metabolome databases (Kabaran, 2018), but only 60 and 8 common nutritional components in the US FoodData and Singapore Health Promotion Board nutrient composition databases, respectively. Barabasi adeptly described this untracked diversity of compounds in foods as the "dark matter of nutrition," which remains largely invisible to epidemiological or hypothesis-driven nutritional studies (Barabási et al., 2020). Considering the chemical diversity of diets in different regions, it is necessary to further our understanding of the full chemical composition of specific foods and complex diets, to be able to map how our highly individualised gut microbiome responds to varying combinations of phytonutrients in geographically or culturally restricted fashions, and how to best employ the gut microbiome for personalised next-generation dietary interventions. In **Supplementary Table 2**, we highlight regional examples of known phytonutrients and functional foods, and their gut microbiome-associated trends that elicit individual and clustered biological responses that could be employed to improve health in an ageing population.

[1]https://foodb.ca

[2]http://phytohub.eu

NEXT GENERATION DIETARY INTERVENTIONS AND PROLONGED HEALTHSPAN: CHALLENGES AND OPPORTUNITIES

An individual's genome may be fixed at conception, but its gut microbiome remains extraordinarily malleable throughout life with a fluctuating genetic composition that is able to respond to both host and dietary cues. By manipulating and utilising the diverse genetic pool of the gut microbiome with targeted dietary interventions, there is an untapped potential to complement and modify otherwise fixed host functions. Most dietary recommendations for the general population represent a "one-size-fits-all approach," which does not ensure that everyone has adequate exposure to health-promoting constituents of foods. For example, dieticians have been using standardised glycaemic indices of food items to guide dietary recommendations in the last decade, but Zeevi et al. (2015) recently showed that glycaemic response to identical foods is in fact highly variable between people and largely dependent on their gut microbiome.

From experience, we have learned to tailor-make food products to certain age and/or vulnerable groups. For example, nutrient-rich milk formula supplemented with essential fatty acids and nucleotides have been designed to meet nutritional demands for the growth spurt of infants, and a selection of ready-to-eat weaning and solid products (e.g., soft vegetable or fruit puree) have been developed for toddlers to transition into whole diets. At the other bookend of life, liquid or pureed food products spiked with nutrients and dietary fibres have been developed to cater to the elderly's reduced capacity for nutrient absorption, decreased taste and smell perception, as well as dental problems resulting in chewing difficulties. Crucially, these existing food products for the elderly are limited in scope to alleviate nutritional shortcomings. A large potential exists in shifting the perspective towards developing a next generation of dietary intervention that shapes, nurtures, and utilises the gut microbiome to complement naturally deteriorating host functions in ageing (**Figure 5**).

Leveraging on the structural composition of food matrices (e.g., whole, intact foods, slowly digestible complex plant carbohydrates, and less processed/liquified foods) is therefore a promising approach to promote longer retention time in the large intestine (Low et al., 2021) to stimulate microbial diversity, richness and a shift in microbial metabolism from protein catabolism towards carbohydrate fermentation

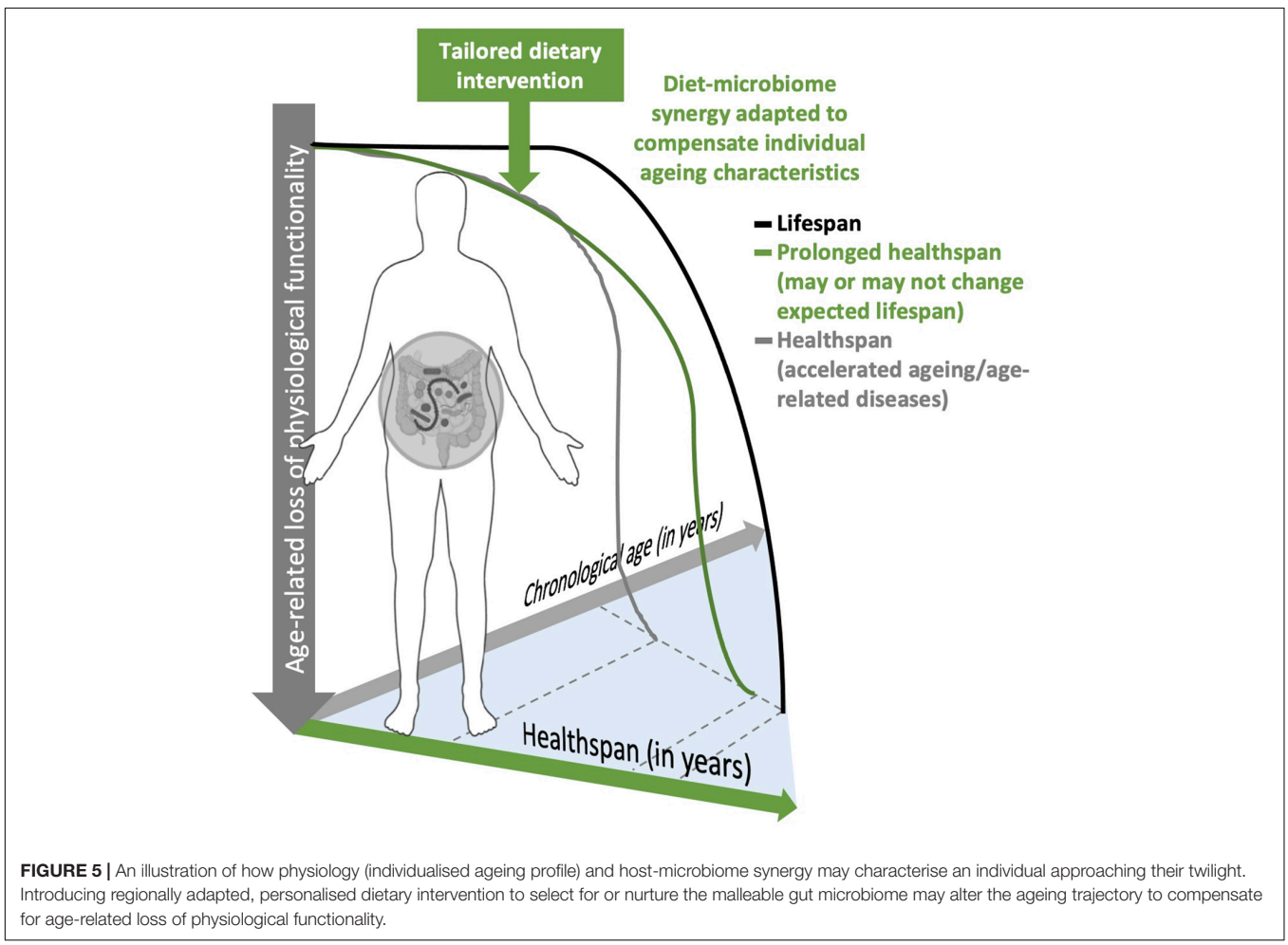

FIGURE 5 | An illustration of how physiology (individualised ageing profile) and host-microbiome synergy may characterise an individual approaching their twilight. Introducing regionally adapted, personalised dietary intervention to select for or nurture the malleable gut microbiome may alter the ageing trajectory to compensate for age-related loss of physiological functionality.

(Grant et al., 2019). To develop guidelines for personalised (and next generation) dietary interventions, there is an urgent need to allocate sufficient resources for basic and translational research that aim to unravel the mechanisms of how physical factors of food matrices alter gut microbial composition and function.

Developing a next generation of dietary interventions for prolonged healthspan requires documentation of individual variation at a systems biology level. A compilation of clinical parameters, socio-economic status, and multi-omics analyses measuring host, diet, and microbial-derived metabolites and microbiome configuration will allow a more accurate determination of an elderly individual's body physiology relevant to the chronological age and estimation of healthspan trajectory. Recent developments in food metabolome databases and innovative metabotyping technologies present opportunities for improving profiling of habitual dietary patterns. A deep characterisation of predictors of individuals' varied response to dietary components can inform well-defined targets or stratified population clusters and guide food companies for the future design of next generation food products. It may be a central component of personalised dietary intervention studies to generate and meaningfully analyse vast amounts of systems level data. This can be challenging since enormous effort, sufficiently large well-phenotyped cohorts, and big data software architecture are required for feeding into machine learning algorithms to be able to recognise patterns that characterise an individual's health and vulnerability (e.g., absence of certain gut microbial communities or lack of consumption of certain food components), and to predict their response to the corresponding dietary interventions. Moreover, it is essential to consider the effect of regional differences in food cultures and lifestyle on baseline microbial and physiological features that will describe the response to new dietary interventions.

The ageing population presents a suitable target for implementing dietary interventions as they have been demonstrated to be receptive towards changing their dietary habits to achieve specific health outcomes (Berendsen et al., 2018). Furthermore, they represent a population of high risk of disease mortality as critically illustrated by the COVID-19 pandemic, which may be alleviated by administering tailored dietary interventions to ensure a strong and vigilant immune system ready to defend the human host under conditions of metabolic homeostasis and optimal inter-organ crosstalk, thus making humans more resilient to emerging stressors.

In summary, we must strive to better define individuals across an otherwise invariant chronological age range, to identify those at greater risk of accelerated ageing for healthspan intervention. Modifiable environmental and lifestyle factors such as our diet and gut microbiome represent accessible targets for prolonging healthspan, where we may introduce personalised dietary interventions to select for or to nurture the highly malleable gut microbiome to alter the ageing trajectory. The design of future diet intervention strategies should consider the chemical diversity of foods and inter-individual variability in biological responses with an emphasis on regional- and cultural-specific context.

AUTHOR CONTRIBUTIONS

DL, SH, and SvP contributed to conception of the review. DL and SH drafted the manuscript. All authors reviewed and approved the manuscript.

FUNDING

DL received the support of Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, through the award of the Presidential Postdoctoral Fellowship (Grant No. 001991-00001).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.659465/full#supplementary-material

REFERENCES

Ahadi, S., Zhou, W., Schüssler-Fiorenza Rose, S. M., Sailani, M. R., Contrepois, K., Avina, M., et al. (2020). Personal aging markers and ageotypes revealed by deep longitudinal profiling. *Nat. Med.* 26, 83–90. doi: 10.1038/s41591-019-0719-5

Barabási, A.-L., Menichetti, G., and Loscalzo, J. (2020). The unmapped chemical complexity of our diet. *Nat. Food* 1, 33–37. doi: 10.1038/s43016-019-0005-1

Barbagallo, C. M., Cavera, G., Sapienza, M., Noto, D., Cefalù, A. B., Polizzi, F., et al. (2002). Nutritional characteristics of a rural southern Italy population: the Ventimiglia di Sicilia project. *J. Am. Coll. Nutr.* 21, 523–529. doi: 10.1080/07315724.2002.10719250

Berendsen, A. A. M., van de Rest, O., Feskens, E. J. M., Santoro, A., Ostan, R., Pietruszka, B., et al. (2018). Changes in dietary intake and adherence to the NU-AGE diet following a one-year dietary intervention among European older adults- Results of the NU-AGE randomized trial. *Nutrients* 10:1905. doi: 10.3390/nu10121905

Biagi, E., Franceschi, C., Rampelli, S., Severgnini, M., Ostan, R., Turroni, S., et al. (2016). Gut microbiota and extreme longevity. *Curr. Biol.* 26, 1480–1485. doi: 10.1016/j.cub.2016.04.016

Bian, G., Gloor, G. B., Gong, A., Jia, C., Zhang, W., Hu, J., et al. (2017). The gut microbiota of healthy aged Chinese is similar to that of the healthy young. *mSphere* 2:e00327-17. doi: 10.1128/mSphere.00327-17

Bong-Soo, K., Chong Won, C., Hyoseung, S., Seon-Pil, J., Jung-Soo, B., Mira, H., et al. (2019). Comparison of the gut microbiota of centenarians in longevity villages of South Korea with those of other age groups. *J. Microbiol. Biotechnol.* 29, 429–440. doi: 10.4014/jmb.1811.11023

Buettner, D., and Skemp, S. (2016). Blue zones: lessons from the world's longest lived. *Am. J. Lifestyle Med.* 10, 318–321. doi: 10.1177/1559827616637066

Byndloss, M. X., and Bäumler, A. J. (2018). The germ-organ theory of non-communicable diseases. *Nat. Rev. Microbiol.* 16, 103–110. doi: 10.1038/nrmicro.2017.158

Campisi, J., Kapahi, P., Lithgow, G. J., Melov, S., Newman, J. C., and Verdin, E. (2019). From discoveries in ageing research to therapeutics for healthy ageing. *Nature* 571, 183–192. doi: 10.1038/s41586-019-1365-2

Chong, C. W., Ahmad, A. F., Lim, Y. A. L., Teh, C. S. J., Yap, I. K. S., Lee, S. C., et al. (2015). Effect of ethnicity and socioeconomic variation to the gut microbiota composition among pre-adolescent in Malaysia. *Sci. Rep.* 5:13338. doi: 10.1038/srep13338

Chua, E. G., Loke, M. F., Gunaletchumy, S. P., Gan, H. M., Thevakumar, K., Tay, C. Y., et al. (2019). The influence of modernization and disease on the gastric microbiome of Orang Asli, Myanmars and modern Malaysians. *Microorganisms* 7:174. doi: 10.3390/microorganisms7060174

Chung, W. (2019). *Penang's 5 Great Nyonya/Peranakan Restaurants, A Blend of Chinese and Malay Cuisines [Online]*. Available online at: https://www.scmp.com/magazines/style/travel-food/article/3001711/5-great-nyonya/peranakan-restaurants-blending-chinese (accessed October 21, 2020).

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., et al. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488, 178–184. doi: 10.1038/nature11319

Cortés-Martín, A., Selma, M. V., Tomás-Barberán, F. A., González-Sarrías, A., and Espín, J. C. (2020). Where to look into the puzzle of polyphenols and health? The postbiotics and gut microbiota associated with human metabotypes. *Mol. Nutr. Food Res.* 64:1900952.

De Angelis, M., Ferrocino, I., Calabrese, F. M., De Filippis, F., Cavallo, N., Siragusa, S., et al. (2020). Diet influences the functions of the human intestinal microbiome. *Sci. Rep.* 10:4247. doi: 10.1038/s41598-020-61192-y

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., et al. (2016). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut* 65:1812. doi: 10.1136/gutjnl-2015-309957

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S.A.* 107:14691. doi: 10.1073/pnas.1005963107

Deng, Y., He, K., Ye, X., Chen, X., Huang, J., Li, X., et al. (2012). Saponin rich fractions from *Polygonatum odoratum* (Mill.) Druce with more potential hypoglycemic effects. *J. Ethnopharmacol.* 141, 228–233. doi: 10.1016/j.jep.2012.02.023

Deschasaux, M., Bouter, K. E., Prodan, A., Levin, E., Groen, A. K., Herrema, H., et al. (2018). Depicting the composition of gut microbiota in a population with varied ethnic origins but shared geography. *Nat. Med.* 24, 1526–1531. doi: 10.1038/s41591-018-0160-1

Di Meo, F., Margarucci, S., Galderisi, U., Crispi, S., and Peluso, G. (2019). Curcumin, gut microbiota, and neuroprotection. *Nutrients* 11:2426. doi: 10.3390/nu11102426

Dimidi, E., Cox, S. R., Rossi, M., and Whelan, K. (2019). Fermented foods: Definitions and characteristics, impact on the gut microbiota and effects on gastrointestinal health and disease. *Nutrients* 11:1806. doi: 10.3390/nu11081806

Espín, J. C., Larrosa, M., García-Conesa, M. T., and Tomás-Barberán, F. (2013). Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. *Evid. Bas. Complement. Alternat. Med.* 2013:270418. doi: 10.1155/2013/270418

Fang, W., Ting, Y., Guohong, H., Da, C., Xiaolin, L., Haiyan, S., et al. (2015). Gut microbiota community and its assembly associated with age and diet in chinese centenarians. *J. Microbiol. Biotechnol.* 25, 1195–1204. doi: 10.4014/jmb.1410.10014

Fragiadakis, G. K., Smits, S. A., Sonnenburg, E. D., Van Treuren, W., Reid, G., Knight, R., et al. (2019). Links between environment, diet, and the hunter-gatherer microbiome. *Gut Microb.* 10, 216–227. doi: 10.1080/19490976.2018.1494103

Fukkoshi, Y., Akamatsu, R., and Shimpo, M. (2015). The relationship of eating until 80% full with types and energy values of food consumed. *Eat. Behav.* 17, 153–156. doi: 10.1016/j.eatbeh.2015.03.001

García-Mantrana, I., Calatayud, M., Romo-Vaquero, M., Espín, J. C., Selma, M. V., and Collado, M. C. (2019). Urolithin metabotypes can determine the modulation of gut microbiota in healthy individuals by tracking walnuts consumption over three days. *Nutrients* 11:2483. doi: 10.3390/nu11102483

Grant, L. J., Mikkelsen, D., Ouwerkerk, D., Klieve, A. V., Gidley, M. J., and Williams, B. A. (2019). Whole fruit pulp (mango) and a soluble fibre (pectin) impact bacterial diversity and abundance differently within the porcine large intestine. *Bioact. Carbohydr. Diet. Fibre* 19:100192. doi: 10.1016/j.bcdf.2019.100192

Gunness, P., Williams, B. A., Gerrits, W. J. J., Bird, A. R., Kravchuk, O., and Gidley, M. J. (2016). Circulating triglycerides and bile acids are reduced by a soluble wheat arabinoxylan via modulation of bile concentration and lipid digestion rates in a pig model. *Mol. Nutr. Food Res.* 60, 642–651. doi: 10.1002/mnfr.201500686

Hålldin, E., Eriksen, A. K., Brunius, C., da Silva, A. B., Bronze, M., Hanhineva, K., et al. (2019). Factors explaining interpersonal variation in plasma enterolactone concentrations in humans. *Mol. Nutr. Food Res.* 63:e1801159. doi: 10.1002/mnfr.201801159

Hehemann, J.-H., Correc, G., Barbeyron, T., Helbert, W., Czjzek, M., and Michel, G. (2010). Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. *Nature* 464, 908–912. doi: 10.1038/nature08937

Herskind, A. M., McGue, M., Holm, N. V., Sørensen, T., Harvald, B., and Vaupel, J. W. (1996). he heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870-1900. *Hum. Genet.* 97, 319–323. doi: 10.1007/BF02185763

Jain, A., Li, X. H., and Chen, W. N. (2018). Similarities and differences in gut microbiome composition correlate with dietary patterns of Indian and Chinese adults. *AMB Expr.* 8:104. doi: 10.1186/s13568-018-0632-1

Jung, J. Y., Lee, S. H., Kim, J. M., Park, M. S., Bae, J.-W., Hahn, Y., et al. (2011). Metagenomic analysis of kimchi, a traditional Korean fermented food. *Appl. Environ. Microbiol.* 77, 2264–2274. doi: 10.1128/AEM.02157-10

Kabaran, S. (2018). "Olive oil: antioxidant compounds and their potential effects over health," in *Functional Foods*, ed. V. Lagouri (London: IntechOpen).

Kang, C., Wang, B., Kaliannan, K., Wang, X., Lang, H., Hui, S., et al. (2017). Gut microbiota mediates the protective effects of dietary capsaicin against chronic low-grade inflammation and associated obesity induced by high-fat diet. *mBio* 8:e00470-17. doi: 10.1128/mBio.00470-17

Kaur, S., Panesar, P. S., Bera, M. B., and Kumari, S. (2014). Physicochemical, textural, pasting, and in vitro digestion properties of some Basmati and non-Basmati rice cultivars. *Int. J. Food Propert.* 17, 1055–1066. doi: 10.1080/10942912.2011.642444

Khine, W. W. T., Zhang, Y., Goie, G. J. Y., Wong, M. S., Liong, M., Lee, Y. Y., et al. (2019). Gut microbiome of pre-adolescent children of two ethnicities residing in three distant cities. *Sci. Rep.* 9:7831. doi: 10.1038/s41598-019-44369-y

Kim, S. H., Kim, M. S., Lee, M. S., Park, Y. S., Lee, H. J., Kang, S.-a., et al. (2016). Korean diet: characteristics and historical background. *J. Ethnic Foods* 3, 26–31. doi: 10.1016/j.jef.2016.03.002

Kong, F., Hua, Y., Zeng, B., Ning, R., Li, Y., and Zhao, J. (2016). Gut microbiota signatures of longevity. *Curr. Biol.* 26, R832–R833. doi: 10.1016/j.cub.2016.08.015

Landberg, R., Hanhineva, K., Tuohy, K., Garcia-Aloy, M., Biskup, I., Llorach, R., et al. (2019). Biomarkers of cereal food intake. *Genes Nutrit.* 14:28. doi: 10.1186/s12263-019-0651-9

Le Roy, C. I., Wells, P. M., Si, J., Raes, J., Bell, J. T., and Spector, T. D. (2020). Red wine consumption associated with increased gut microbiota α-diversity in 3 independent cohorts. *Gastroenterology* 158, 270–272. doi: 10.1053/j.gastro.2019.08.024

Lee, J.-H., and Lee, J. (2009). Indole as an intercellular signal in microbial communities. *FEMS Microbiol. Rev.* 34, 426–444. doi: 10.1111/j.1574-6976.2009.00204.x

Lee, M. s., and Moon, K.-H. (2013). Health and functional characteristics of indigenous food in the Korean longevity belt. *FASEB J.* 27:1079.12. doi: 10.1096/fasebj.27.1_supplement.1079.12

Lee, Y. K., Conway, P., Pettersson, S., Nair, G. B., Surono, I., Egayanti, Y., et al. (2017). ILSI Southeast Asia Region conference proceedings: the gut, its microbes and health: relevance for Asia. *Asia Pac. J. Clin. Nutr.* 26, 957–971. doi: 10.6133/apjcn.112016.09

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194–1217. doi: 10.1016/j.cell.2013.05.039

Low, D. Y., Pluschke, A. M., Gerrits, W. J. J., Zhang, D., Shelat, K. J., Gidley, M. J., et al. (2020). Cereal dietary fibres influence retention time of digesta solid and liquid phases along the gastrointestinal tract. *Food Hydrocol.* 104:105739. doi: 10.1016/j.foodhyd.2020.105739

Low, D. Y., Pluschke, A. M., Flanagan, B., Sonni, F., Grant, L. J., Williams, B. A., et al. (2021). Isolated pectin (apple) and fruit pulp (mango) impact gastric emptying, passage rate and short chain fatty acid (SCFA) production differently along the pig gastrointestinal tract. *Food Hydrocol.* 106723. doi: 10.1016/j.foodhyd.2021.106723

Lu, Q.-Y., Rasmussen, A. M., Yang, J., Lee, R.-P., Huang, J., Shao, P., et al. (2019). Mixed spices atculinary doses have prebiotic effects in healthyadults: a pilot study. *Nutrients* 11:1425. doi: 10.3390/nu11061425

Luan, Z., Sun, G., Huang, Y., Yang, Y., Yang, R., Li, C., et al. (2020). Metagenomics study reveals changes in gut microbiota in centenarians: a cohort study of hainan centenarians. *Front. Microbiol.* 11:1474. doi: 10.3389/fmicb.2020.01474

Månberger, A., Verbrugghe, P., Guðmundsdóttir, E. E., Santesson, S., Nilsson, A., Hreggviðsson, G. Ó, et al. (2020). Taxogenomic assessment and genomic characterisation of Weissella cibaria strain 92 able to metabolise oligosaccharides derived from dietary fibres. *Sci. Rep.* 10:5853. doi: 10.1038/s41598-020-62610-x

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., et al. (2017). Health benefits of fermented foods: microbiota and beyond. *Curr. Opin. Biotechnol.* 44, 94–102. doi: 10.1016/j.copbio.2016.11.010

Mayo, B., Vázquez, L., and Flórez, A. B. (2019). Equol: a bacterial metabolite from the daidzein isoflavone and its presumed beneficial health effects. *Nutrients* 11:2231. doi: 10.3390/nu11092231

Melzer, D., Pilling, L. C., and Ferrucci, L. (2020). The genetics of human ageing. *Nat. Rev. Genet.* 21, 88–101. doi: 10.1038/s41576-019-0183-6

Meyer, K. A., and Bennett, B. J. (2016). Diet and gut microbial function in metabolic and cardiovascular disease risk. *Curr. Diab. Rep.* 16, 93–93. doi: 10.1007/s11892-016-0791-x

Naito, Y., Takagi, T., Inoue, R., Kashiwagi, S., Mizushima, K., Tsuchiya, S., et al. (2019). Gut microbiota differences in elderly subjects between rural city Kyotango and urban city Kyoto: an age-gender-matched study. *J. Clin. Biochem. Nutr.* 65, 125–131. doi: 10.3164/jcbn.19-26

Nakayama, J., Watanabe, K., Jiang, J., Matsuda, K., Chao, S.-H., Haryono, P., et al. (2015). Diversity in gut bacterial community of school-age children in Asia. *Sci. Rep.* 5:8397. doi: 10.1038/srep08397

Nishijima, S., Suda, W., Oshima, K., Kim, S.-W., Hirose, Y., Morita, H., et al. (2016). The gut microbiome of healthy Japanese and its microbial and functional uniqueness. *DNA Res. Int. J. Rapid Publ. Rep. Genes Genom.* 23, 125–133. doi: 10.1093/dnares/dsw002

Noh, H., Jang, H.-H., Kim, G., Zouiouich, S., Cho, S.-Y., Kim, H.-J., et al. (2021). Taxonomic composition and diversity of the gut microbiota in relation to habitual dietary intake in Korean adults. *Nutrients* 13:366. doi: 10.3390/nu13020366

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J.-z., et al. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol.* 16:90. doi: 10.1186/s12866-016-0708-5

Partula, V., Mondot, S., Torres, M. J., Kesse-Guyot, E., Deschasaux, M., Assmann, K., et al. (2019). Associations between usual diet and gut microbiota composition: results from the Milieu Intérieur cross-sectional study. *Am. J. Clin. Nutr.* 109, 1472–1483. doi: 10.1093/ajcn/nqz029

Pluta, R., Januszewski, S., and Ułamek-Kozioł, M. (2020). Mutual two-way interactions of curcumin and gut microbiota. *Int. J. Mol. Sci.* 21:1055. doi: 10.3390/ijms21031055

Pudlo, N. A., Pereira, G. V., Parnami, J., Cid, M., Markert, S., Tingley, J. P., et al. (2020). Extensive transfer of genes for edible seaweed digestion from marine to human gut bacteria. *bioRxiv* [Preprint]. doi: 10.1101/2020.06.09.142968

Rafii, F. (2015). The role of colonic bacteria in the metabolism of the natural isoflavone daidzin to equol. *Metabolites* 5, 56–73. doi: 10.3390/metabo5010056

Rampelli, S., Soverini, M., D'Amico, F., Barone, M., Tavella, T., Monti, D., et al. (2020). Shotgun metagenomics of gut microbiota in humans with up to extreme longevity and the increasing role of xenobiotic degradation. *mSystems* 5:e00124-20. doi: 10.1128/mSystems.00124-20

Robine, J.-M., and Cubaynes, S. (2017). Worldwide demography of centenarians. *Mech. Ageing Dev.* 165, 59–67. doi: 10.1016/j.mad.2017.03.004

Roman, B., Carta, L., Martínez-González, M. A., and Serra-Majem, L. (2008). Effectiveness of the Mediterranean diet in the elderly. *Clin. Interven. Aging* 3, 97–109. doi: 10.2147/cia.s1349

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., et al. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature* 555, 210–215. doi: 10.1038/nature25973

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., et al. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *Eur. J. Nutr.* 57, 1–24. doi: 10.1007/s00394-017-1445-8

Ruby, J. G., Wright, K. M., Rand, K. A., Kermany, A., Noto, K., Curtis, D., et al. (2018). Estimates of the heritability of human longevity are substantially inflated due to assortative mating. *Genetics* 210, 1109–1124. doi: 10.1534/genetics.118.301613

Ruiz-Ruiz, S., Sanchez-Carrillo, S., Ciordia, S., Mena, M. C., Méndez-García, C., Rojo, D., et al. (2020). Functional microbiome deficits associated with ageing: chronological age threshold. *Aging Cell* 19:e13063. doi: 10.1111/acel.13063

Salazar, N., Arboleya, S., Fernández-Navarro, T., de los Reyes-Gavilán, C. G., Gonzalez, S., and Gueimonde, M. (2019). Age-associated changes in gut microbiota and dietary components related with the immune system in adulthood and old age: a cross-sectional study. *Nutrients* 11:1765. doi: 10.3390/nu11081765

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nat. Rev. Gastroenterol. Hepatol.* 16, 605–616. doi: 10.1038/s41575-019-0173-3

Sender, R., and Milo, R. (2021). The distribution of cellular turnover in the human body. *Nat. Med.* 27, 45–48. doi: 10.1038/s41591-020-01182-9

Shen, L., Liu, L., and Ji, H.-F. (2017). Regulative effects of curcumin spice administration on gut microbiota and its pharmacological implications. *Food Nutr. Res.* 61:1361780. doi: 10.1080/16546628.2017.1361780

Steves, C. J., Spector, T. D., and KJackson, S. H. D. (2012). Ageing, genes, environment and epigenetics: what twin studies tell us now, and in the future. *Age Ageing* 41, 581–586. doi: 10.1093/ageing/afs097

Tourism Exchange Division of Wakayama Prefecture (2020). *Flavors of Wakayama [Online]*. Available online at: https://en.visitwakayama.jp/themes/flavors-of-wakayama/ (accessed October 21, 2020).

Tuikhar, N., Keisam, S., Labala, R. K., Imrat Ramakrishnan, P., Arunkumar, M. C., et al. (2019). Comparative analysis of the gut microbiota in centenarians and young adults shows a common signature across genotypically non-related populations. *Mech. Ageing Dev.* 179, 23–35. doi: 10.1016/j.mad.2019.02.001

Tulipani, S., Urpi-Sarda, M., García-Villalba, R., Rabassa, M., López-Uriarte, P., Bulló, M., et al. (2012). Urolithins are the main urinary microbial-derived phenolic metabolites discriminating a moderate consumption of nuts in free-living subjects with diagnosed metabolic syndrome. *J. Agricult. Food Chem.* 60, 8930–8940. doi: 10.1021/jf301509w

Unno, T., and Osakabe, N. (2018). Green tea extract and black tea extract differentially influence cecal levels of short-chain fatty acids in rats. *Food Sci. Nutr.* 6, 728–735. doi: 10.1002/fsn3.607

Vázquez, L., Guadamuro, L., Giganto, F., Mayo, B., and Flórez, A. B. (2017). Development and use of a real-time quantitative PCR method for detecting and quantifying equol-producing bacteria in human faecal samples and slurry cultures. *Front. Microbiol.* 8:1155–1155. doi: 10.3389/fmicb.2017.01155

Wang, D., Nguyen, L., Li, Y., Yan, Y., Ma, W., Rinott, E., et al. (2020). The gut microbiome modifies the protective effects of a Mediterranean diet against cardiometabolic disease risk. *Curr. Dev. Nutr.* 4(Suppl. 2), 1597–1597. doi: 10.1093/cdn/nzaa062_054

Wilmanski, T., Diener, C., Rappaport, N., Patwardhan, S., Wiedrick, J., Lapidus, J., et al. (2020). Gut microbiome pattern reflects healthy aging and predicts extended survival in humans. *bioRxiv* [Preprint]. doi: 10.1101/2020.02.26.966747

Wu, L., Zeng, T., Zinellu, A., Rubino, S., Kelvin, D. J., and Carru, C. (2019). A cross-sectional study of compositional and functional profiles of gut microbiota in Sardinian centenarians. *mSystems* 4:e00325-19. doi: 10.1128/mSystems.00325-19

Yeo, L.-F., Aghakhanian, F. F., Tan, J. S. Y., Gan, H. M., and Phipps, M. E. (2019). Health and saliva microbiomes of a semi-urbanized indigenous tribe in Peninsular Malaysia. *F1000Res.* 8, 1–19. doi: 10.12688/f1000research.17706.3

Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., et al. (2015). Personalized nutrition by prediction of

glycemic responses. *Cell* 163, 1079–1094. doi: 10.1016/j.cell.2015.11.001

Zhang, N., and Ma, G. (2020). Nutritional characteristics and health effects of regional cuisines in China. *J. Ethnic Foods* 7:7. doi: 10.1186/s42779-020-0045-z

Zhernakova, A., Kurilshikov, A., Bonder, M. J., Tigchelaar, E. F., Schirmer, M., Vatanen, T., et al. (2016). Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* 352:565. doi: 10.1126/science.aad3369

Zicasso (2021). *Food of Emilia-Romagna – The Emilia-Romagna Food Guide [Online]*. Available online at: https://www.zicasso.com/italy/cuisines/north/emilia-romagna (accessed October 21, 2020).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 10 June 2021
doi: 10.3389/fmicb.2021.662159

Effect of Fecal Microbiota Transplantation Combined With Mediterranean Diet on Insulin Sensitivity in Subjects With Metabolic Syndrome

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OPEN ACCESS

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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 31 January 2021
Accepted: 29 April 2021
Published: 10 June 2021

Citation:
Koopen AM, Almeida EL, Attaye I, Witjes JJ, Rampanelli E, Majait S, Kemper M, Levels JHM, Schimmel AWM, Herrema H, Scheithauer TPM, Frei W, Dragsted L, Hartmann B, Holst JJ, O'Toole PW, Groen AK and Nieuwdorp M (2021) Effect of Fecal Microbiota Transplantation Combined With Mediterranean Diet on Insulin Sensitivity in Subjects With Metabolic Syndrome. Front. Microbiol. 12:662159. doi: 10.3389/fmicb.2021.662159

Background: Recent studies demonstrate that a Mediterranean diet has beneficial metabolic effects in metabolic syndrome subjects. Since we have shown that fecal microbiota transplantation (FMT) from lean donors exerts beneficial effects on insulin sensitivity, in the present trial, we investigated the potential synergistic effects on insulin sensitivity of combining a Mediterranean diet with donor FMT in subjects with metabolic syndrome.

Design: Twenty-four male subjects with metabolic syndrome were put on a Mediterranean diet and after a 2-week run-in phase, the subjects were randomized to either lean donor (n = 12) or autologous (n = 12) FMT. Changes in the gut microbiota composition and bacterial strain engraftment after the 2-week dietary regimens and 6 weeks post-FMT were the primary endpoints. The secondary objectives were changes in glucose fluxes (both hepatic and peripheral insulin sensitivity), postprandial plasma incretin (GLP-1) levels, subcutaneous adipose tissue inflammation, and plasma metabolites.

Results: Consumption of the Mediterranean diet resulted in a reduction in body weight, HOMA-IR, and lipid levels. However, no large synergistic effects of combining the diet with lean donor FMT were seen on the gut microbiota diversity after 6 weeks. Although we did observe changes in specific bacterial species and plasma metabolites, no significant beneficial effects on glucose fluxes, postprandial incretins, or subcutaneous adipose tissue inflammation were detected.

Conclusions: In this small pilot randomized controlled trial, no synergistic beneficial metabolic effects of combining a Mediterranean diet with lean donor FMT on glucose metabolism were achieved. However, we observed engraftment of specific bacterial species. Future trials are warranted to test the combination of other microbial interventions and diets in metabolic syndrome.

Keywords: gut microbiota, fecal microbiota transplantation, mediterranean diet, metabolic syndrome, insulin sensitivity

INTRODUCTION

With the continuous rise in obesity prevalence (NCD Risk Factor Collaboration, 2016a), the number of obese adults with metabolic syndrome and type 2 diabetes mellitus (DM2) has quadrupled over the last decade (NCD Risk Factor Collaboration, 2016b). The most important driver of metabolic syndrome is excess intestinal uptake of energy. Our gut microbiota is one of the factors that play an important role in the regulation of energy harvest and storage, in which gut microbiota from obese subjects has been postulated to yield more energy from diet compared to gut microbiota from lean subjects (Bäckhed et al., 2004; Turnbaugh et al., 2006). Colonization of germ-free mice with microbiota from obese littermates or obese humans has been shown to cause an increase in body weight, demonstrating that this obese phenotype is transmissible *via* fecal microbiota transplantation (FMT) (Turnbaugh et al., 2006; Ridaura et al., 2013). In humans, several studies have demonstrated that the gut microbiota composition of healthy subjects differs from that of subjects with metabolic syndrome and DM2 (Ley et al., 2006; Turnbaugh et al., 2006; Qin et al., 2012; Karlsson et al., 2013; Castaner et al., 2018). Furthermore, we have previously shown that transplantation of lean donor fecal microbiota can transiently improve insulin sensitivity in obese subjects with metabolic syndrome (Vrieze et al., 2012; Kootte et al., 2017). In recent years, our group has also demonstrated that gut microbiota diversity at baseline is an important determinant of the engraftment of donor bacteria, which subsequently affects the magnitude of metabolic response after FMT (Li et al., 2016; Kootte et al., 2017).

The composition of the gut microbiota is governed by a complex interplay of many independent factors, such as exercise, concomitant medication use, aging, and diet (Dominguez-Bello et al., 2010; Yatsunenko et al., 2012; Clarke et al., 2014; Falony et al., 2016). Dietary composition and intake are considered the most important contributing factors to the altered diversity of intestinal microbes (De Filippo et al., 2010; Gentile and Weir, 2018). Indeed, prior data have clearly shown that the gut microbiota rapidly changes after alterations in diet (David et al., 2014; Zeevi et al., 2015), with an increase in gut microbiota diversity upon a healthy (high-fiber) diet (Cotillard et al., 2013). A Mediterranean diet is such a high-fiber diet that has been associated with many beneficial health effects, such as reduced cardiovascular (Estruch et al., 2018) and DM2 risk (Salas-Salvadó et al., 2011; Haro et al., 2016). Also, beneficial changes in the microbiota composition after a Mediterranean diet have been reported (De Filippis et al., 2015; Haro et al., 2016; Ghosh et al., 2020). A recent randomized controlled trial has investigated the effects of an 8-week adherence to a Mediterranean diet in 82 obese subjects (Meslier et al., 2020). In line with earlier studies, a beneficial effect on the plasma cholesterol levels was observed as well as a number of changes in fecal microbiota, while the effect on insulin sensitivity was marginal. Although many studies have demonstrated that the type of diet has a great impact on the gut microbiota composition, none of these previous intervention studies combine modulation of dietary intake with reestablishment of a healthy microbiome by means of lean donor FMT.

In the present study, we therefore hypothesized that a Mediterranean diet may help beneficial microbes to better engraft, enabling long-term and greater effects after FMT. We thus performed lean healthy donor FMT preceded by a controlled Mediterranean diet and studied possible synergistic effects on the gut microbiota composition in subjects with metabolic syndrome. Secondary objectives were intervention-driven changes in both hepatic and peripheral insulin sensitivity, postprandial plasma concentrations of the incretin glucagon-like peptide-1 (GLP-1), subcutaneous adipose tissue (AT) inflammation, and plasma metabolites.

MATERIALS AND METHODS

Study Population

We recruited obese treatment-naive Caucasian male subjects (age, 21–65 years) with a body mass index (BMI) between 30 and 43 kg/m^2 with otherwise normal health *via* local newspaper advertisements. All subjects had to meet the inclusion criteria of metabolic syndrome (Alberti et al., 2006); requiring to meet at least three out of the following five criteria: fasting plasma glucose $\geq$ 5.6 mmol/L, triglycerides $\geq$ 1.7 mmol/L, waist circumference $\geq$ 102 cm, high-density lipoprotein (HDL) cholesterol $\leq$ 1.04 mmol/L, and/or blood pressure $\geq$ 130/85 mmHg. The main exclusion criteria were the use of any type of medication, smoking, alcohol abuse, a history of cardiovascular event or cholecystectomy, and being unmotivated or unable to adhere to the diet. Moreover, eligible FMT donors were healthy Caucasian men (age, 18–65 years) with a BMI between 18.5 and 25 kg/m^2. Donors were thoroughly screened as previously described (Kootte et al., 2017) for the presence of infectious diseases in blood and feces and completed questionnaires regarding medical, sexual, family and travel history, and bowel habits, as previously

described (Cammarota et al., 2017). Written informed consent was obtained from all subjects. The study was approved by the local Institutional Review Board of the Amsterdam University Medical Center (Amsterdam UMC) in Amsterdam, the Netherlands, and conducted at the AMC in accordance with the Declaration of Helsinki. The study was registered at the Dutch Trial Register (NTR 5983).

Study Design

The design of the study is presented in **Figure 1**. We conducted a double-blind randomized controlled single-center trial. All male metabolic syndrome subjects adhered to a Mediterranean diet for a total of 8 weeks with a 2-week run-in period (week −2 to week 0). After these 2 weeks, the subjects were randomized (using computerized randomization) to either an allogeneic FMT (receiving the feces of a lean healthy donor) or autologous FMT (receiving their own feces). At weeks −2, 0, and 6 we collected anthropomorphic data and blood and fecal samples. Three weeks after the FMT, we collected an additional fecal sample, and at week 12 (6 weeks after cessation of the diet), the subjects visited our center for the last time to provide a final fecal sample. In week 0, we performed tests to determine postprandial lipid metabolism, subcutaneous adipose tissue inflammation, and insulin sensitivity, which we repeated at week 6. The sample size of $n = 12$ was based on data from our previous FMT clinical trials in which absolute improvements in insulin sensitivity from 26.2 to 45.3 μmol L^{-1} min^{-1} (Vrieze et al., 2012) and from 25.8 to 28.8 μmol L^{-1} min^{-1} (Kootte et al., 2017) were observed upon lean donor FMT.

Week −2: Start of Mediterranean Diet

The Mediterranean diet guidelines used in this study were based on the PREDIMED Study (Estruch et al., 2018), although adapted slightly to Dutch settings in consultation with the clinical dietitian who was part of the research team. The diet contained the following components: two or more daily servings of vegetables, two or more daily servings of fresh fruits, three or more weekly servings of legumes, two or more weekly servings of fish or seafood, abundant use of olive oil for cooking and dressing dishes, and daily consumption of nuts. Negative recommendations were given about red meat, cream, butter, carbonated and/or sugared beverages, and pastries and industrial bakery products (see **Supplementary File 1**). To enhance adherence to the Mediterranean diet, the subjects were provided food boxes developed in collaboration with a Dutch food box company (de Krat, Amsterdam, Netherlands). The boxes were suited for two adults so that meals for partners did not have to be prepared separately, thereby facilitating compliance. The food boxes contained all the ingredients for five main meals, five times lunch, and extra supplementation of fruit, extra virgin olive oil (one bottle of 500 ml per week), and raw unsalted mixed nuts (500 g per week). The recipes and contents of the boxes met our Mediterranean guidelines and were all checked by the clinical dietitian. For the remaining 2 days of the week, subjects could prepare their own food, provided that they adhered to the Mediterranean guidelines provided by the dietitian. The same held true for breakfast and any snacks.

All subjects visited the Amsterdam UMC clinical dietitian before they started the Mediterranean diet. During this intake, the subjects were counseled on the Mediterranean diet plus food boxes and received personal advice based on the online nutritional diary[1] they completed beforehand. During the 2-week run-in period that followed, the decision was made whether a subject could continue with the study based on motivation and adherence. During the diet phase, the subjects were asked to fill out the online food diary at three different time points for at least 2 days (which were checked by the dietitian) and had telephone calls with the dietitian for at least two occasions and more if necessary. The 3 days before the last visit, the subjects completed the nutritional diary for a final time. From the online nutritional diary, the total energy, carbohydrate, protein, fiber, total fat, and saturated fat intakes were calculated for the pre-diet, during, and post-diet stages.

Mixed Meal Test

Mixed meal test (MMT) was performed at week 0 (study day 1) and repeated at week 6. After an overnight fast, baseline blood samples were drawn from an inserted intravenous catheter in a distal arm vein. Hereafter, the subjects immediately ingested a standardized liquid high-fat meal containing 626 kcal (61% fat, 33% carbohydrates, and 6% proteins) within 5 min, as previously described (Reijnders et al., 2016; Kootte et al., 2017). For the next 4 h, blood samples were drawn every 30 min for postprandial metabolism and were stored at −80°C. Before the start of the MMT, abdominal subcutaneous adipose tissue was aspirated as previously described (De Groot et al., 2019). All subjects provided a fecal sample for gut microbiota and short-chain fatty acid (SCFA) analysis on this day or study day 2.

Two-Step Hyperinsulinemic Euglycemic Clamp

On the second study day of week 0 and week 6, a two-step hyperinsulinemic euglycemic clamp test with stable isotopes was performed to measure hepatic and peripheral insulin sensitivity, as described previously (Vrieze et al., 2012; Kootte et al., 2017). After an overnight fast, intravenous catheters were inserted into a distal vein of both arms. One catheter was used for arterialized venous blood withdrawal using a heated-hand box (60°C); the other catheter was used for infusion of the glucose tracer, glucose, and insulin. Prior to infusion, baseline blood samples were drawn to determine background isotope enrichment. Hereafter, a continuous infusion of [6,6-$^2H_2$] glucose (prime, 11 μmol kg^{-1}; continuous, 0.11 μmol kg^{-1} min^{-1}) was started and continued until the end of the experiment. After blood withdrawal at 2 h of equilibration, infusion of insulin (Actrapid; Novo Nordisk Farma B.V., Alphen aan den Rijn, Netherlands) at a rate of 20 mU m^{-2} min^{-1} was started. Plasma glucose concentrations were measured every 10 min and infusion of a 20% glucose solution enriched with 1% [6,6-$^2H_2$] glucose was started to maintain a plasma glucose concentration of 5 mmol/L. After 2 h of insulin infusion, five repetitive blood samples were drawn with an interval of 5 min to determine glucose enrichments,

[1] https://mijn.voedingscentrum.nl/nl/eetmeter/

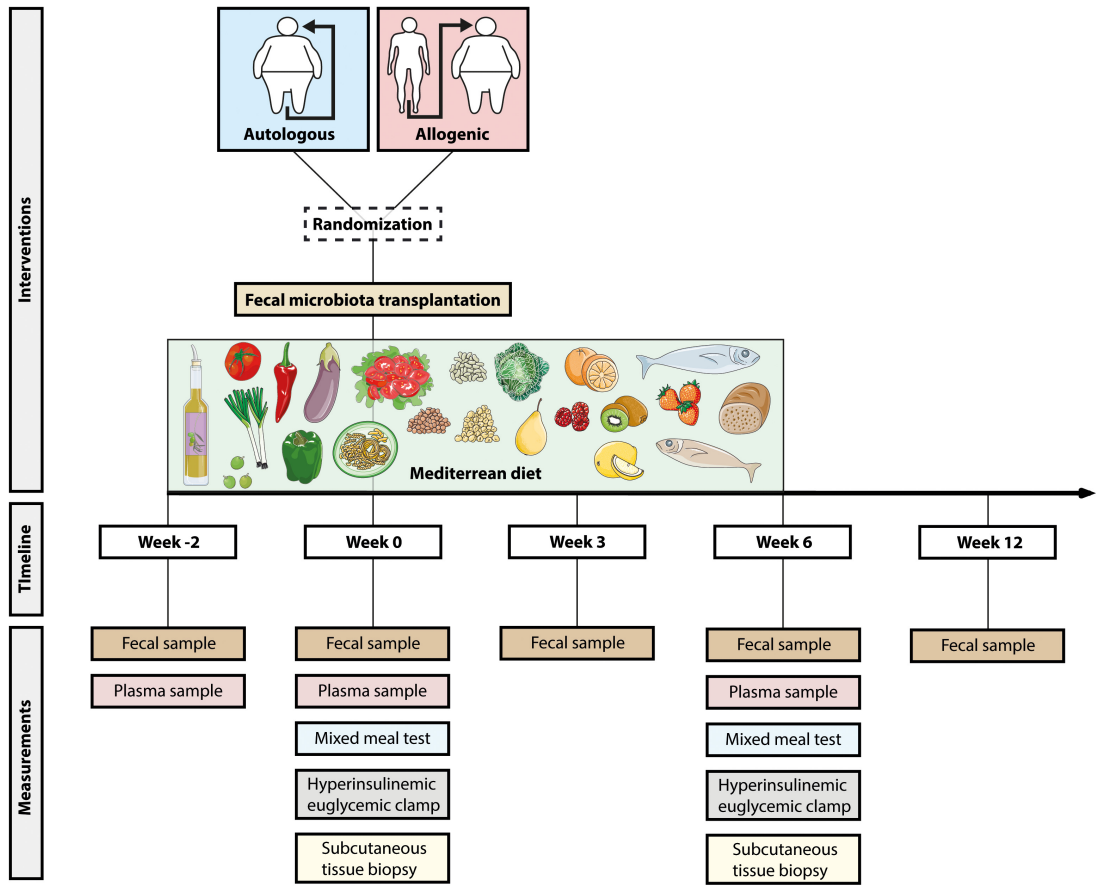

FIGURE 1 | Study design. Overview of the study. All subjects adhered to the Mediterranean diet for 8 weeks (from W–2 to W6). After 2 weeks (W0), the subjects were randomized to autologous or allogeneic fecal microbiota transplantation. Before the start of the diet (W–2), the first fecal and blood samples were collected. At W0 (before fecal microbiota transplantation, FMT) and W6, we performed a mixed meal test, hyperinsulinemic euglycemic clamp, and a subcutaneous tissue biopsy.

glucoregulatory hormones, and free fatty acids. Hereafter, the insulin infusion rate was increased to 60 mU m^{-2} min^{-1} and continued for another 2 h, after which five repetitive blood samples were drawn again. Plasma samples were stored at −80°C for later analyses. Resting energy expenditure (REE) was measured using indirect calorimetry during the basal and hyperinsulinemic state. Oxygen and carbon dioxide productions were measured for 20 min using a ventilated hood system (Vmax Encore 29; SensorMedics, Anaheim, CA, United States).

Gene Expression of Subcutaneous Tissue Biopsy

At both week 0 and week 6, abdominal subcutaneous adipose tissue was aspirated using a hollow needle and a 50-ml syringe. TriPure Isolation Reagent was used to isolate RNA according to the protocol of the manufacturer (Roche, Mannheim, Germany). SensiFAST cDNA Synthesis Kit (Bioline, London, United Kingdom) was used to prepare complementary DNA (cDNA) and SensiFAST SYBR No-ROX Kit (Bioline, London, United Kingdom) was used to measure mRNA expression. The expression levels were normalized to RPLP0 (ribosomal protein lateral stalk, subunit P0). The primers for *IL-10*, *CCL2*, *CD68*, *CD11c*, *IRS1*, *TNF*, and *IL-6* are presented in **Supplementary Table 1**.

Fecal Microbiota Transplantation

Fecal microbiota transplantation was performed on the third study day of week 0. Gastroduodenoscopy and the positioning of the nasoduodenal tube and FMT on the third day of the first week were all performed as described previously (Kootte et al., 2017). *Via* gastroduodenoscopy, a nasoduodenal tube was placed and its position was checked by an abdominal X-ray. Hereafter, bowel lavage was started with infusion of (usually 2–3 L) dissolved macrogol/electrolytes (Klean-Prep) through the nasoduodenal tube to clean the intestines of fecal material. At this point, the subject was randomized in a double-blinded fashion to receive either the feces from the assigned lean donor (allogeneic FMT) or his own feces (autologous FMT), both delivered as fresh fecal sample the same morning. An independent colleague with access to the randomization list made sure that the researcher used the (blinded) container with the assigned feces. The feces was mixed with a saline solution (0.9% NaCl) until fully homogenized and sieved to remove all debris. The obtained homogenous solution

was subsequently stored in a sterile 500-ml bottle. After complete bowel lavage, 500 ml of the dissolved fecal content was infused *via* the nasoduodenal tube. All procedures (MMT, hyperinsulinemic clamp test, and subcutaneous tissue biopsy) were performed at both week 0 and week 6, with the exception of the FMT.

Gut Microbiota Sequencing Analysis

DNA Extraction

Genomic DNA was extracted from 0.25 g of fecal samples using the repeat bead beating (RBB) method of Yu and Morrison[2], with the following modifications. Three types of sterile zirconia beads (Thistle Scientific, Glasgow, United Kingdom) were used (0.5 g in total; one 3.0-mm bead, 0.1 g of 0.5-mm beads, and 0.3 g of 0.1-mm beads). Fecal samples were homogenized three times for 60 s at maximum speed on a Mini-Beadbeater-24^{TM} (Thistle Scientific, Glasgow, United Kingdom), with the samples cooled on ice for 60 s in between bead-beating cycles. The supernatants of two bead-beating rounds were pooled and incubated with 350 μl of 7.5 M ammonium acetate (Sigma, St. Louis, MO, United States) on ice. The extraction proceeded as per the RBB protocol using Qiagen's DNeasy® Blood & Tissue Kit (Qiagen, West Sussex, United Kingdom) according to the manufacturer's instructions for the final DNA purification (without the lysis steps and eluted in 100 μl of AE buffer).

DNA Library Preparation

Genomic DNA was quantified using the Qubit dsDNA high-sensitivity assay kit (Invitrogen – Carlsbad, California, United States). Samples were prepared for shotgun metagenomic sequencing using the Illumina Nextera XT library preparation kit and following the manufacturer's instructions. Unique Nextera XT 8-nt dual indices were used for multiplexing (Illumina, San Diego, CA, United States). Libraries were pooled to an equimolar concentration and sequenced by Edinburgh Genomics (Edinburgh, United Kingdom) using a 2 × 150-bp paired-end method on an Illumina NovaSeq 6000 platform and aiming to achieve ∼4–5 Gbp of sequencing data per sample.

The raw reads were initially processed using the KneadData tool (version 0.7.2[3]) for read quality trimming, filtering, and also the removal of potential contaminant reads with the recommended settings. Microbial community composition profiling was performed using the MetaPhlAn 2.0 pipeline (Truong et al., 2015), while the HUMAnN 2.0 pipeline (Franzosa et al., 2018) was employed for functional profiling and, more specifically, for gene count determination. The resulting data from these pipelines were processed using R (version 4.0.2) (R Core Team, 2020) in the RStudio IDE (version 1.3.1093) (R Studio Team, 2020). Alpha diversity indices were computed using the *phyloseq* (version 1.34.0) (McMurdie and Holmes, 2013) and *vegan* (version 2.5–6) (Oksanen et al., 2017) packages, and beta diversity was computed using Spearman's distances between samples.

[2]https://doi.org/10.2144/04365ST04

[3]https://github.com/biobakery/kneaddata

Fecal Short-Chain Fatty Acid and Bile Acid Measurements

Fecal SCFA levels were measured using high-performance liquid chromatography (HPLC) with UV detection according to the method of De Baere (De Baere et al., 2013). In addition, for all samples, the dry weights were determined after freeze drying a homogenized fecal aliquot for 24 h. The SCFA measurements were corrected for the difference in the wet and dry weights for each sample. We also measured the concentrations of seven bile acids and the neutral sterols cholesterol, dihydrocholesterol, and coprostanol in the stool samples, as previously described (Jakulj et al., 2016).

Plasma Metabolites and GLP1 Levels

Untargeted metabolomics profiling was performed by Metabolon (Durham, NC, United States) using ultra high-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), as previously described (Koh et al., 2018). This resulted in 756 annotated plasma metabolites. Raw data were normalized and subsequently rescaled to set the median equal to 1. Missing values, mainly caused by measurements below the detection limits, were imputed. With regard to plasma GLP-1, the concentrations were determined in postprandial samples in the first 2 h of the mixed meal test (at 0, 30, and 120 min). The levels of total GLP-1 were measured by Holst group with ELISA (cat no. 10-1278-01; Mercodia, Sweden). All quality controls provided by the manufacturer were within the allowed limits. All samples from the same individual were measured in the same assay run.

Statistical Analysis

The clinical parameters measured were not normally distributed and are thus presented as medians and interquartile ranges. Non-parametric tests were used for statistical testing: Wilcoxon signed-rank test was used for within-group comparisons and the Mann–Whitney *U* test was used for between-group comparisons. Postprandial results are described as incremental area under the curves (iAUC). Statistical analyses were performed using SPSS Statistics software, version 25. P-values < 0.05 were considered statistically significant.

Statistical analyses of the microbiome and metabolome data were performed in R (R Core Team, 2020) using the RStudio IDE (R Studio Team, 2020). Statistically significant differences between multiple groups were computed employing the non-parametric Kruskal–Wallis' test, with pairwise comparisons performed using the Wilcoxon's test, using the *ggpubr* package (version 0.4.0) (Kassambara, 2020). Multiple hypothesis testing p-values were adjusted for false discovery rate using the Benjamini and Hochberg method, as indicated. Principal coordinates analysis plots were computed using the *ade4* package (version 1.7–16) (Thioulouse et al., 2018), and statistically significant differences between groups were determined employing permutational multivariate analysis of variance (MANOVA) *via* the *adonis* function from the *vegan* package (Oksanen et al., 2017). Unless indicated otherwise, p-values < 0.05 were considered statistically significant. Plots were generated in R using the *ggplot2*

(version 3.3.2) (Wickham, 2016), *ggpubr* (Kassambara, 2020), and *ComplexHeatmap* (version 2.6.0) (Gu et al., 2016) packages.

RESULTS

We included a total of 28 male metabolic syndrome subjects from November 2016 until September 2018, of whom four were excluded due to technical difficulties with clinical measurements (two subjects), non-compliance to the Mediterranean diet (one subject), and withdrawal because of personal reasons before the start of the diet (one subject). The baseline characteristics of the 24 randomized patients are presented in **Table 1**. There were no significant differences in baseline characteristics between subjects who later received an autologous or allogeneic FMT (**Table 1**). Moreover, we included five healthy subjects who served as FMT donors in multiple metabolic syndrome subjects, with eventually a range of one to four recipients of allogeneic FMT per single donor.

Adherence to Mediterranean Diet and Effects on Gut Microbiota Composition

Macronutrient intake before, during, and after the Mediterranean diet period were extracted from the self-reported online nutritional diaries (**Figure 2** and **Supplementary Table 2**). The total energy intake of the whole group during the first 2 weeks on Mediterranean diet was significantly reduced compared to that of the habitual diet [from 2,193 (1,734–2,644) to 1,820 kcal (1,602–2,009) daily, $p = 0.01$]. During the diet phase, the subjects consumed significantly less saturated fat [from 28 (19–39) to 20 g (17–23), $p = 0.01$], with an unchanged total fat intake [from 85 (68–109) to 81 g (66–100), $p = 0.67$]. The subjects also significantly reduced their total amount of carbohydrates [from 216 (167–272) to 158 g (148–191), $p = 0.01$], but increased their fiber intake [from 20 (17–25) to 24 g (23–28), $p = 0.01$]. The decrease in protein intake was not significant [from 90 (73–115) to 81 g (75–94), $p = 0.12$] (**Supplementary Table 2**). When the subjects who were later randomized to the allogeneic FMT were compared with those in the autologous FMT group, no differences in the Mediterranean diet-induced changes in energy, fat, saturated fat, carbohydrate, and fiber intakes were observed.

With regard to any effect on the gut microbiota composition due to the Mediterranean diet, the values for alpha diversity (Shannon index) and gene counts are presented in **Figures 3A,B**. No effect of the Mediterranean diet on microbiota Shannon diversity or metagenome gene count was detectable over the 2-week period from the start of the Mediterranean diet for the whole group (W−2 to W0, $p = 0.12$ and $p = 0.56$, respectively). Furthermore, no clear shift in the overall microbiome relatedness (beta diversity) was observed upon the Mediterranean diet (W−2 to W0; **Figure 3C**). Following FMT, however, we did observe a clustering according to the source of FMT (**Figure 3D**).

Although we did not observe great changes in the overall microbial species richness and composition after 2 weeks of Mediterranean diet, subsequent gut microbiota analysis demonstrated marked changes in fecal abundance of several species 2 weeks after the start of the Mediterranean diet (**Figure 4A**). Adherence to the Mediterranean diet resulted in an increased abundance of several species, including *Bacteroides* species, *Akkermansia muciniphila*, and SCFA butyrate-producing *Roseburia hominis*, as well as a reduced fecal abundance of *Collinsella aerofaciens*.

Following allogeneic FMT (from W0 to W6), we observed in the recipient fecal microbiota an increase in several bacterial species, which were also present in the healthy donor microbiota (**Figure 4C**). Moreover, a significant differential enrichment in the gut microbial species was detected between the group receiving autologous FMT or allogeneic donor FMT, with the donor-derived microbiota being enriched in *Bifidobacterium pseudocatenulatum*, *Gordonibacter pamelaeae*, and *Bacteroides*

TABLE 1 | Baseline characteristics at week−2 for the study subjects and separated by fecal microbiota transplantation (FMT) group.

	Total group ($n = 24$)	Autologous ($n = 12$)	Allogeneic ($n = 12$)
Male gender (%)	100	100	100
Age (years)	51.5 (47–58.8)	52.5 (47.3–54.8)	50.5 (46.3–60.0)
Weight (kg)	118.7 (104.2–129.3)	119.6 (108.9–129.3)	116.2 (100.2–129.4)
BMI (kg/m^2)	34.0 (31.8–37.3)	35.4 (32.7–40.0)	33.06 (30.8–37.2)
Waist circumference (cm)	120 (113–131)	121.5 (112.3–129.5)	118 (111.8–132.5)
Blood pressure: systolic (mmHg)	150 (138–89)	152 (144–172)	147 (132–156)
Blood pressure: diastolic (mmHg)	93 (89–105)	96 (91–110)	91 (84–102)
Fasting glucose (mmol/L)	5.8 (5.5–6.4)	5.90 (5.53–6.36)	5.75 (5.53–6.53)
Insulin (pmol/L)	102 (62–124)	104 (67.2–120.8)	91 (53.1–192.2)
HOMA-IR	3.7 (2.3–4.6)	3.65 (2.63–4.48)	3.55 (2.20–7.23)
HbA1c (mmol/mol)	40 (36–41)	39.5 (37–40)	40 (36–41)
Cholesterol: total (mmol/L)	5.7 (4.6–6.3)	5.97 (4.80–6.36)	5.16 (4.59–6.24)
Cholesterol: HDL (mmol/L)	1.3 (1.1–1.5)	1.28 (0.97–1.52)	1.32 (1.10–1.45)
Cholesterol: LDL (mmol/L)	3.6 (2.9–4.2)	4.08 (2.69–4.48)	3.12 (2.92–4.12)
Cholesterol: triglycerides (mmol/L)	1.3 (1.0–1.8)	1.35 (1.15–2.12)	1.32 (1.01–1.77)

Data are expressed as medians and interquartile ranges. There were no statistically significant differences in the baseline characteristic between subjects who later received an autologous or allogeneic FMT.

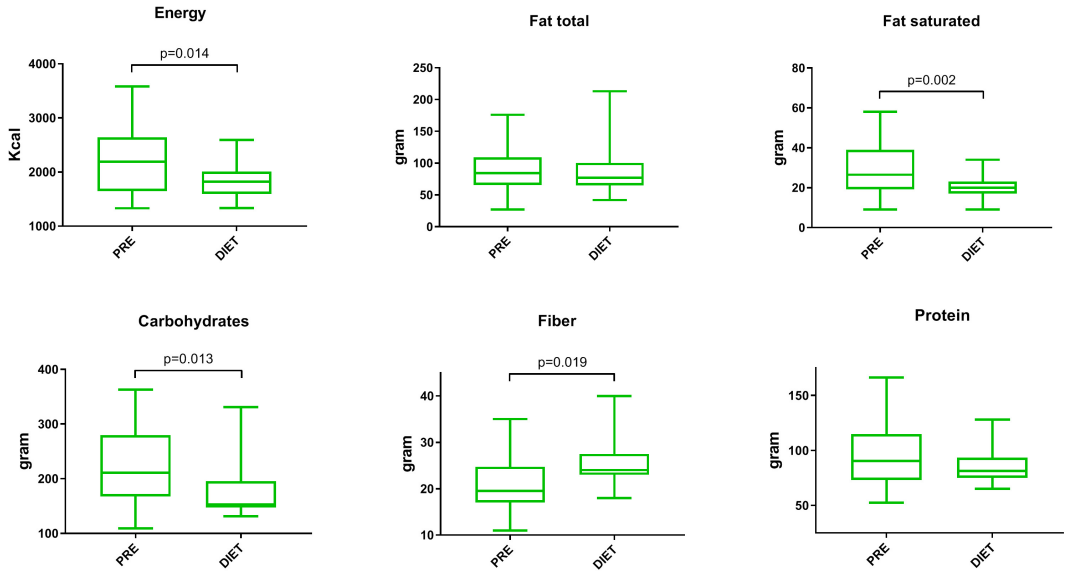

FIGURE 2 | Changes in macronutrient intake following the Mediterranean diet. Changes in the daily macronutrient intake comparing the habitual diet (*PRE*) to the Mediterranean diet (*DIET*). For comparison, a paired Wilcoxon signed-rank test was used. Only statistically significant *p*-values are shown. *Box* and *whisker*, min–max.

dorei and deprived in *Desulfovibrio piger* with respect to autologous microbiota (**Figures 4B,C**).

Effect of Combining FMT With Mediterranean Diet on Metabolic Parameters

Changes in the metabolic parameters caused by the Mediterranean diet alone (from W−2 to W0) and, subsequently, the addition of the FMT (from W0 to W6) are presented in **Table 2**, with a subset of markers visualized in **Figure 5**. In the first 2 weeks of the diet, before the FMT, the body weight of the subjects significantly decreased [118.7 kg (104.2–129.3) at baseline vs. 117.7 kg (100.6–127.1) after a 2-week diet, $p < 0.001$]. Likewise, we found a significant reduction in the fasting blood glucose level [from 5.8 (5.5–6.4) to 5.6 mmol/L (5.3–6.2), $p = 0.02$], a numeric, but non-significant, decrease in the fasting insulin levels [from 102 (62–124) to 83 (57–107), $p = 0.25$], and a borderline significant decrease in homeostatic model assessment of insulin resistance (HOMA-IR) [from 3.7 (2.3–4.6) to 2.8 (2.2–3.8), $p = 0.056$]. Moreover, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were all significantly reduced after 2 weeks of Mediterranean diet intake. Similarly, dietary intervention diminished the total leukocyte counts and the systemic liver injury markers gamma-glutamyl transferase (GGT) and alkaline phosphatase (AP). After randomization to either an autologous or allogeneic FMT (from W0 to W6), both groups continued losing a significant amount of weight. The glucose levels remained stable, as well as the insulin levels and HOMA-IR. There was a small but statistically significant reduction in glycated hemoglobin HbA1c in the 6 weeks after both allogeneic and autologous FMT (8 weeks after starting the diet; **Table 2**).

Lastly, as low-grade systemic inflammation is an important pathologic feature of insulin resistance and adiposity, we examined the impact of FMT on inflammation at the systemic (plasma) and local (subcutaneous adipose tissue biopsies) levels. Independently of the source, FMT did not lead to alterations in circulating C-reactive protein (CRP) and leukocyte count (**Table 2**), nor did it affect the expressions of the inflammatory genes (*IL6*, *IL10*, *CD68*, *CCL2*, *IRS1*, *CD11c*, and *TNFA*) within subcutaneous AT (**Supplementary Table 1** and **Supplementary Figure 1**).

Effect of FMT on Insulin Sensitivity and Postprandial Metabolism

Next, we evaluated the impact of FMT interventions on peripheral and hepatic insulin sensitivity. Peripheral insulin sensitivity, assessed as the insulin-induced glucose disposal rate (R_d), was unaltered after either autologous or donor FMT (**Supplementary Figure 2A**): autologous R_d from 39.7 (33.2–53.1) to 44.4 μmol kg^{-1} min^{-1} (32.4–56.2, $p = 0.88$); allogeneic R_d from 41.0 (24.0–48.3) to 41.7 μmol kg^{-1} min^{-1} (34.8–49.5, $p = 0.48$). Similarly, hepatic insulin sensitivity measured as insulin-mediated suppression of endogenous glucose production (EGP) did not change after autologous [from 72.9% (61.7–81.4) to 65.2% (43.3–85.2, $p = 0.35$)] or allogeneic FMT [from 70.1% (62.9–75.4) to 67.2% (43.0–84.00, $p = 0.53$)] (**Supplementary Figure 2B**).

We further performed correlation analysis to study the link between the FMT-induced changes in metabolic markers (weight, cholesterol levels, HOMA-IR, fasting blood glucose, and R_d) and the Bray–Curtis distance, a measure of microbiome dissimilarity

TABLE 2 | Clinical parameters expressed as medians and interquartile ranges.

	Diet only			Diet + autologous FMT			Diet + allogeneic FMT		
	W−2 (*n* = 24)	**W0 (*n* = 24)**	***p***	**W0 (*n* = 12)**	**W6 (*n* = 12)**	***p***	**W0 (*n* = 12)**	**W6 (*n* = 12)**	***p***
Weight (kg)	118.7 (104.2–129.3)	117.7 (100.6–127.1)	0.01	118.6 (106.9–127.1)	111.9 (105.3–123.7)	0.01	115.8 (97.6–128.5)	114.5 (95.1–124.4)	**0.01**
Fasting glucose (mmol/L)	5.8 (5.5–6.4)	5.6 (5.3–6.2)	**0.02**	5.6 (5.0–6.0)	5.5 (5.3–6.1)	0.62	5.9 (5.3–6.3)	5.6 (5.3–6.2)	0.40
Insulin (pmol/L)	102 (62–124)	83 (57–107)	0.2	81 (61–97)	69 (61–98)	0.46	86 (50–140)	100 (57–149)	0.72
HOMA-IR	3.7 (2.3–4.6)	2.8 (2.2–3.8)	**0.05**	2.7 (2.2–3.6)	2.5 (1.9–3.5)	0.64	3.0 (2.0–5.0)	3.5 (2.2–5.1)	0.39
HbA1c (mmol/mol)	40 (36–41)	39 (37–40)	0.2	38 (36–39)	36 (34–38)	**0.01**	40 (38–42)	37 (36–40)	**0.01**
Cholesterol: total (mmol/L)	5.7 (4.6–6.3)	4.7 (4.4–5.4)	**0.01**	4.7 (4.4–5.2)	4.5 (4.0–5.7)	0.2	4.6 (4.4–5.4)	4.4 (4.0–5.1)	**0.02**
Cholesterol: HDL (mmol/L)	1.3 (1.1–1.5)	1.1 (1.0–1.2)	**0.01**	1.1 (0.9–1.4)	1.2 (0.9–1.4)	0.50	1.1 (1.0–1.2)	1.1 (0.9–1.3)	0.53
Cholesterol: LDL (mmol/L)	3.6 (2.9–4.2)	3.0 (2.5–3.6)	**0.01**	3.1 (2.5–3.5)	3.1 (2.2–3.8)	0.43	3.0 (2.4–3.6)	2.9 (2.4–3.4)	0.06
Cholesterol: triglycerides (mmol/L)	1.3 (1.0–1.8)	1.2 (1.0–1.6)	0.5	1.3 (1.0–1.6)	0.9 (0.8–1.5)	0.07	1.2 (1.0–1.6)	1.1 (0.8–1.3)	0.31
CRP (mg/L)	2.2 (1.2–5.3)	2.6 (1.6–4.0)	0.96	2.6 (1.6–3.8)	2.5 (1.9–3.9)	0.66	2.7 (1.7–5.9)	3.5 (1.1–6.0)	0.37
Leukocytes (10^9/L)	6.8 (5.5–7.6)	6.3 (4.7–7.3)	**0.01**	6.2 (4.9–7.2)	5.5 (4.7–6.3)	0.25	6.5 (4.2–7.9)	6.7 (4.7–8.7)	0.28
AP (U/L)	75 (68–96)	74 (63–84)	**0.01**	74 (65–91)	74 (63–88)	0.06	73 (58–78)	73 (62–84)	0.15
γ-GT (U/L)	38 (34–61)	37 (27–55)	**0.01**	30 (22–41)	27 (19–42)	0.24	49 (29–75)	44 (25–60)	0.08
ASAT (U/L)	25 (21–28)	26 (24–31)	0.16	26 (24–31)	26 (22–31)	0.86	27 (23–32)	25 (23–26)	**0.03**
ALAT (U/L)	32 (22–39)	33 (27–43)	0.28	33 (27–26)	30 (23–33)	**0.02**	37 (23–46)	32 (20–44)	**0.02**

Effect of Mediterranean diet only (W−2 vs. W0, whole group) and with the addition of fecal microbiota transplantation (FMT) (W0 vs. W6, split into autologous FMT and allogeneic FMT). There were no significant differences in any of the parameters between the autologous and allogeneic FMT groups. Significant differences ($p < 0.05$) emphasized in bold.

(from W0 to W6). We found a positive correlation between the Bray–Curtis distances and the changes in R_d in only the allogeneic FMT group ($\rho = 0.622$, $p = 0.03$; **Supplementary Figure 3**), whereas no other significant correlations were noted between variations in the metabolic markers and microbiota diversity.

Finally, we did not observe differences in the postprandial triglyceride and GLP1 levels between week 0 and week 6 in either the autologous or the allogeneic FMT group, measured during a 2-h mixed meal test (**Supplementary Figures 4**, **5**).

Effect of Treatments on Fecal Bile Acids and Short-Chain Fatty Acids

To measure fecal cholesterol elimination, the cholesterol and bile acid levels were assessed in 24-h fecal samples at week −2, week 0, and week 6 (**Supplementary Table 3**). After 2 weeks of Mediterranean diet, we observed a substantial reduction in the 24-h fecal excretion levels of cholesterol ($p < 0.001$), dihydrocholesterol ($p < 0.001$), and coprostanol ($p = 0.01$; from W−2 to W0) (**Supplementary Figure 6**). However, neither form of FMT (autologous or allogeneic) induced further reductions (from W0 to W6) (**Supplementary Table 3**). Furthermore, no significant changes were detected in the fecal concentrations of SCFA acetate, propionate, and butyrate at any of the three time points (**Supplementary Table 3**).

Impact of Mediterranean Diet and FMT Interventions on Plasma Metabolome

The Mediterranean diet induced significant changes in the levels of several fasting plasma metabolites (from W−2 to W0; **Figure 6**). For example, acetylphenylalanine, *N*-palmitoyl-sphingoadenine, and suberoylcarnitine were significantly increased after 2 weeks of Mediterranean diet. In contrast, ethyl glucuronide, a metabolite of ethanol, was decreased. It is not clear whether this ethanol may be of endogenous (microbial) source. Interestingly, the plant sterol campesterol, as well as derivatives of plasma amino acid metabolites lysine, leucine, and isoleucine were decreased upon Mediterranean diet. However, treatment with either autologous or allogeneic donor FMT did not differentially affect the overall plasma metabolite composition (**Supplementary Figure 7**). In contrast to the donor bacterial strain engraftment, we did not see that specific metabolites were transferred by the donor FMT, nor did we observe significant differences in the plasma metabolite levels at W6 between the autologous and allogeneic FMT groups (data not shown).

DISCUSSION

In this double-blind randomized trial, we investigated the potential synergistic effects of combining a Mediterranean diet with lean donor FMT on the gut microbiota composition, hepatic and peripheral insulin sensitivity, and plasma metabolites in treatment-naive obese subjects with metabolic syndrome. We confirm that a Mediterranean diet *per se* has a beneficial effect on metabolic parameters and results in increased relative abundances of several beneficial bacterial strains including

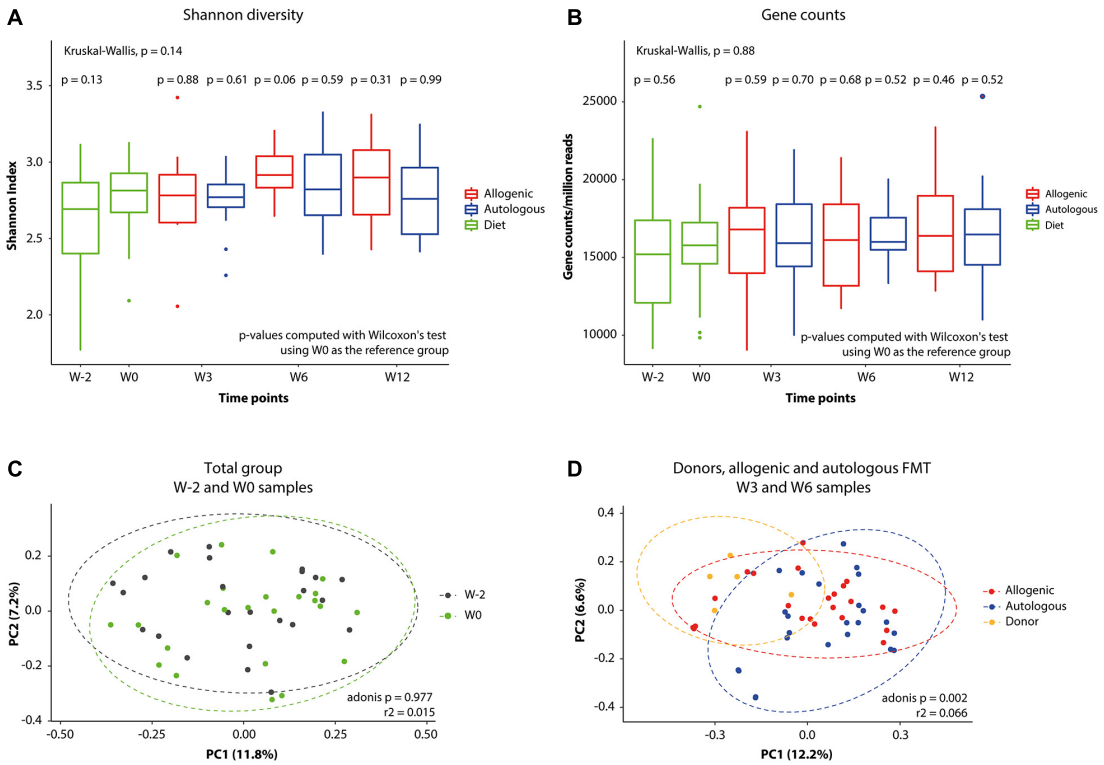

FIGURE 3 | Changes in gut microbiota global measures. Differences in fecal microbiota alpha diversity. **(A)** Shannon index values. **(B)** Normalized gene counts of bacterial species. **(C,D)** Principal coordinates analysis (PCoA) plots graphically representing the microbiota compositional changes after **(C)** Mediterranean diet (from W–2 to W0) and after **(D)** fecal microbiota transplantation (FMT) on top of the Mediterranean diet.

A. muciniphila. The combination of this diet with lean donor FMT seemingly resulted in the engraftment of some specific FMT donor-derived bacterial species, including *Bifidobacterium* and *Bacteroides* (primary endpoint), yet failed to improve insulin sensitivity (secondary endpoint). Therefore, further studies are warranted to explore whether a longer adherence time to a Mediterranean diet or other beneficial diets (e.g., vegan or low-protein diet) together with multiple healthy donor FMTs can provide significant and durable clinical metabolic improvements to metabolic syndrome subjects.

Introduction of the Mediterranean diet was associated with alterations in specific fecal bacterial species including an increase in the levels of *A. muciniphila*. This well-studied bacterial species has previously been linked with improved glucose and cholesterol metabolism (Dao et al., 2016; Depommier et al., 2019) as well as healthy and Mediterranean diets (Liu et al., 2019; Rinott et al., 2020). Moreover, the increase in the butyrate-producing *R. hominis* corroborates other studies on Mediterranean diets (Haro et al., 2016; Ghosh et al., 2020; Meslier et al., 2020; Rinott et al., 2020) and also has been associated with beneficial effects on glucose homeostasis (Karlsson et al., 2013; Zhang et al., 2013; Meslier et al., 2020). The decrease in *C. aerofaciens* levels is in line with previous studies showing increased levels associated with DM2 and high serum cholesterol (Lahti et al., 2013; Lambeth et al., 2015) and low dietary fiber intake (Gomez-Arango et al., 2018). Upon FMT, we observed the engraftment of healthy donor-derived bacterial strains, including increased *B. pseudocatenulatum* and *G. pamelaeae* as well as *B. dorei*, which are all linked to anti-inflammatory and beneficial metabolic effects (Moya-Pérez et al., 2015; Selma et al., 2017; Yoshida et al., 2018; Sanchis-Chordà et al., 2019). In contrast, following donor FMT, decreased levels of the opportunistic pathogen *D. piger* often seen in DM2 were observed in recipients (Qin et al., 2012; Doumatey et al., 2020). Despite the increased fiber intake, the Mediterranean diet did not result in increased fecal SCFA levels in this study. Although a Mediterranean diet has previously been associated with higher fecal SCFA levels (De Filippis et al., 2015), others corroborate our findings (Wu et al., 2016; Meslier et al., 2020). Wu et al. (2016) proposed the concept of a restrictive microbiota associated with westernized societies as an explanation for the lack of increased SCFA formation despite additional substrate intake. Moreover, the unchanged fecal SCFA levels could be caused by an increased utilization or rapid absorption by the colonocytes (Sakata, 2019).

Our group previously demonstrated an increase in peripheral insulin sensitivity (R_d) upon lean donor FMT (Vrieze et al., 2012; Kootte et al., 2017) while subjects were adhering to their habitual (omnivorous) diet. To our surprise, we observed no synergistic effect of combining Mediterranean diet with lean donor FMT on metabolic parameters including peripheral and hepatic insulin sensitivity, although a large standard deviation (**Supplementary Figure 2**) was observed, suggesting responders

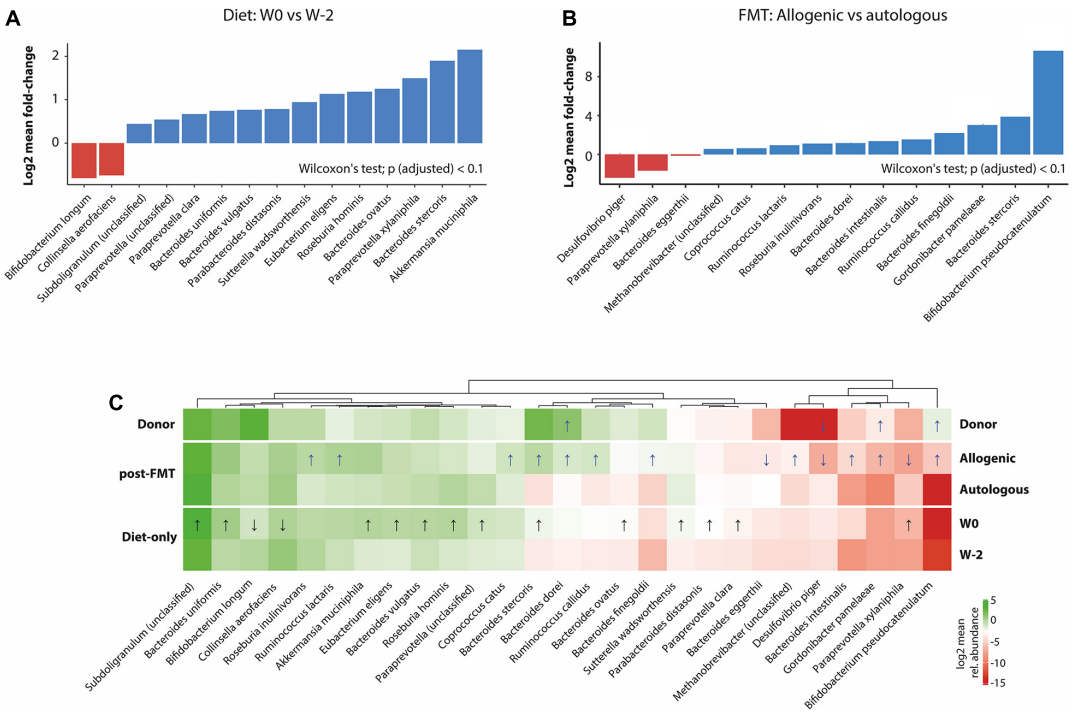

FIGURE 4 | Alterations in the gut microbiota associated with treatments. **(A)** Significant changes ($p_{adjusted} < 0.1$) in the species relative abundance after the Mediterranean diet (W0 vs. W2). **(B)** Differences ($p_{adjusted} < 0.1$) between the allogeneic and autologous fecal microbiota transplantation (FMT) groups. **(C)** Heat map representing the log2-transformed 10% trimmed means of the bacterial species relative abundances in the different groups (donor, allogeneic FMT, autologous FMT, W0, and W–2). Only showing species with a statistically significant differential abundance between at least two groups (Wilcoxon's test of significance with a $p_{adjusted} < 0.1$), after filtering out rare taxa (at least 0.01% abundance in at least 50% of the samples within each group). Statistically significant differential abundance values between W0 vs. W–2 (diet effect) are indicated with *black arrows*, while those between donor and allogeneic vs. autologous (W3, W6, and W12) (FMT effect) are indicated by *blue arrows*. Directionality of differential abundance (greater or less) is indicated either with an *arrow pointing up* or *arrow pointing down*, respectively. Interestingly, when comparing the donor and the allogeneic groups with the autologous group, some overlap is observed, including the direction of differential abundance, strongly indicating that these species could have been acquired from the donor group or are being antagonized by species acquired from the donor group.

and non-responders upon this combined intervention. However, the sample size precluded further *post hoc* analyses. Previous data have demonstrated that the effect of a FMT is controlled by the baseline microbiota composition (Li et al., 2016; Kootte et al., 2017) and that subjects with a low baseline diversity have greater metabolic improvement upon interventions (Cotillard et al., 2013; Kootte et al., 2017; Yu et al., 2020). Despite the short-period of dietary intervention (as compared to other studies, e.g., those of Meslier et al. (2020) with 8 weeks of Mediterranean diet intervention and Ghosh et al., 2020 with 1 year), the Mediterranean diet intake provoked clear changes in the gut microbiota composition, whereas the introduction of a healthy donor microbiota resulted in modest changes in the microbiome and did not significantly increase the microbiome species diversity.

Thus, we postulate that the diet-induced changes in the gut microbiota (enrichment in the beneficial commensal species *Bacteroides*, *A. muciniphila*, and *R. hominis*) and metabolic profiles (decrease in fasting glucose, cholesterol, body weight, and a trend toward lower HOMA-IR index) render the host less susceptible to FMT-induced changes and therefore attenuates the (previously observed) beneficial effect of a lean donor FMT on both hepatic and peripheral insulin sensitivity. We hypothesize that the gut microbial system is "locked" after the introduction of a beneficial Mediterranean diet, precluding additional beneficial metabolic effects of donor FMT. Indeed, a higher biodiversity in an ecosystem is associated with increased resilience, thus higher tolerance to environmental perturbations (in this case represented by engraftment of exogenous bacterial species) (Mosca et al., 2016). This hypothesis is underscored by the fact that only a few donor strains were able to engraft in the gut as compared to other studies without dietary interventions prior to FMT (Li et al., 2016). Finally, we observed a significant positive correlation between the changes (between W0 and W6) in the Bray–Curtis distance, a measure of microbiome dissimilarity, and insulin sensitivity (R_d changes) solely after lean donor FMT and not in the autologous FMT group. This indicates that, when the microbiota composition/diversity still exhibits room for change, we do observe greater alterations in insulin sensitivity after lean donor FMT.

Moreover, we cannot exclude that the difference in the effects of allogeneic or autologous FMT is masked by the introduction of a "less diabetogenic" microbiota in the autologous FMT group after 2 weeks on Mediterranean diet. In line with this,

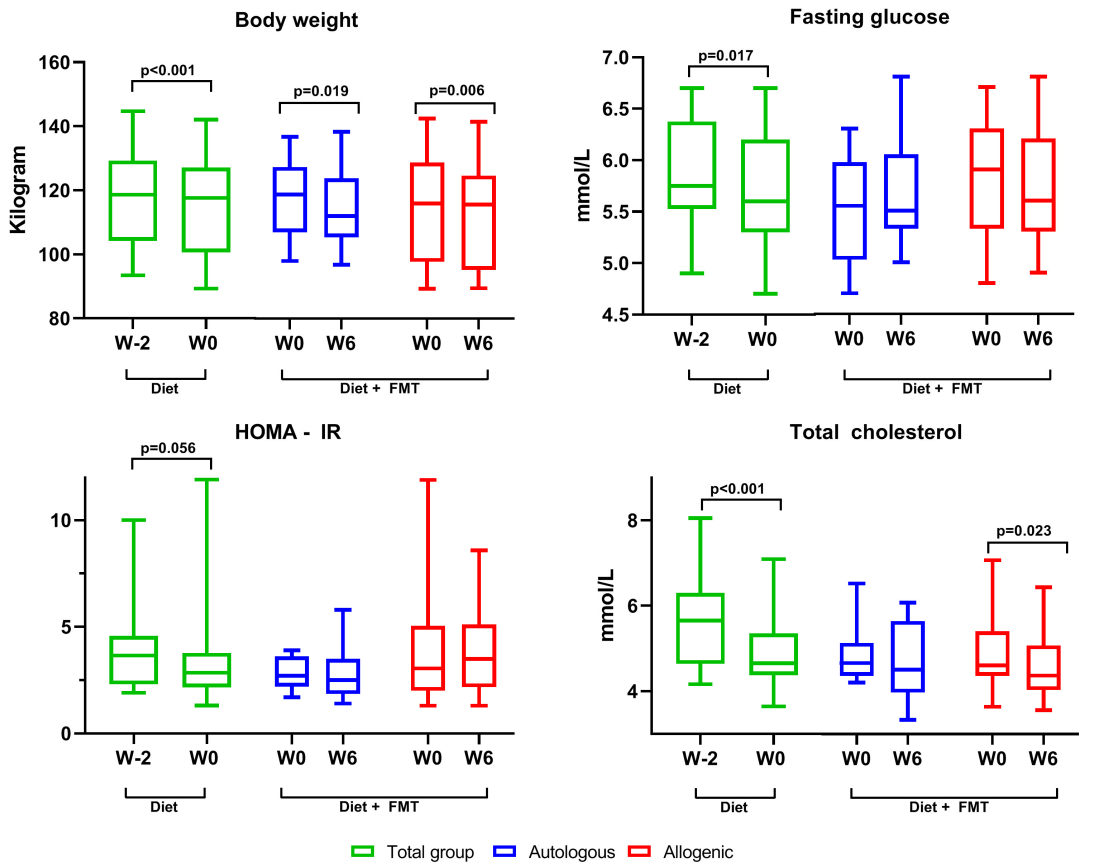

FIGURE 5 | Changes in the clinical parameters. This figure shows changes in a subset of the clinically relevant parameters. In *green* are the changes in the first 2 weeks after initiation of the Mediterranean diet (for the whole group, since the subjects were not yet randomized). In *blue* and *red* are the changes after autologous or allogeneic fecal microbiota transplantation (FMT), respectively. *Box* and *whisker*, min–max. Only statistically significant *p*-values are shown. There were no significant differences between the autologous and allogeneic groups.

Rinott et al. (2020) reported that autologous FMT utilizing fecal samples collected at the end of a 6-month Mediterranean diet intervention ameliorated the metabolic profiles of obese dyslipidemic patients until 8 months afterward. Of note is that the microbiome intervention in the present study consisted of a single fresh (non-processed) duodenal lean donor FMT, in line with previous studies from our group (Vrieze et al., 2012; Kootte et al., 2017). More recent microbiota intervention studies in obese populations, however, investigated the effect of oral encapsulated (processed) feces, repeated transplantations, and the usage of multiple donors (Allegretti et al., 2020; Leong et al., 2020; Yu et al., 2020). Although the metabolic effects in these studies were variable, the microbial shifts might be more pronounced and durable. Perhaps, in the present study, a more robust microbiota intervention (e.g., multiple FMTs or lean donor FMTs prior to the dietary intervention) could have resulted in a more distinct effect of the lean donor microbiota on top of the rigorous change in diet.

It is known that different levels of plasma metabolites corroborate with the changes in food intake. Hereof, the plasma levels of lysine, leucine, and isoleucine derivatives (being the amino acids mainly found in meat and cheese) were significantly lowered after 2 weeks of Mediterranean diet. In line with this, previous studies also described a decrease in branched-chain amino acids after a Mediterranean or a vegan diet (Draper et al., 2018; Meslier et al., 2020).

Similarly to what we observed for the metabolic parameters after FMT, no additive effects of FMT on plasma metabolome were detected, indicating that the Mediterranean diet-induced changes in the metabolic profiles overrides the effect of donor bacterial species engraftment. The observed decrease in fecal plant sterol levels (most specifically campesterol) suggests a change in intestinal cholesterol absorption, which was surprising in view of the major decrease in total sterol excretion. Although the Mediterranean diet is low in cholesterol, the observed decrease in the total sterol output was more than 1,000 mg/day (**Supplementary Figure 4**). As a normal cholesterol intake amounts to around 300 mg/day, this changed intake cannot account for the effect, and we therefore hypothesize that cholesterol synthesis itself must have decreased, accounting for the decrease in the plasma levels of total cholesterol, LDL, and HDL. In contrast to the recent study of Meslier et al. (2020), we did not observe changes in the fecal bile acid output. Perhaps a 2-week exposure to the Mediterranean diet was not enough

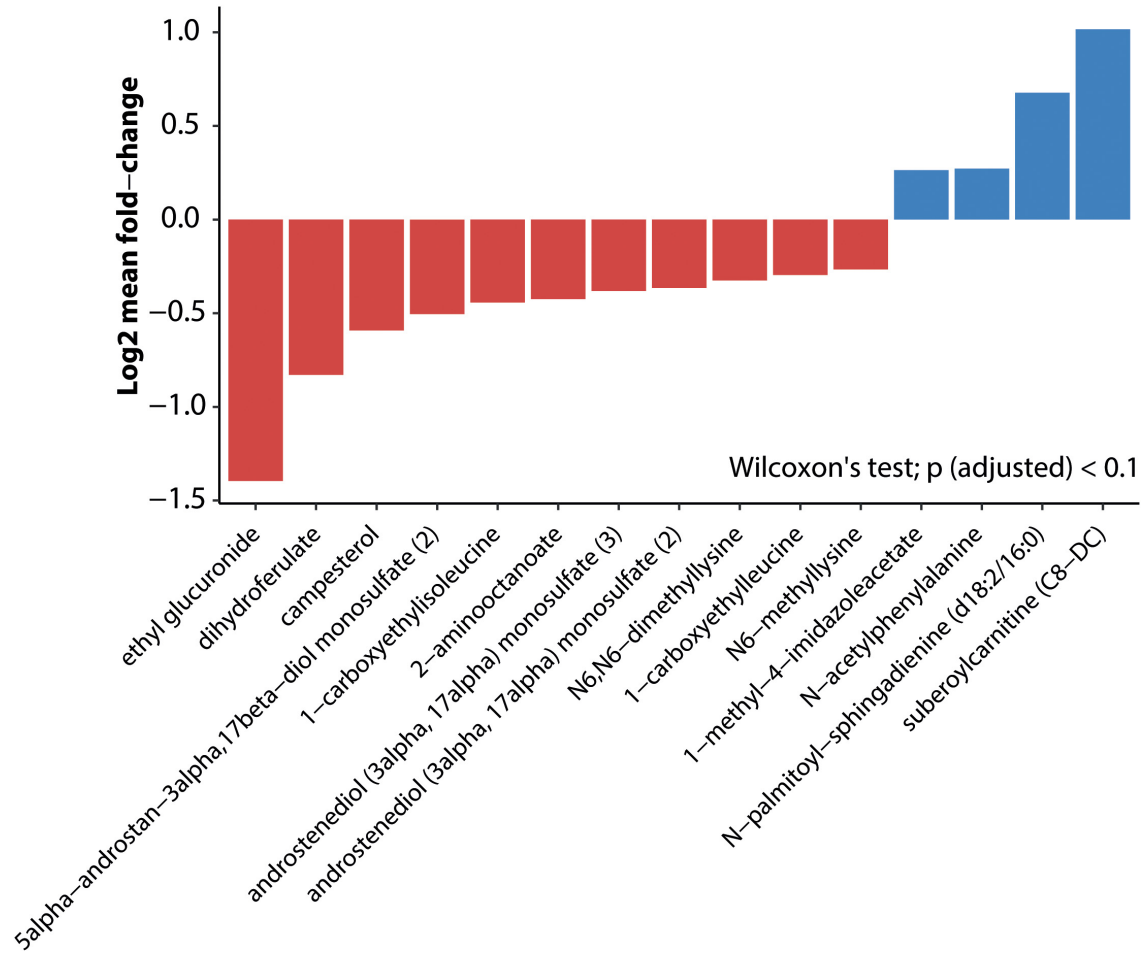

FIGURE 6 | Mediterranean diet-induced changes in plasma metabolites. Log2 mean fold changes in plasma metabolites that differ significantly ($p_{adjusted} < 0.1$) between W0 (before diet) and W2 (2 weeks after initiation of the Mediterranean diet).

to translate the decreased cholesterol synthesis to a decrease in bile acid synthesis.

Our study has several limitations. Firstly, a notable difference between our current and previous FMT studies is the insulin sensitivity prior to the administration of FMT (Vrieze et al., 2012; Kootte et al., 2017). Although these former studies had comparable inclusion criteria and the metabolic profiles at baseline were largely comparable, the median R_d after 2 weeks of Mediterranean diet just before applying donor FMT (week 0) was much higher in the current study (25.8 and 26.2 vs. 41.0 μmol kg^{-1} min^{-1}), although still in the range found in obese individuals (Vrieze et al., 2012; Ter Horst et al., 2015). In line with the significant increase in HOMA-IR between week −2 and week 0, we can speculate that this higher R_d results from a 2-week Mediterranean diet consumption. Nonetheless, ideally, we should have determined insulin sensitivity at W−2 as well (thus, before starting the Mediterranean diet) in order to test this hypothesis; however, due to ethical restrictions, we were only allowed to perform the hyperinsulinemic–euglycemic clamps twice. Secondly, in line with our previous studies (Vrieze et al., 2012; Kootte et al., 2017), we included Caucasian male subjects only. Although this was done to minimize the well-known effects of ethnicity and sex (hormones) on the gut microbiota composition (Deschasaux et al., 2018; Yoon and Kim, 2021), this may have reduced generalization to the general population. Thirdly, although the subjects were monitored by a hospital dietitian for dietary adherence, data on the actual dietary intake were retrieved from self-reported online nutritional diaries. Prior studies have demonstrated that self-administered dietary records may underestimate the energy intake in overweight and obese individuals (Lichtman et al., 1990; Schoeller, 1995; Bedard et al., 2004; Freedman et al., 2014). However, as all dietary records were checked by the dietitian on the same week, abnormalities were discussed immediately with the subject and corrected if necessary. Finally, despite the fact that no total calorie restriction was promoted, we observed a decrease in total energy intake in the first 2 weeks. Although the total amount of weight loss in these weeks was

not that pronounced and other studies corroborate the beneficial metabolic health effects of the Mediterranean diet (including non-calorie-restricted diets) (Salas-Salvadó et al., 2011; Estruch et al., 2018), the decreased total energy intake *per se* may have influenced the metabolic outcomes.

CONCLUSION

We confirm previous studies showing a beneficial effect of a Mediterranean diet on metabolic markers and gut microbiota composition in metabolic syndrome subjects. Although we observed (donor FMT-induced) engraftment of some bacterial species in the feces on top of this diet, we did not observe synergistic beneficial metabolic effects in this small randomized controlled trial. Therefore, these data warrant further study by combining specific diets with more frequently administered and/or different microbiome interventions (like repeated FMTs, specific bacterial species, or donor selection) to optimize microbiota changes and potential metabolic effects. Alternatively, given the previous indications that a low baseline microbiota diversity results in a greater metabolic response, pretreatment with antibiotics before microbial intervention is still an interesting, although challenging, concept.

DATA AVAILABILITY STATEMENT

The data have been deposited at the European Nucleotide Archive (ENA) under the accession number PRJEB44237.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by METC AMC (Amsterdam University Medical Center, Amsterdam, Netherlands). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AK, AG, and MN designed the study. AK, IA, JW, WF, ER, JH, SM, MK, JL, AS, HH, TS, LD, BH, JH, and PO'T performed the research. AK and EA performed the statistical analysis. AK, AG, and MN drafted the manuscript. All authors critically reviewed the manuscript.

FUNDING

This study was supported by a JPI HDHL consortium grant (DINAMIC; Diet-Induced Arrangement of the Gut Microbiome for Improvement of Cardiometabolic Health 529051011).

ACKNOWLEDGMENTS

We would like to thank J. A. Haverkamp (dietitian, Amsterdam UMC) for dietary assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.662159/full#supplementary-material

Supplementary Figure 1 | Subcutaneous tissue inflammation. Change in gene expression in subcutaneous adipose tissue between week 0 and week 6 for both the autologous and allogeneic FMT group. Each line represents an individual subject. None of the genes in our panel changed significantly (rest of the genes not shown). CCL2, chemokine-ligand 2; IL-10, interleukin-10; IRS1, insulin receptor substrate 1; CD11, cluster of Differentiation 11, TNF, tumor necrosis factor; IL-6, interleukin-6, CD68, cluster of differentiation 68.

Supplementary Figure 2 | Peripheral and hepatic insulin sensitivity. This figure shows changes in insulin sensitivity between weeks 0 and 6 for both the autologous and allogeneic treatment group. In both groups there were no statistically significant changes in peripheral (R_d) or hepatic (endogenous glucose production –EGP- suppression) insulin sensitivity. There were also no differences between the autologous and allogeneic FMT group. Box and whisker, 10–90th percentile.

Supplementary Figure 3 | Correlation change in R_d and Bray–Curtis distance. This figure depicts the positive correlation between Bray Curtis distance (a measure of microbiome dissimilarity) and change in R_d (a measure of peripheral insulin sensitivity) in only the allogeneic FMT group (Rho 0.622, $p = 0.03$).

Supplementary Figure 4 | Postprandial triglycerides. This figure shows triglyceride excursions after mixed meal for week 0 and week 6 for both autologous and allogeneic FMT group (line plot, median + IQR). The box and whisker plots (min–max) on the right represent area under curves (AUC) for both groups at the different time points. There were no significant differences between the two groups and within the groups at any time point.

Supplementary Figure 5 | Postprandial GLP-1. This figure shows GLP-1 excursions after mixed meal for week 0 and week 6 for both autologous and allogeneic FMT group (line plot, median + IQR). The box and whisker plots (min–max) on the right represent area under curves (AUC) for both groups at the different time points. There were no significant differences between the two groups and within the groups at any time point.

Supplementary Figure 6 | 24 h fecal sterol excretion. This figure shows 24-h fecal cholesterol, coprostanol and DiH-cholesterol excretion in the first 2 weeks of the Mediterranean diet (whole group). Box and whisker plots, min–max.

Supplementary Figure 7 | Fecal microbiota transplantation (FMT) induced changes in plasma metabolome. Effect of treatment with either autologous **(A)** or allogeneic donor **(B)** FMT on overall plasma metabolite composition.

Supplementary Table 1 | Primers used to measure the relative expression of *IL-10, CCL2, CD68, CD11c, IRS1, TNF,* and *IL-6*.

Supplementary Table 2 | Macronutrient intake before-, during- and after the Mediterranean diet, assessed using self-reported only nutritional diaries. Diet-induced changes in energy, fat, saturated fat, carbohydrate and fiber intake did not significantly differ between the two FMT groups.

Supplementary Table 3 | Fecal bile acid, -cholesterol and -short chain fatty acid concentrations, expressed as medians and interquartile ranges. Effect of Mediterranean diet only (W−2 to W0, whole group) and with addition of FMT (W0 to W6, split to autologous FMT and allogeneic FMT). There were no statistically significant differences in any of the above parameters between the autologous and allogeneic group at any time point.

REFERENCES

Alberti, K. G. M. M., Zimmet, P., and Shaw, J. (2006). Metabolic syndrome – a new world-wide definition. A consensus statement from the International Diabetes Federation. *Diabet. Med.* 23, 469–480. doi: 10.1111/j.1464-5491.2006.01858.x

Allegretti, J. R., Kassam, Z., Mullish, B. H., Chiang, A., Carrellas, M., Hurtado, J., et al. (2020). Effects of fecal microbiota transplantation with oral capsules in obese patients. *Clin. Gastroenterol. Hepatol.* 18, 855–863.e2. doi: 10.1016/j.cgh.2019.07.006

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., et al. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15718–15723. doi: 10.1073/pnas.0407076101

Bedard, D., Shatenstein, B., and Nadon, S. (2004). Underreporting of energy intake from a self-administered food-frequency questionnaire completed by adults in Montreal. *Public Health Nutr.* 7, 675–681. doi: 10.1079/phn2003578

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., Kump, P., Satokari, R., et al. (2017). European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* 66, 569–580. doi: 10.1136/gutjnl-2016-313017

Castaner, O., Goday, A., Park, Y., Lee, S., Magkos, F., Shiow, S. T. E., et al. (2018). Review article the gut microbiome profile in obesity: a systematic review. *Int. J. Endocrinol.* 2018:4095789.

Clarke, S. F., Murphy, E. F., O'Sullivan, O., Lucey, A. J., Humphreys, M., Hogan, A., et al. (2014). Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* 63, 1913–1920. doi: 10.1136/gutjnl-2013-306541

Cotillard, A., Kennedy, S. P., Kong, L. C., Prifti, E., Pons, N., Le Chatelier, E., et al. (2013). Dietary intervention impact on gut microbial gene richness. *Nature* 500, 585–588. doi: 10.1038/nature12480

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., et al. (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* 65, 426–436. doi: 10.1136/gutjnl-2014-308778

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi: 10.1038/nature12820.Diet

De Baere, S., Eeckhaut, V., Steppe, M., De Maesschalck, C., De Backer, P., Van Immerseel, F., et al. (2013). Development of a HPLC-UV method for the quantitative determination of four short-chain fatty acids and lactic acid produced by intestinal bacteria during in vitro fermentation. *J. Pharm. Biomed. Anal.* 80, 107–115. doi: 10.1016/j.jpba.2013.02.032

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., et al. (2015). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut* 65, 1812–1821. doi: 10.1136/gutjnl-2015-309957

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14691–14696. doi: 10.1073/pnas.1005963107

De Groot, P., Scheithauer, T., Bakker, G. J., Prodan, A., Levin, E., Khan, M. T., et al. (2019). Donor metabolic characteristics drive effects of faecal microbiota transplantation on recipient insulin sensitivity, energy expenditure and intestinal transit time. *Gut* 69, 502–512. doi: 10.1136/gutjnl-2019-318320

Depommier, C., Everard, A., Druart, C., Plovier, H., Van Hul, M., Vieira-Silva, S., et al. (2019). Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat. Med.* 25, 1096–1103. doi: 10.1038/s41591-019-0495-2

Deschasaux, M., Bouter, K. E., Prodan, A., Levin, E., Groen, A. K., Herrema, H., et al. (2018). Depicting the composition of gut microbiota in a population with varied ethnic origins but shared geography. *Nat. Med.* 24, 1526–1531. doi: 10.1038/s41591-018-0160-1

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11971–11975. doi: 10.1073/pnas.1002601107

Doumatey, A. P., Adeyemo, A., Zhou, J., Lei, L., Adebamowo, S. N., Adebamowo, C., et al. (2020). Gut microbiome profiles are associated with type 2 diabetes in urban Africans. *Front. Cell. Infect. Microbiol.* 10:63. doi: 10.3389/fcimb.2020.00063

Draper, C. F., Vassallo, I., Di Cara, A., Milone, C., Comminetti, O., Monnard, I., et al. (2018). A 48-hour vegan diet challenge in healthy women and men induces a BRANCH-chain amino acid related, health associated, metabolic signature. *Mol. Nutr. Food Res.* 62:1700703. doi: 10.1002/mnfr.201700703

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M.-I., Corella, D., Arós, F., et al. (2018). Primary prevention of cardiovascular disease with a mediterranean diet supplemented with extra-virgin olive oil or nuts. *N. Engl. J. Med.* 378:e34. doi: 10.1056/NEJMoa1800389

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., et al. (2016). Population-level analysis of gut microbiome variation. *Science* 352, 560–564. doi: 10.1126/science.aad3503

Franzosa, E. A., McIver, L. J., Rahnavard, G., Thompson, L. R., Schirmer, M., Weingart, G., et al. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* 15, 962–968. doi: 10.1038/s41592-018-0176-y

Freedman, L. S., Commins, J. M., Moler, J. E., Arab, L., Baer, D. J., Kipnis, V., et al. (2014). Pooled results from 5 validation studies of dietary self-report instruments using recovery biomarkers for energy and protein intake. *Am. J. Epidemiol.* 180, 172–188. doi: 10.1093/aje/kwu116

Gentile, C. L., and Weir, T. L. (2018). The gut microbiota at the intersection of diet and human health. *Science* 362, 776–780. doi: 10.1126/science.aau5812

Ghosh, T. S., Rampelli, S., Jeffery, I. B., Santoro, A., Neto, M., Capri, M., et al. (2020). Mediterranean diet intervention alters the gut microbiome in older people reducing frailty and improving health status: the NU-AGE 1-year dietary intervention across five European countries. *Gut* 69, 1218–1228. doi: 10.1136/gutjnl-2019-319654

Gomez-Arango, L. F., Barrett, H. L., Wilkinson, S. A., Callaway, L. K., McIntyre, H. D., Morrison, M., et al. (2018). Low dietary fiber intake increases *Collinsella* abundance in the gut microbiota of overweight and obese pregnant women. *Gut Microbes* 9, 189–201. doi: 10.1080/19490976.2017.1406584

Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32, 2847–2849. doi: 10.1093/bioinformatics/btw313

Haro, C., Montes-Borrego, M., Rangel-Zúñiga, O. A., Alcalá-Díaz, J. F., Gómez-Delgado, F., Pérez-Martínez, P., et al. (2016). Two healthy diets modulate gut microbial community improving insulin sensitivity in a human obese population. *J. Clin. Endocrinol. Metab.* 101, 233–242. doi: 10.1210/jc.2015-3351

Jakulj, L., van Dijk, T. H., de Boer, J. F., Kootte, R. S., Schonewille, M., Paalvast, Y., et al. (2016). Transintestinal cholesterol transport is active in mice and humans and controls ezetimibe-induced fecal neutral sterol excretion. *Cell Metab.* 24, 783–794. doi: 10.1016/j.cmet.2016.10.001

Karlsson, F. H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C. J., Fagerberg, B., et al. (2013). Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* 498, 99–103. doi: 10.1038/nature12198

Kassambara, A. (2020). *ggpubr: "ggplot2" Based Publication Ready Plots. R Package Version 0.4.0*. Available online at: https://CRAN.R-project.org/package=ggpubr (accessed September, 2020).

Koh, A., Molinaro, A., Ståhlman, M., Khan, M. T., Schmidt, C., Mannerås-Holm, L., et al. (2018). Microbially produced imidazole propionate impairs insulin signaling through mTORC1. *Cell* 175, 947–961.e17. doi: 10.1016/j.cell.2018.09.055

Kootte, R. S., Levin, E., Salojärvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., et al. (2017). Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. *Cell Metab.* 26, 611–619.e6. doi: 10.1016/j.cmet.2017.09.008

Lahti, L., Salonen, A., Kekkonen, R. A., Salojärvi, J., Jalanka-Tuovinen, J., Palva, A., et al. (2013). Associations between the human intestinal microbiota, *Lactobacillus rhamnosus* GG and serum lipids indicated by integrated analysis of high-throughput profiling data. *PeerJ* 1:e32. doi: 10.7717/peerj.32

Lambeth, S. M., Carson, T., Lowe, J., Ramaraj, T., Leff, J. W., Luo, L., et al. (2015). Composition, diversity and abundance of gut microbiome in prediabetes and type 2 diabetes. *J. Diabetes Obes.* 2, 1–7. doi: 10.15436/2376-0949.15.031

Leong, K. S. W., Jayasinghe, T. N., Wilson, B. C., Derraik, J. G. B., Albert, B. B., Chiavaroli, V., et al. (2020). Effects of fecal microbiome transfer in adolescents with obesity: the gut bugs randomized controlled trial. *JAMA Netw. Open* 3:e2030415. doi: 10.1001/jamanetworkopen.2020.30415

Ley, R., Turnbaugh, P., Klein, S., and Gordon, J. (2006). Microbial ecology: human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/nature4441021a

Li, S. S., Zhu, A., Benes, V., Costea, P. I., Hercog, R., Hildebrand, F., et al. (2016). Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science* 352, 586–589. doi: 10.1126/science.aad8852

Lichtman, S. W., Pisarska, K., Berman, E. R., Pestone, M., Dowling, H., Offenbacher, E., et al. (1990). Discrepancy between self-reported and actual caloric intake and exercise in obese subjects. *N. Engl. J. Med.* 322, 1098–1105.

Liu, Y., Ajami, N. J., El-Serag, H. B., Hair, C., Graham, D. Y., White, D. L., et al. (2019). Dietary quality and the colonic mucosa-associated gut microbiome in humans. *Am. J. Clin. Nutr.* 110, 701–712. doi: 10.1093/ajcn/nqz139

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Meslier, V., Laiola, M., Roager, H. M., De Filippis, F., De Filippis, F., Roume, H., et al. (2020). Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and causes changes in the gut microbiome and metabolome independently of energy intake. *Gut* 69, 1258–1268. doi: 10.1136/gutjnl-2019-320438

Mosca, A., Leclerc, M., and Hugot, J. P. (2016). Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem? *Front. Microbiol.* 7:455. doi: 10.3389/fmicb.2016.00455

Moya-Pérez, A., Neef, A., and Sanz, Y. (2015). Bifidobacterium pseudocatenulatum CECT 7765 reduces obesity-associated inflammation by restoring the lymphocyte-macrophage balance and gut microbiota structure in high-fat diet-fed mice. *PLoS One* 10:e0126976. doi: 10.1371/journal.pone.0126976

NCD Risk Factor Collaboration (2016a). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet* 387, 1377–1396. doi: 10.1016/S0140-6736(16)30054-X

NCD Risk Factor Collaboration (2016b). Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants. *Lancet* 387, 1513–1530. doi: 10.1016/S0140-6736(16)00618-8

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2017). *vegan: Community Ecology Package*.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490, 55–60. doi: 10.1038/nature11450

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

R Studio Team (2020). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, PBC.

Reijnders, D., Goossens, G. H., Hermes, G. D. A., Neis, E. P. J. G., van der Beek, C. M., Most, J., et al. (2016). Effects of gut microbiota manipulation by antibiotics on host metabolism in obese humans: a randomized double-blind placebo-controlled trial. *Cell Metab.* 24, 63–74. doi: 10.1016/j.cmet.2016.06.016

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, L., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice gut microbiota from twins metabolism in mice. *Science* 341:1241214. doi: 10.1126/science.1241214

Rinott, E., Youngster, I., Meir, A. Y., Tsaban, G., Zelicha, H., Kaplan, A., et al. (2020). Effects of diet-modulated autologous fecal microbiota transplantation on weight regain. *Gastroenterology* 160, 158–173.e10. doi: 10.1053/j.gastro.2020.08.041

Sakata, T. (2019). Pitfalls in short-chain fatty acid research: a methodological review. *Anim. Sci. J.* 90, 3–13. doi: 10.1111/asj.13118

Salas-Salvadó, J., Bullo, M., Babio, N., Martínez-González, M. Á, Ibarrola-Jurado, N., Basora, J., et al. (2011). Reduction in the incidence of type 2 diabetes with the Mediterranean diet. *Diabetes Care* 34, 14–19. doi: 10.2337/dc10-1288

Sanchis-Chordà, J., del Pulgar, E. M. G., Carrasco-Luna, J., Benítez-Páez, A., Sanz, Y., and Codoñer-Franch, P. (2019). Bifidobacterium pseudocatenulatum CECT 7765 supplementation improves inflammatory status in insulin-resistant obese children. *Eur. J. Nutr.* 58, 2789–2800. doi: 10.1007/s00394-018-1828-5

Schoeller, D. A. (1995). Limitations in the assessment of dietary energy intake by self-report. *Metabolism* 44(2 Suppl. 2), 18–22. doi: 10.1016/0026-0495(95)90204-X

Selma, M. V., Beltrán, D., Luna, M. C., Romo-Vaquero, M., García-Villalba, R., Mira, A., et al. (2017). Isolation of human intestinal bacteria capable of producing the bioactive metabolite isourolithin a from ellagic acid. *Front. Microbiol.* 8:1521. doi: 10.3389/fmicb.2017.01521

Ter Horst, K. W., Gilijamse, P. W., Koopman, K. E., De Weijer, B. A., Brands, M., Kootte, R. S., et al. (2015). Insulin resistance in obesity can be reliably identified from fasting plasma insulin. *Int. J. Obes.* 39, 1703–1709. doi: 10.1038/ijo.2015.125

Thioulouse, J., Dray, S., Dufour, A.-B., Siberchicot, A., Jombart, T., and Pavoine, S. (2018). *Multivariate Analysis of Ecological Data with ade4*. New York, NY: Springer New York. doi: 10.1007/978-1-4939-8850-1

Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., et al. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat. Methods* 12, 902–903. doi: 10.1038/nmeth.3589

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., Kootte, R. S., Bartelsman, J. F. W. M., et al. (2012). Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 143, 913–916.e7. doi: 10.1053/j.gastro.2012.06.031

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag New York.

Wu, G. D., Compher, C., Chen, E. Z., Smith, S. A., Shah, R. D., Bittinger, K., et al. (2016). Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production. *Gut* 65, 63–72. doi: 10.1136/gutjnl-2014-308209

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Yoon, K., and Kim, N. (2021). Roles of sex hormones and gender in the gut microbiota. *J. Neurogastroenterol. Motil.* doi: 10.5056/jnm20208 [Epub ahead of print].

Yoshida, N., Emoto, T., Yamashita, T., Watanabe, H., Hayashi, T., Tabata, T., et al. (2018). *Bacteroides vulgatus* and *Bacteroides dorei* reduce gut microbial lipopolysaccharide production and inhibit atherosclerosis. *Circulation* 138, 2486–2498. doi: 10.1161/CIRCULATIONAHA.118.033714

Yu, E. W., Gao, L., Stastka, P., Cheney, M. C., Mahabamunuge, J., Soto, M. T., et al. (2020). Fecal microbiota transplantation for the improvement of metabolism in obesity: the fmt-trim double-blind placebo-controlled pilot trial. *PLoS Med.* 17:e1003051. doi: 10.1371/JOURNAL.PMED.1003051

Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., et al. (2015). Personalized nutrition by prediction of glycemic responses. *Cell* 163, 1079–1094. doi: 10.1016/j.cell.2015.11.001

Zhang, X., Shen, D., Fang, Z., Jie, Z., Qiu, X., Zhang, C., et al. (2013). Human gut microbiota changes reveal the progression of glucose intolerance. *PLoS One* 8:e71108. doi: 10.1371/journal.pone.0071108

Conflict of Interest: MN is in the Scientific Advisory Board of Caelus Pharmaceuticals, Netherlands and Kaleido, United States. None of these are directly relevant to the current paper.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 30 June 2021
doi: 10.3389/fmicb.2021.637282

Coffee Consumption Modulates Amoxicillin-Induced Dysbiosis in the Murine Gut Microbiome

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The microbiome is essential for host health, and perturbations resulting from antibiotic use can lead to dysbiosis and disease. Diet can be a powerful modulator of microbiome composition and function, with the potential to mitigate the negative effects of antibiotic use. Thus, it is necessary to study the impacts of diet and drug interactions on the gut microbiome. Coffee is a commonly consumed beverage containing many compounds that have the potential to affect the microbiome, including caffeine, polyphenols, and fiber. We supplemented mice with caffeinated and decaffeinated coffee in conjunction with amoxicillin, and used 16S rRNA amplicon sequencing of fecal samples to investigate changes in diversity and composition of the murine fecal microbiome. We found that antibiotics, regardless of coffee supplementation, caused significant disruption to the murine fecal microbiome, enriching for Proteobacteria, Verrucomicrobia, and Bacteroidetes, but reducing Firmicutes. While we found that coffee alone did not have a significant impact on the composition of the fecal microbiome, coffee supplementation did significantly affect relative abundance metrics in mice treated with amoxicillin. After caffeinated coffee supplementation, mice treated with amoxicillin showed a smaller increase in Proteobacteria, specifically of the family Burkholderiaceae. Correspondingly we found that *in vitro*, *Burkholderia cepacia* was highly resistant to amoxicillin, and that it was inhibited by concentrations of caffeine and caffeinated coffee comparable to levels of caffeine in murine ceca. Overall, this work shows that coffee, and possibly the caffeine component, can impact both the microbiome and microbiome members during antibiotic exposure.

Keywords: microbiome, gut, coffee, caffeine, antibiotics

OPEN ACCESS

Edited by:
Katia Sivieri,
São Paulo State University, Brazil

Reviewed by:
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Michael Gänzle,
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *03 December 2020*
Accepted: *02 June 2021*
Published: *30 June 2021*

Citation:
Diamond E, Hewlett K, Penumutchu S, Belenky A and Belenky P (2021) Coffee Consumption Modulates Amoxicillin-Induced Dysbiosis in the Murine Gut Microbiome. Front. Microbiol. 12:637282. doi: 10.3389/fmicb.2021.637282

INTRODUCTION

The human gut is home to trillions of bacteria which are vital for many host processes, including energy extraction from food, synthesis of important molecules, and protection from pathogens (Becattini et al., 2016). These microorganisms and their genetic content are collectively referred to as the gut microbiome. We now understand that the gut microbiota plays a key role in host health (Knight et al., 2017). Thus, studying the dynamic factors that affect microbiome composition and function is key to understanding and promoting health.

Most of the bacteria comprising the gut microbiome live in the colon, where they ferment substances indigestible to the host, such as fiber and polyphenols (Korpela, 2018). Fermentation leads to the production of short chain fatty acids (SCFAs) and this in turn increases the anaerobicity of the colon (Jha et al., 2019). Treatment with antibiotics decreases microbiome diversity and can cause antibiotic induced dysbiosis (AID). Under dysbiosis, the oxygen content of the gut increases, leading to decreased fermentation by obligate anaerobes and diminished SCFA production. This in turn decreases gut barrier integrity, which leads to inflammation and promotes the growth of facultative aerobic bacteria such as Proteobacteria (Jha et al., 2019). Some members of this phylum are opportunistic pathogens and are highly immunogenic, such as *Escherichia coli* and *Klebsiella* spp., and have the potential for expansion under dysbiotic conditions (Becattini et al., 2016; Kim et al., 2017; Zarrinpar et al., 2018).

Antibiotic use and AID not only lead to increased risk of secondary infections, but also are associated with chronic conditions including inflammatory bowel disease, asthma, diabetes, obesity, and other metabolic syndromes (Blaser, 2011; Cabral D. et al., 2020). As antibiotic use continues to rise worldwide, with tens of billions of daily doses taken each year, it is now more important than ever to investigate ways to prevent AID (Klein et al., 2018).

Several remedies for AID have been proposed and are in use, including the administration of probiotics and prebiotics. Prebiotics are substrates, such as fiber and polyphenols, that are selectively used by native bacteria to promote health, as opposed to probiotics which repopulate the gut with exogenous microbes (Preidis and Versalovic, 2009; Gibson et al., 2017; Rezende et al., 2021). Probiotics have the potential to increase abundance of the probiotic species rather than the original native population of the gut. In fact, supplementing the microbiome with probiotics after antibiotic use delays microbiome reconstitution as compared to spontaneous reconstitution (Suez et al., 2018). Thus, prebiotics may be a safer way to address AID.

Many substances have been studied for their potential as prebiotic compounds. Coffee is a common caffeinated beverage that contains dietary fibers like galactomannan and type II arabinogalactan, as well as polyphenols such as chlorogenic acid, which may act as an antioxidant (Gniechwitz et al., 2007; Liang and Kitts, 2015; Win et al., 2019). One study in rats showed that coffee stimulated smooth muscle contractility in the intestine in a dose-dependent manner (Hegde et al., 2019). Related studies have shown that this effect of caffeine can lead to a decreased transit time of nutrients in the gut, which has been shown to change microbiome composition by affecting water and nutrient availability throughout the gut (Brown et al., 1990; Kashyap et al., 2013). Caffeine has also been previously associated with a richer gut microbiome and may reduce the prevalence of inflammatory bacteria (Gurwara et al., 2019).

Several studies have explored the effects of coffee and its components on the gut microbiome. One study gave mice filtered coffee for 3 days and found that relative abundance of *Eubacteria, E. coli, Enterococcus,* and *Clostridium* decreased, while *Bifidobacteria* and *Lactobacillus* increased (Nakayama and Oishi, 2013). A similar study in humans found that after 3 weeks of consistent coffee intake, relative abundance of most bacterial species remained unchanged, with some increase in *Bifidobacteria* (Jaquet et al., 2009). While both coffee consumption and antibiotic administration have the potential to change the composition of the microbiome, the joint impacts of these perturbations are not established *in vivo*. Previous *in vitro* experiments have studied interactions between caffeine and antibiotics, finding that caffeine can lower the efficacy of many first-line antibiotics. Some possible explanations for this are competitive binding between caffeine and the antibiotic or formation of caffeine-antibiotic complexes (Olajuyigbe et al., 2017).

Here we to investigate the effects of coffee, both caffeinated and decaffeinated, on AID in the murine microbiome. We gave mice caffeinated and decaffeinated coffee for 12 days, in conjunction with amoxicillin for 7 days and analyzed gut microbiome samples by 16S rRNA sequencing. We found that compared to controls, mice treated with amoxicillin and given caffeinated coffee exhibited a decrease in the abundance of potentially pro-inflammatory bacteria, such as Proteobacteria.

MATERIALS AND METHODS

Mouse Experiments

All animal work was approved by Brown University's Institutional Animal Care and Use Committee (IACUC) under protocol number 1706000283. Four-week-old female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, United States) and given a 2-week habituation period immediately following arrival at Brown University's Animal Care Facility, during which they were given standard chow (Laboratory Rodent Diet 5001, St. Louis, MO, United States) and water *ad libitum*. After habituation and for the duration of the experiment, mice were given daily oral gavage of caffeinated coffee (Starbucks Via Instant Italian Roast, 1.5 mg caffeine/400 μL), decaffeinated coffee (Starbucks Via Instant Decaf Italian Roast, 0.14 mg caffeine/400 μL), or water (400 μL) (n = 12 per group), per the protocol described in Nakayama and Oishi (2013). After 5 days of gavage, half of each group (n = 6 per group) were administered antibiotics in the drinking water (amoxicillin 25 mg/kg/day) for 7 days. Oral gavage of coffee or water was continued throughout antibiotic treatment. Mice were sacrificed on day 12 of the experiment. Fecal samples were collected for 16S rRNA analysis on days 0 (before first oral gavage), 5 (before first antibiotic treatment), 7, 10, and 12 (**Figure 1A**).

Mass Spectrometry for Caffeine Quantification

Eighteen additional mice were randomly assigned to receive oral gavage of caffeinated coffee, decaffeinated coffee, or water control (n = 6). One hour after gavage, mice were sacrificed and their blood and ceca were removed. 30 μL of plasma was extracted from the blood and combined with 94 μL of methanol and 6 μL of 0.01 mg/mL internal standard (IS) caffeine solution (caffeine labeled with two C13, MW 188). For every 1 mg of cecum, 10 μL

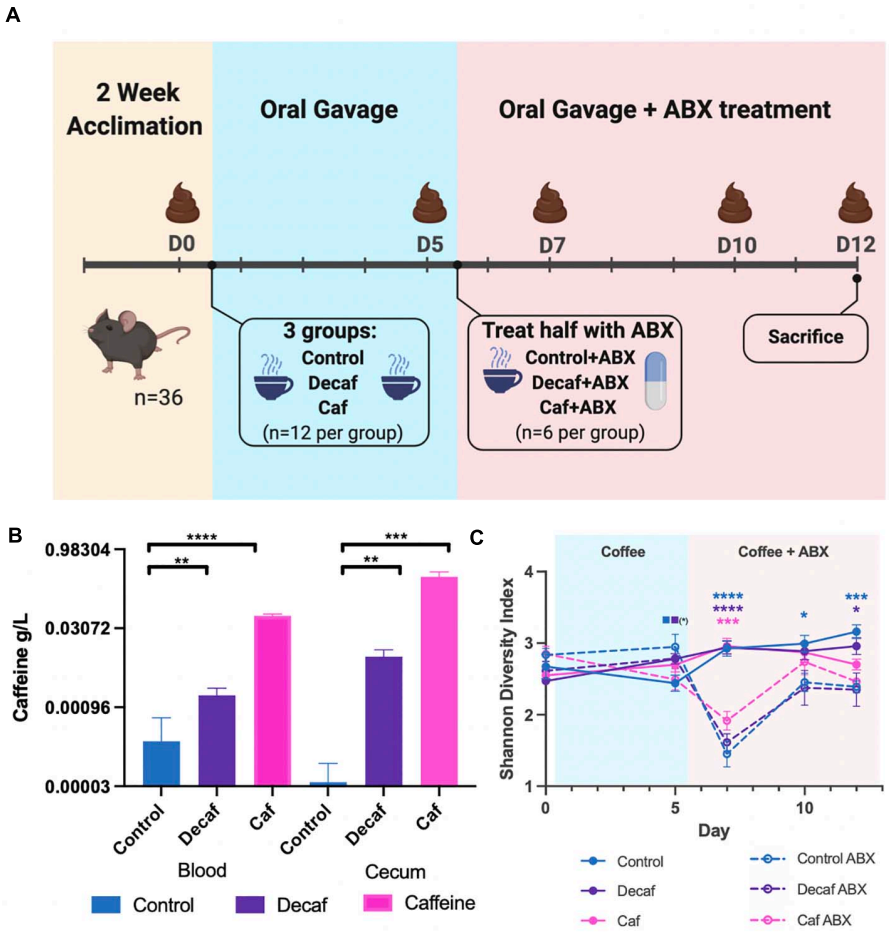

FIGURE 1 | (A) Mouse experimental design and timeline. Figure was created with biorender.com. **(B)** Quantification of caffeine content in blood and cecum 1-h post-oral gavage ($n = 6$). **(C)** Alpha diversity of experimental groups as measured by the Shannon Diversity Index. Data are represented as mean ± standard deviation (SD). Significance between each group and its antibiotic counterpart is denoted by stars (*) with the color coordinating to control (ctrl, blue), decaffeinated coffee (decaf, purple), or caffeinated coffee (caf, pink). Significance between different experimental groups is denoted by squares color-coded according to the experimental groups being compared. Significance was determined by the Benjamini, Krieger and Yekutieli test to correct for False discoveries with adjusted p-value < 0.05. (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $0.0001 < P < 0.001$; ****, $P < 0.0001$). Solid squares represent groups without antibiotics, open squares represent groups given antibiotics. Each comparison of two experimental groups has its corresponding significance denoted in stars (*) next to it. $n = 6$. For example, on Day 5, the solid blue square next to the solid purple square represents a significant difference between the control non-antibiotic group and the decaf non-antibiotic group, at a significance level of 1 star (shown in parentheses). On Day 12, the 3 blue stars represent a significant difference between the control non-antibiotic group and the control antibiotic group, at a significance level of 3 stars. The 1 purple star represents a significant difference between the decaf non-antibiotic group and the decaf antibiotic group at a significance level of 1 star.

of methanol with IS caffeine solution was added. Each mixture was vortexed for 5 min at 1,175 rpm, then centrifuged for 5 min at 17,900 × g. 15 μL of supernatant was removed for analysis.

Caffeine in biological samples after spiking with IS and extraction was quantitated with HPLC-MS on an Ultimate 3000, Dionex coupled to Q Executive Classic (Thermo) with ESI interface. Data acquisition and processing was performed by Excalibur software. The chromatographic separation was achieved on a Xselect CSH C18 2.5 μm; 2.1 × 30 mm (Waters, Milford, MA, United States), at 60°C. Mobile phase consisted of water for phase A and 50/50 methanol/acetonitril for phase B, both containing 0.2% FA. Separation was optimized using a fast gradient method with mobile phase A/B set to 90%/10% from 0.00 to 0.20 min and 10%/90% from 0.21 to 1.4 min and then back after 1.7 min for equilibration at 90%/10% from 1.7 to 6.00 min. with the flow 0.35 ml/min. The mass spectrometer was operated in the positive ion mode in Full MS; at resolution 70,000; AGT target 5 E5 and Scan range 170–200 m/z. Spray voltage and source temperature were set at 3,500 volts, and 320°C, respectively. CAF-2C13 was the IS used for quantification of CAF.

Nucleic Acid Extraction and Quantification

Total nucleic acids (DNA) were extracted from samples using the ZymoBIOMICS DNA Miniprep Kit from Zymo Research

(R2002, Irvine, CA, United States) using the extraction protocol as per the manufacturer instructions. Total DNA was eluted in nuclease-free water and quantified using the dsDNA-HS on a QubitTM 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) before use in amplicon preparations.

16S rRNA Amplicon Preparation and Sequencing

The 16S rRNA V4 hypervariable region was amplified from total DNA using the barcoded 515F forward primer and the 806R reverse primers from the Earth Microbiome Project (Thompson et al., 2017). Amplicons were generated using 5X Phusion High-Fidelity DNA Polymerase under the following cycling conditions: initial denaturation at 98°C for 30 s, followed by 25 cycles of 98°C for 10 s, 57°C for 30 s, and 72°C for 30 s, then a final extension at 72°C for 5 min. After amplification, samples were visualized via gel electrophoresis and pooled in equimolar amounts. The pooled amplicon library was submitted to the Rhode Island Genomics and Sequencing Center at the University of Rhode Island (Kingston, RI, United States) for sequencing on the Illumina MiSeq platform. Amplicons were paired-end sequenced (2 × 250 bp) using the 600-cycle kit with standard protocols. We obtained an average of 27,563 reads per sample. Raw reads were deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA682275.

Analysis of 16S rRNA Sequencing Reads

Raw 16S rRNA reads were subjected to quality filtering, trimming, de-noising with DADA2 (Callahan et al., 2016) (via q2-dada2), and merging using the Qiime2 pipeline (version 2019.10) (Bolyen et al., 2019). Ribosomal sequence variants were aligned with mafft (Katoh et al., 2002) (via q2-alignment), and phylogenetic tree construction was done with fasttree2 (Price et al., 2010) (via q2-phylogeny). Taxonomic assignment was conducted using the pre-trained Naive Bayes classifier and the q2-feature-classifier (Bokulich et al., 2018a) trained on the SILVA 132 99% database (Quast et al., 2013). Alpha diversity (Shannon, Faith's phylogenetic diversity) and beta diversity (Bray-Curtis dissimilarity) (Bray and Curtis, 1957; Bokulich et al., 2018b) were calculated using the phyloseq package (version 1.30.0) in R (version 3.6.2) (Faith, 1992; Lozupone and Knight, 2005; Lozupone et al., 2007). Significance was determined by the Benjamini, Krieger and Yekutieli test to correct for False discoveries with adjusted p-value < 0.05 (Benjamini and Hochberg, 1995). Linear Discriminant analysis was conducted using the LEfse package (version 1.0) on the galaxy server[1] (Segata et al., 2011; Afgan et al., 2018). Raw 16S rRNA reads were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA682275.

Minimal Inhibitory Concentration Determination

Minimal inhibitory concentrations (MICs) were determined using the broth dilution method (Wiegand et al., 2008). Overnight cultures of *B. cepacia* or *E. coli* were diluted 100-fold into TSB and LB media, respectively. Cultures were incubated with various concentrations of caffeine (Sigma-Aldrich, St. Louis, MO, United States), caffeinated coffee or decaffeinated coffee (Starbucks). Amoxicillin (Sigma-Aldrich, St. Louis, MO, United States) was added at varying concentrations to cell culture media and serially diluted two-fold. Cells were then incubated aerobically at 37°C until controls reached their maximum OD_{600} reading (18 h).

B. cepacia Growth Rate Determination With Caffeinated and Decaffeinated Coffee

Overnight cultures of *B. cepacia* grown in TSB were pelleted and resuspended in 1X PBS. Cells were subsequently diluted to an optical density at 600 nm (OD_{600}) of approximately 0.01 into TSB supplemented with caffeinated coffee, decaffeinated coffee or caffeine. Cells were incubated at 37°C under aerobic conditions and growth was monitored by taking OD_{600} readings at 10-min intervals. To determine the doubling time in each condition, the growth curves were fitted to an exponential growth function using default settings in Prism (version 8.4.2).

RESULTS

Oral Gavage With Coffee Leads to Significant Increases in Murine Blood and Cecum Caffeine Levels

To determine the effect of coffee and its components on the murine gut microbiome, female C57BL/6J mice were randomly assigned to receive oral gavage of caffeinated coffee (caf), decaffeinated coffee (decaf), or water (control) (n = 12) (**Figure 1A**). Mice given caffeinated coffee received 1.5 mg caffeine at each gavage (per manufacturer-reported caffeine content), equivalent to 3 cups of coffee according to FDA human to animal dose conversion guidelines (Nair and Jacob, 2016). Mice given an equal volume of decaffeinated coffee received 0.14 mg caffeine at each gavage. After 5 days, half of the mice from each group additionally received amoxicillin in their drinking water (n = 6) for 7 days (Cabral D. et al., 2020). Fecal samples were taken on days 0, 5, 7, 10, and 12 for 16S rRNA amplicon sequencing of the fecal microbiome. We utilized mass spectrometry to determine the maximum level of caffeine present in the ceca and blood of mice 1-h post-oral gavage, using 6 additional mice (Teekachunhatean et al., 2013). Caffeine supplementation led to a significant increase in cecum and blood caffeine levels (**Figure 1B**). Blood caffeine levels were approximately 0.0530 g/L and cecum levels were approximately 0.293 g/L. While these likely represent peak caffeine levels, based on the rapid metabolic activity of mice we expect that this level would drop significantly between gavages (Teekachunhatean et al., 2013). This also indicates that while some caffeine was absorbed into the bloodstream, the majority of the caffeine was unabsorbed and available to the microbiota. As described above, coffee is a complex mixture of fibers and polyphenols

[1] https://huttenhower.sph.harvard.edu/galaxy/

and, like caffeine, these molecules would also become available to gut microbes.

Antibiotics and Coffee Supplementation Lead to Changes in Bacterial Diversity in the Fecal Microbiome

The 16S rRNA v4 hypervariable region was sequenced to determine the bacteria present in the fecal microbiome of mice. Overall, the average sequencing depth was approximately 27,563 and the data were analyzed using Qiime2 to determine metrics of bacterial diversity as well as composition. We did not detect an impact of coffee administration on the diversity of the fecal microbiome as measured by the Shannon Diversity Index over the entire length of the experiment (**Figure 1C**). Conversely, antibiotics caused a significant drop in microbial diversity, as shown by the significant decrease in diversity in the antibiotic-supplemented groups at 2 days post antibiotic administration (**Figure 1C**). The caf group showed a smaller antibiotic-induced decrease in diversity ($p = 0.064$), potentially indicating that caffeinated coffee supplementation in this amount may reduce the drop in microbial diversity.

We used the Bray-Curtis beta-diversity metric to assess the degree of dissimilarity between our samples. After 5 days of oral coffee gavage, caf and decaf mice did not cluster separately from mice gavaged with the vehicle. This indicates that compounds in coffee or caffeine alone do not significantly alter the overall microbiome composition (**Figures 2A,B**). As other studies have found, antibiotics led to significant changes in microbiome composition in mice, regardless of supplementation (Cabral D. et al., 2020; Cabral D. J. et al., 2019). Over the course of the experiment, our samples formed clusters mainly based on amoxicillin treatment (**Figures 2C,D**).

Antibiotics Alter Fecal Microbiome Composition by Reducing Firmicutes and Increasing Bacteroidetes, Proteobacteria, and Verrucomicrobia

While the overall beta-diversity indicated that antibiotic exposure had a profound impact on microbial composition, it appears that coffee had a very modest impact on composition in the broad sense. To directly determine which microbial families were affected throughout the experiment, we plotted their relative abundance over time and utilized LEfSe to determine which bacteria were significantly associated with each experimental group. From visual analysis of the data, we found that the greatest perturbation in composition occurred 2 days after antibiotic exposure (day 7) (**Figure 3** and **Supplementary Tables 1**, **2**). After this point, the microbiome began to return to a new altered baseline as antibiotic therapy progressed. For example, *Burkholderiaceae* from the Proteobacteria phylum had a peak of expansion on day 7, and then returned back to baseline. Similarly, *Bacteroidaceae* from the Bacteroidetes phylum peaked at day 7. This trend was inverted for *Muribaculaceae*, which collapsed at day 7. Finally, *Akkermansiaceae* began to increase at day 7 and then expanded throughout the rest of the experiment. Looking at these data, we can also see that supplementing with caffeinated or decaffeinated coffee has an impact on the intensity of these changes. For example, the expansion of *Burkholderiaceae* appears to be greater in the control and decaf groups than in the caf group (**Figure 3** and **Supplementary Table 1**).

In order to gain an appreciation for the statistical significance of these changes, we utilized LEfSe to determine drug-induced and supplement-induced changes at the phylum and family levels on day 7, since this was the time point with the greatest perturbations. In all groups, amoxicillin exposure caused an increase in Proteobacteria (t-test $P < 0.05$; LEfSe Kruskal–Wallis alpha = 0.05, LDA Threshold = 2.0) (**Figure 4A** and **Supplementary Figures 3A,C,E**). This expansion was matched by a reduction in Firmicutes (**Figure 4B**). While Proteobacteria expanded in all treatment groups, the level of expansion differed based on supplementation. Comparing the control antibiotic (control ABX) treated group to the caffeinated coffee antibiotic (caf ABX) treated group, we found that Proteobacteria were significantly associated with the control ABX group (LEfSe) (**Figure 4A** and **Supplementary Figure 3J**). This differential association was not observed when we compared the decaf antibiotic (decaf ABX) group to the control ABX group (LEfSe) (**Supplementary Figure 3H**), possibly indicating that caffeinated but not decaffeinated coffee reduced the antibiotic-induced expansion in Proteobacteria. Bacteroidetes relative abundance was significantly higher in the control ABX and caf ABX mice 2 days after amoxicillin treatment as compared to their non-antibiotic counterparts. The caf ABX group was the only one to stay significantly elevated as compared to its control counterpart throughout the rest of the experiment (t-test $P < 0.05$) (**Figure 4C**). Visually, Verrucomicrobia began to expand at day 7, although this expansion appeared to be delayed in the control group. This perceived delay is followed by a significant association of Verrucomicrobia with the decaf ABX group, but not the control ABX group at day 7 (LEfSe) (**Figures 4D,H** and **Supplementary Figure 3G**).

Biomarker analysis using LEfSe at lower taxonomic levels showed that the strongest associations observed at the phylum level could be traced down to specific families (**Supplementary Figure 3**). Within the Bacteroidetes phylum, we found that an increase in the relative abundance of *Bacteroidaceae* was significantly associated with all three antibiotic-treated groups compared to their non-antibiotic counterparts at day 7 (LEfSe) (**Figure 4E** and **Supplementary Figures 3B,D,F**). This expansion was reversed for *Muribaculaceae*, which was significantly associated with the non-antibiotic treated groups at the same time point (LEfSe) (**Figure 4F** and **Supplementary Figures 3B,D,F**). The only family found to be significantly different between experimental groups for Proteobacteria was *Burkholderiaceae*, which was significantly increased in the antibiotic-treated groups as compared to their non-antibiotic counterparts at day 7 (t-test $P < 0.05$; LEfSe) (**Figure 4G** and **Supplementary Figures 3B,D,F**). This likely explains the differences in Proteobacteria observed at the phylum level. As we observed in Proteobacteria, this antibiotic-related increase in *Burkholderiaceae* was not significantly associated with the caf group, but was significant in the control and decaf group (LEfSe) (**Figure 4G** and **Supplementary Figure 3I**). As expected, the

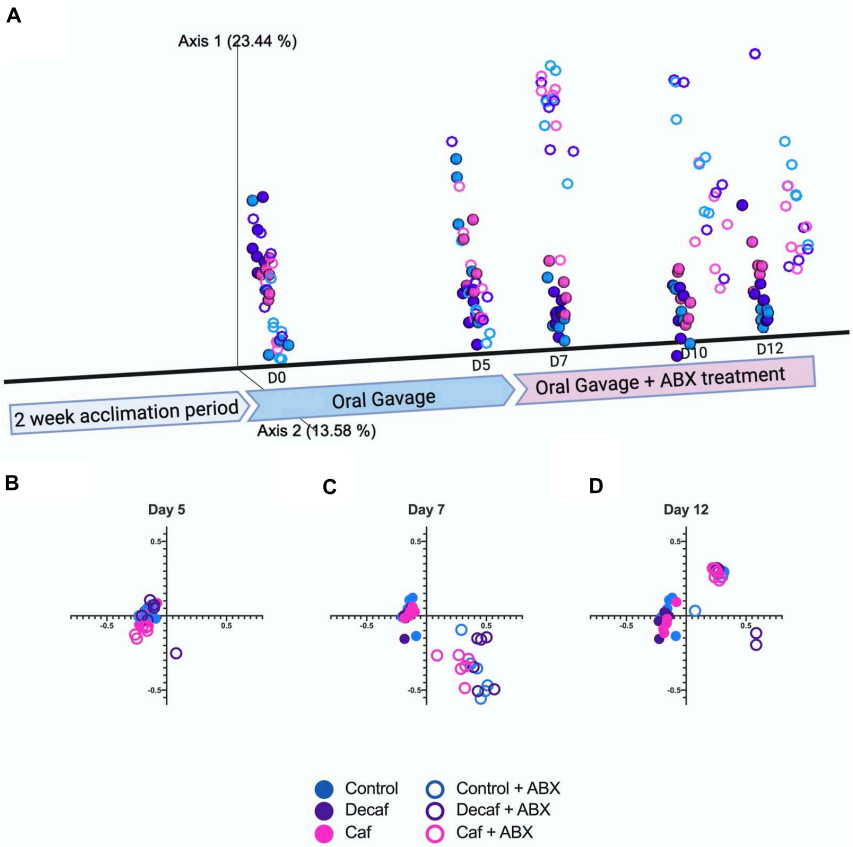

FIGURE 2 | (A) Beta diversity of experimental groups over time represented on a three-dimensional principal coordinate analysis plot. Figure was created with biorender.com. **(B)** Beta diversity metrics using Bray Curtis Distance Matrices at 5 days of coffee gavage, **(C)** 2 days of antibiotic treatment and 7 days coffee gavage, **(D)** 7 days of antibiotic treatment and 12 days coffee gavage; $n = 6$.

expansion in *Burkholderiaceae* was not impacted by decaffeinated coffee (**Figure 4G** and **Supplementary Figure 3H**). Within the Verrucomicrobia phylum, *Akkermansiaceae* was significantly associated with the caf ABX and decaf ABX groups as compared to the control ABX group at Day 7 (LEfSe) (**Figure 4D** and **Supplementary Figures 3B,D,F**). By day 10, all three antibiotic-treated groups had significantly more *Akkermansiaceae* than their non-antibiotic counterparts, confirming the delayed expansion in the control group observed at the phylum level (*t*-test $P < 0.05$; LEfSe) (**Figure 4H** and **Supplementary Figures 4A–C**).

Burkholderia cepacia Is Resistant to Amoxicillin

Because Proteobacteria are strongly associated with dysbiotic microbiomes (Becattini et al., 2016), we further investigated the impact of coffee and caffeine on *Burkholderiaceae in vitro*. 16S analysis does not allow us to accurately characterize bacteria down to the species level, so we chose to utilize *B. cepacia* as a model from the *Burkholderiaceae* family to investigate the impact of coffee and amoxicillin. *B. cepacia* is a common member of the murine gut microbiome and has been associated with inflammation (Armstrong et al., 2019). It is also an opportunistic pathogen found in the lungs of cystic fibrosis patients (Scoffone et al., 2017). We determined the minimal inhibitory concentration (MIC_{90}) of amoxicillin for *B. cepacia*, and found it to be 4000 μ/mL, indicating that it is essentially resistant to this drug. As a control, we determined the MIC_{90} of *E. coli* was much lower, at 10 μ/mL (**Figure 5A**). Other studies have also found that *B. cepacia* is highly resistant to amoxicillin treatment (Everaert and Coenye, 2016). This resistance is a possible explanation for the significant increase in *Burkholderiaceae* relative abundance immediately after amoxicillin treatment in all three groups (**Figure 4G**). However, this does not explain the fact that *Burkholderiaceae* did not expand to the same extent in the caf ABX group as compared to the decaf and control ABX groups. Other data indicate that some species of *Burkholderiaceae* have the capacity to degrade caffeine, so we next investigated the impact of caffeine on *B. cepacia*'s growth rate (Win et al., 2019).

We measured the doubling time of *B. cepacia in vitro* when exposed to increasing concentrations of caffeinated coffee, decaffeinated coffee, and pure caffeine. We found that the doubling time of *B. cepacia* was significantly greater when exposed to the two highest concentrations of caffeinated coffee, as compared to the control (6.7 h vs. 4.8 h) (**Figure 5B**). This

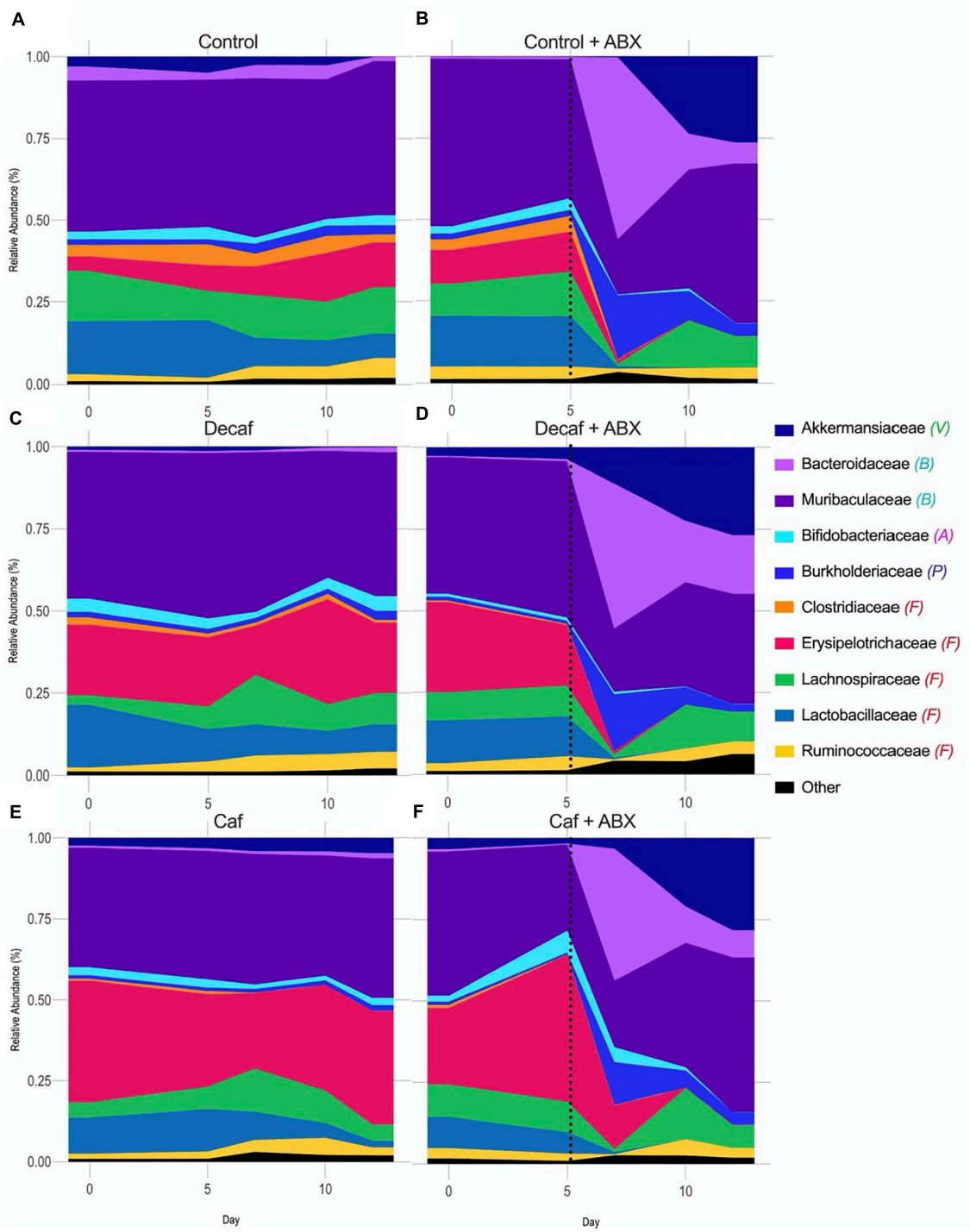

FIGURE 3 | Relative abundance of bacterial families over the course of the experiment. Phylum denoted by: V, Verrucomicrobia; B, Bacteroidetes; A, Actinobacteria; P, Proteobacteria; F, Firmicutes. Dotted line denotes beginning of antibiotic treatment. **(A)** Control, **(B)** Control + amoxicillin days 5–12, **(C)** Decaf, **(D)** Decaf + amoxicillin days 5–12, **(E)** Caf, **(F)** Caf + amoxicillin days 5–12; $n = 6$.

significant inhibition in growth also occurred at the highest concentration of pure caffeine (**Figure 5C**). We did not observe any significant changes in doubling time at any concentration of decaffeinated coffee, although there was an overall upward trend (**Figure 5D**). Although some species of *Burkholderiaceae* may utilize caffeine, because these experiments were performed in rich media, the pure caffeine is likely not acting as a primary carbon source. In fact, other work shows that high levels of

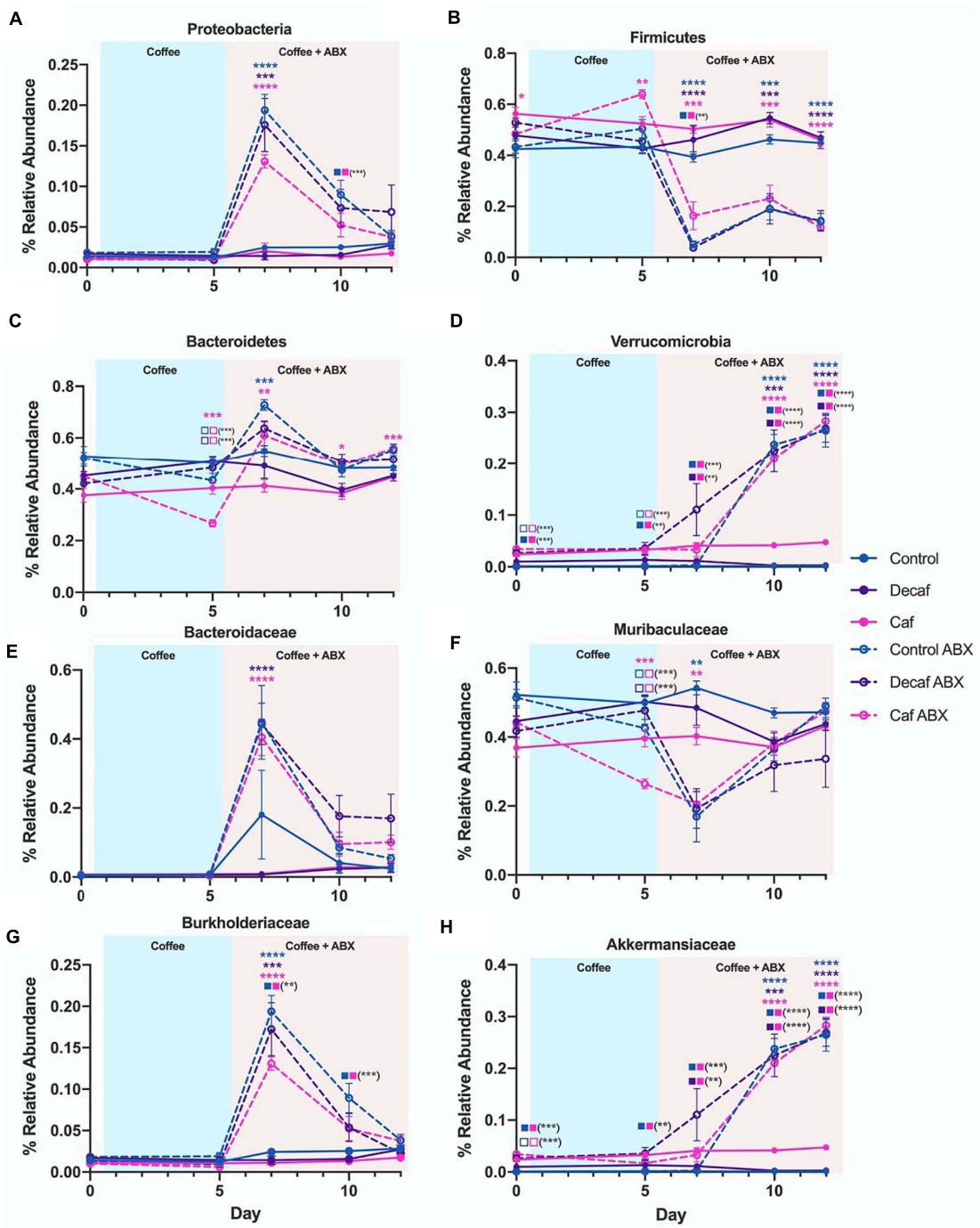

FIGURE 4 | (A–H) Relative abundance of notable phyla and families over the course of the experiment. Data are represented as mean ± SD. Significance was determined by the Benjamini, Krieger and Yekutieli test to correct for False discoveries with adjusted *p*-value < 0.05 (*, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01; ***, 0.0001 < *P* < 0.001; ****, *P* < 0.0001). Significance between each experimental group and its antibiotic counterpart is denoted by stars (*) with the color coordinating to control (ctrl, blue), decaffeinated coffee (decaf, purple), or caffeinated coffee (caf, pink). Significance between different experimental groups is denoted by squares color-coded according to the experimental groups being compared. Solid squares represent groups without antibiotics, open squares represent groups given antibiotics. Each comparison of two experimental groups has its corresponding significance denoted in stars (*) next to it. For example, in the Bacteroidetes graph **(C)** on Day 5, the 3 pink stars represent a significant difference between the caf no-antibiotic group and the caf antibiotic group, at a significance level of 3 stars. Below that, the open blue square next to the open pink square represents a significant difference between the control antibiotic group and the caf antibiotic group, at a significance level of 3 stars (shown in parentheses). Below that, the open purple square next to the open pink square represents a significant difference between the decaf antibiotic group and the caf antibiotic group, at a significance level of 3 stars (shown in parentheses).

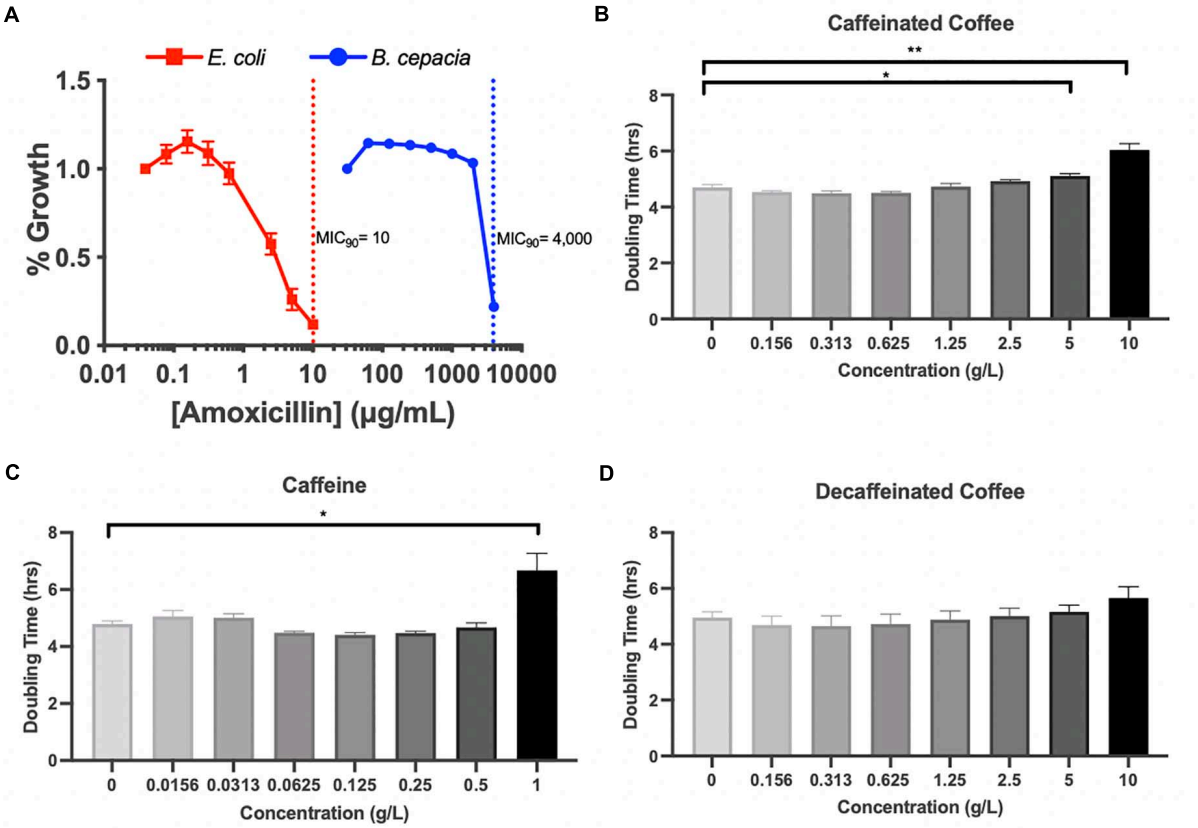

FIGURE 5 | (A) MIC assessment of *B. cepacia*, *E. coli* with amoxicillin in TSB or MGAM, respectively, containing varying concentrations of amoxicillin. MIC_{90} indicated by dotted lines. Doubling time calculated from growth curves of *B. cepacia* cultured in TSB with varying concentrations of **(B)** caffeinated coffee, **(C)** caffeine, and **(D)** decaffeinated coffee; $n = 3$ (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; unpaired *t* test).

caffeine may be inhibitory to some species of *Burkholderiaceae*, as reflected in our findings (Win et al., 2019). Thus, *in vitro*, we see that caffeine and caffeinated coffee are factors that can affect bacterial behavior. Overall, these data indicate that coffee, and even possibly caffeine, may have an inhibitory impact on these bacteria in the gut, possibly explaining the observed reduction in relative abundance during the antibiotic-induced bloom.

DISCUSSION

Coffee is made up of several components that individually have the potential to change the gut microbiome, including caffeine, polyphenols, and fiber (Gniechwitz et al., 2007; Liang and Kitts, 2015). Previous studies have investigated the effect of coffee on the microbiome, but to our knowledge there has not been extensive research investigating the combined effects of coffee and antibiotics on the gut microbiome (Jaquet et al., 2009; Nakayama and Oishi, 2013; Gurwara et al., 2019). Food and drug interactions are important to study, as they have the potential to impact microbiome composition, function, and host health in ways that cannot necessarily be predicted (Cabral D. et al., 2020; Cabral D. J. et al., 2019). Coffee and antibiotics are both commonly consumed, presumably together, and therefore it is important to investigate their combined effects on the gut microbiota (Klein et al., 2018).

In this study, we found that neither caffeinated nor decaffeinated coffee alone has a major impact on the diversity or composition of the murine fecal microbiome. However, we did find that coffee supplementation has some impact on the taxonomic composition of the microbiome in mice treated with amoxicillin, although this impact is not reflected in statistically significant changes in alpha or beta diversity. We found that, according to LEfSe analysis, *Akkermansiaceae* was significantly associated with both coffee groups in mice treated with antibiotics, and not with the control mice. This may indicate that a component common to caffeinated and decaffeinated coffee is directly or indirectly affecting this population. In addition, we observed fewer Proteobacteria in amoxicillin-treated mice given caffeinated coffee in the relative abundance analysis, as compared to mice given antibiotics and decaffeinated coffee or the vehicle (**Figures 3B,D,F**), an observation which was confirmed by LEfSe analysis of significance. We tested LDA effect size at the phylum and family level and determined that there were significant changes at the level of the Proteobacteria phylum and the *Burkholderiaceae* family, which changed in response to

amoxicillin and coffee treatment. Proteobacteria are associated with inflammation and dysbiosis due to antibiotics, so a reduction in this phylum may represent a beneficial effect of caffeinated coffee on the microbiome. Some species of *Burkholderiaceae* that reside in the gut have pathogenic potential, and *B. cepacia* has been associated with irritable bowel syndrome (Rizzatti et al., 2017; Armstrong et al., 2019). *B. cepacia* is also an important opportunistic pathogen found in cases of cystic fibrosis (Scoffone et al., 2017).

While coffee alone did not seem to impact overall microbial composition, some differences could be observed. However, the main difference in *Akkermansiaceae* levels appears to be an artifact of individual differences in baseline microbiota composition. *Akkermansiaceae* levels were significantly higher in mice given caffeinated coffee in comparison to mice given decaffeinated coffee or the vehicle in the absence of amoxicillin. However, at day 0 before any coffee supplementation, these mice had more of this bacterial family than controls, so this finding may not be attributable to coffee supplementation. While we did find some other significant differences in relative abundance of certain bacterial phyla and families between coffee experimental groups, these often only were significant for a single time point and did not remain consistent throughout the rest of the experiment.

Using MIC assays and growth curves, we further investigated the response of *B. cepacia* to amoxicillin and coffee. As we expected based on previous studies, *B. cepacia* was highly resistant to amoxicillin (Everaert and Coenye, 2016). We also found that it was significantly inhibited by caffeinated coffee and pure caffeine at concentrations slightly above the maximum concentration detected in the ceca of our mice. While this result does not directly demonstrate that *Burkholderiaceae* inhibition *in vivo* is related to caffeine content, this conclusion is still possible since the concentration dependence and dynamics of inhibition may be different in the natural environment of the cecum compared to artificial media. In addition, the *in vitro* doubling time was measured over a relatively short period of time and a single exposure to the caffeine, whereas in the mouse, the microbiome was exposed to caffeine multiple times and over a much longer period of time. The metabolic environment is a known determinant of antimicrobial susceptibility to antibiotics and other toxins (Allison et al., 2011; Belenky et al., 2015; Lobritz et al., 2015; Cabral D. et al., 2020). This indicates caffeine has the potential to slow the growth of *B. cepacia in vitro*, which may explain why there were fewer *Burkholderiaceae* in the caffeinated coffee-supplemented mice after antibiotic treatment. However, caffeinated and decaffeinated coffee do not differ exclusively in caffeine content. Our coffee was decaffeinated by washing coffee beans with methylene chloride and then roasting the coffee to evaporate all methylene chloride out of the product, a common decaffeination method (How Is Coffee Decaffeinated? | Britannica, 2020). Caffeine is soluble in methylene chloride, along with chlorogenic acid, one of the main polyphenols in coffee, meaning decaffeinated coffee has fewer polyphenols. Chlorogenic acid has the potential to act as an antioxidant and can be metabolized by gut bacteria (Couteau et al., 2001; Liang and Kitts, 2015). Higher levels of chlorogenic acid in caffeinated coffee could also partly explain the different levels of Proteobacteria seen in our mice after antibiotic exposure. Thus, the differences observed between caffeinated and decaffeinated coffee may be the result of other components of coffee besides caffeine. In addition, physiological effects of coffee consumption such as decreased transit time of nutrients in the gut could be occurring *in vivo* leading to changes in microbial abundance (Brown et al., 1990; Kashyap et al., 2013; Gurwara et al., 2019).

The work presented here is an early step in determining the interaction between coffee consumption and antibiotic-induced dysbiosis. Additionally, the study presented here is the first, to our knowledge, to show the combined effects of antibiotics and coffee consumption on the gut microbiome *in vivo*. Based on these results, we may make some predictions to drive future research. Our results lead us to conclude that consuming even a high amount of coffee does not significantly impact the murine microbiome, and if this translates to humans, indicate that coffee is not a source of microbiome disruption. Our data also show that while amoxicillin clearly disturbs the microbiome, adding coffee consumption does not drastically exacerbate this perturbation. In fact, coffee supplementation might even be beneficial to the gut, as shown by the reduction in the bloom of Proteobacteria in the caffeinated coffee-treated groups. Since coffee is one of the most polyphenol-rich and caffeine-rich foods we consume (Leonard et al., 2020), these results may also give us information about the extent to which polyphenols, particularly chlorogenic acid, and caffeine individually impact the composition of the microbiome. This is an important step in increasing our knowledge of dietary means to prevent or treat antibiotic-induced dysbiosis and its associated diseases, an increasing problem as antibiotic use continues to rise worldwide (Klein et al., 2018).

Overall, we found that coffee alone has very little impact on the diversity and structure of the mouse fecal microbiome. This finding is surprising for several reasons. Firstly, we administered a relatively large amount of coffee for 12 days, and other studies have found that coffee and its components can significantly alter the composition of the microbiome (Jaquet et al., 2009; Nakayama and Oishi, 2013). Additionally, coffee can directly impact gastrointestinal physiology (González et al., 2020). For example, in a rat model, coffee was shown to stimulate intestinal smooth muscle contractility in a dose-dependent manner (Hegde et al., 2019). The reduction of colonic transit time has the capacity to shape the microbiota, as the speed at which substances travel through the gut can change water and nutrient availability, as well as the rate of luminal washout (Müller et al., 2018). *In vitro* models have also confirmed that decreasing transit time can change microbial composition, leading to a decrease in certain bacterial populations (Child et al., 2006). Fiber, a component of coffee, also has laxation effects in the gut (Müller et al., 2018). However, we did not observe a change in microbiome composition despite these potential effects. Broadly speaking, this study may indicate that one of the most widely consumed substances in the world does not significantly impact microbiome composition (Manchón et al., 2013).

There are some limitations to this study that must be taken into account when interpreting the results and designing

future experiments. While we did not observe major changes in microbial composition or diversity in mice supplemented with coffee alone, we did observe some significant differences in mice supplemented with coffee and treated with antibiotics for a few phyla. We found that the microbiome was the most affected on the second day of antibiotic treatment (day 7), and a general but incomplete recovery in diversity and composition was observed through the end of the experiment (day 12). Conducting statistical testing on Shannon diversity using the Holm-Šídák method revealed that decaf (adjusted p-value = 0.0202), control ABX (adjusted p-value = 0.0237), and caf ABX (adjusted p-value = 0.0307) were still significantly different at day 12 compared to day 0. In addition to these diversity differences, we also observed taxonomic differences between the treated and untreated groups at day 12 using LEfSe (**Supplementary Figure 6**). Due to this partial recovery and the robust changes observed early on, we focused on day 7 to analyze significant changes in microbial composition due to antibiotics. While the initial impact is clearly important for long-term disruption of the microbiome, this later time point should also be considered as it can impact recovery after treatment.

The effects of coffee on the microbiome may differ depending on the method of coffee production, the type of beans used, whether it is brewed or instant coffee, and the method of decaffeination, as all of these can affect the levels of caffeine, polyphenols, and fiber within coffee. We used only one brand and type of coffee, Starbucks instant coffee, and thus captured only the impact of this specific formulation. Future studies could investigate the impact of coffee's individual components, especially polyphenols, on the fecal microbiome. In addition, humans typically consume coffee intermittently throughout the day, unlike through a single oral gavage dose that we utilized in our mice. This may affect how well our results can be extrapolated to humans. We used only one concentration of caffeine and amoxicillin in our *in vivo* experiments, so nuances in the effect of concentration on the microbiome may have been missed. There was also no recovery period for mice treated with antibiotics, so we were unable to determine if there was any difference in microbiome recovery for mice given different treatments. We were also limited in our studies due to the fact that 16S analysis cannot accurately tell us the genus or species of the bacteria we observed, so we could not get a clear picture of the microbiome at lower taxonomic levels than those shown here. Since the microbiome cannot solely inform us of the state of someone's health, it would be interesting to see future studies on host physiology post-coffee and antibiotic administration. Such studies could include histology of the intestine to look for changes in gut permeability induced by treatment and qPCR to determine expression levels of important gut barrier genes.

In conclusion, as antibiotic use continues to be a necessary treatment, there remains a need to investigate dietary components with potential to impact antibiotic-induced dysbiosis, such as coffee and caffeine. The results of this study show that coffee consumption alone does not perturb the microbiome within the timeframe of the study. In addition, we found that in the context of antibiotic administration, coffee consumption does impact the response of the microbiome to amoxicillin. Although the observed changes at the taxonomic level were not dramatic, they may point to a potentially beneficial modulation. Thus, we believe that this work should be followed up with additional research looking at other drugs, timing of administration, and the *in vitro* impact of coffee and caffeine on other bacterial taxa.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA682275.

ETHICS STATEMENT

The animal study was reviewed and approved by Brown University's Institutional Animal Care and Use Committee (IACUC) under protocol number 1706000283.

AUTHOR CONTRIBUTIONS

PB, ED, KH, and SP conceptualized the project and assisted in experimentation, writing, and editing. AB conducted MS analysis. All authors have reviewed and approved the manuscript for final version.

FUNDING

ED and KH were supported by a Karen T. Romer Undergraduate Teaching and Research Award (UTRA) and a BrownConnect Collaborative Short-term Projects for Research, Internships, and Teaching (SPRINT) Award. SP was supported by the Graduate Research Fellowship Program from the National Science Foundation under award number 1644760. PB was supported by the National Center of the National Institutes of Health under award number R21 AT010366 and the National Institute of Diabetes and Digestive and Kidney Diseases under award number RO1 DK25382. The funding agencies had no role in the design and preparation of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.637282/full#supplementary-material

Supplementary Figure 1 | Phylogenetic diversity of experimental groups as measured by the Faith's Phylogenetic Diversity Index. Data are represented as mean ± standard deviation (SD). Significance was determined by the Benjamini, Krieger and Yekutieli test to correct for False discoveries with adjusted p-value < 0.05. (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $0.0001 < P < 0.001$; ****, $P < 0.0001$). Significance between each group and its antibiotic counterpart is denoted by stars (*) with the color coordinating to control (ctrl, blue), decaffeinated

coffee (decaf, purple), or caffeinated coffee (caf, pink). Significance between different experimental groups is denoted by squares color-coded according to the experimental groups being compared. Solid squares represent groups without antibiotics, open squares represent groups given antibiotics. Each comparison of two experimental groups has its corresponding significance denoted in stars (*) next to it. $n = 6$.

Supplementary Figure 2 | Relative abundance of bacterial phyla over the course of the experiment. Dotted line denotes beginning of antibiotic treatment. **(A)** Control, **(B)** Control + amoxicillin days 5–12, **(C)** Decaf, **(D)** Decaf + amoxicillin days 5–12, **(E)** Caf, **(F)** Caf + amoxicillin days 5–12. $n = 6$.

Supplementary Figure 3 | Linear discriminant analysis effect size (LEfSe) analysis between treatment groups on day 7 at the phylum level **(A,C,E,G,I)**, and at the family level **(B,D,F,H,J)**. Histogram of LDA scores plotted on a log 10 scale.

Supplementary Figure 4 | (A–C) Linear discriminant analysis effect size (LEfSe) analysis between antibiotic treated and control groups on day 10 at the family level. Histogram of LDA scores plotted on a log 10 scale.

Supplementary Figure 5 | *Burkholderiaceae* relative abundance, with control and antibiotic samples interleaved by ranked abundance of each taxon. Means represented by dotted (ABX) or dashed (Control) lines. Relative abundance shown for **(A)** Day 7, **(B)** Day 10, **(C)** Day 12. Significance between each group and its antibiotic counterpart is denoted by vertical bars. Solid horizontal bars denote significance between non-antibiotic treated groups (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $0.0001 < P < 0.001$; ****, $P < 0.0001$; unpaired *t* test).

Supplementary Figure 6 | (A–C) Linear discriminant analysis effect size (LEfSe) analysis between antibiotic treated and control groups on day 12 at the family level. Histogram of LDA scores plotted on a log 10 scale.

REFERENCES

Afgan, E., Baker, D., Batut, B., Van Den Beek, M., Bouvier, D., Ech, M., et al. (2018). The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46, W537–W544.

Allison, K. R., Brynildsen, M. P., and Collins, J. J. (2011). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* 473, 216–220. doi: 10.1038/nature10069

Armstrong, H., Alipour, M., Valcheva, R., Bording-Jorgensen, M., Jovel, J., Zaidi, D., et al. (2019). Host immunoglobulin G selectively identifies pathobionts in pediatric inflammatory bowel diseases. *Microbiome* 7:1. doi: 10.1186/s40168-018-0604-3

Becattini, S., Taur, Y., and Pamer, E. G. (2016). Antibiotic-induced changes in the intestinal microbiota and disease. *Trends Mol. Med.* 22, 458–478. doi: 10.1016/j.molmed.2016.04.003

Belenky, P., Ye, J. D., Porter, C. B. M., Cohen, N. R., Lobritz, M. A., Ferrante, T., et al. (2015). Bactericidal antibiotics induce toxic metabolic perturbations that lead to cellular damage. *Cell Rep.* 13, 968–980. doi: 10.1016/j.celrep.2015.09.059

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Blaser, M. (2011). Antibiotic overuse: stop the killing of beneficial bacteria. *Nature* 476, 393–394. doi: 10.1038/476393a

Bokulich, N. A., Dillon, M. R., Zhang, Y., Rideout, J. R., Bolyen, E., Li, H., et al. (2018a). q2-longitudinal: longitudinal and paired-sample analyses of microbiome data. *mSystems* 3:e00219-18.

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018b). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6:90.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857.

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Brown, S. R., Cann, P. A., and Read, N. W. (1990). Effect of coffee on distal colon function. *Gut* 31, 450–453. doi: 10.1136/gut.31.4.450

Cabral, D. J., Penumutchu, S., Reinhart, E. M., Zhang, C., Korry, B. J., Wurster, J. I., et al. (2019). Microbial metabolism modulates antibiotic susceptibility within the murine gut microbiome. *Cell Metab.* 30, 800–823.e7.

Cabral, D., Wurster, J., Korry, B., Penumutchu, S., and Belenky, P. (2020). Consumption of a Western-style diet modulates the response of the murine gut microbiome to ciprofloxacin. *mSystems* 5, e317–e320.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Child, M. W., Kennedy, A., Walker, A. W., Bahrami, B., Macfarlane, S., and Macfarlane, G. T. (2006). Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques. *FEMS Microbiol. Ecol.* 55, 299–310. doi: 10.1111/j.1574-6941.2005.00016.x

Couteau, D., McCartney, A. L., Gibson, G. R., and Williamson, G. (2001). Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. *J. Appl. Microbiol.* 90, 873–881. doi: 10.1046/j.1365-2672.2001.01316.x

Everaert, A., and Coenye, T. (2016). Effect of β-Lactamase inhibitors on in vitro activity of β-Lactam antibiotics against Burkholderia cepacia complex species. *Antimicrob. Resist. Infect. Control* 5:44.

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14, 491–502. doi: 10.1038/nrgastro.2017.75

Gniechwitz, D., Reichardt, N., Blaut, M., Steinhart, H., and Bunzel, M. (2007). Dietary fiber from coffee beverage: degradation by human fecal microbiota. *J. Agric. Food Chem.* 55, 6989–6996. doi: 10.1021/jf070646b

González, S., Salazar, N., Ruiz-Saavedra, S., Gómez-Martín, M., de los Reyes-Gavilán, C. G., and Gueimonde, M. (2020). Long-term coffee consumption is associated with fecal microbial composition in humans. *Nutrients* 12:1287. doi: 10.3390/nu12051287

Gurwara, S., Dai, A., Ajami, N., El-Serag, H. B., Graham, D. Y., and Jiao, L. (2019). 196 caffeine consumption and the colonic mucosa-associated gut microbiota. *Off. J. Am. Coll. Gastroenterol.* 114, S119–S120.

Hegde, S., Shi, D., Lin, Y.-M., and Shi, X.-Z. P. (2019). Su1625 – in vivo and in vitro effects of coffee on gut microbiota and smooth muscle contractility in rats. *Gastroenterology* 156:S-587.

How Is Coffee Decaffeinated? | Britannica (2020). Available Online at: https://www.britannica.com/story/how-is-coffee-decaffeinated (accessed October 4, 2020).

Jaquet, M., Rochat, I., Moulin, J., Cavin, C., and Bibiloni, R. (2009). Impact of coffee consumption on the gut microbiota: a human volunteer study. *Int. J. Food Microbiol.* 130, 117–121. doi: 10.1016/j.ijfoodmicro.2009.01.011

Jha, R., Fouhse, J. M., Tiwari, U. P., Li, L., and Willing, B. P. (2019). Dietary fiber and intestinal health of monogastric animals. *Front. Vet. Sci.* 6:48.

Kashyap, P. C., Marcobal, A., Ursell, L. K., Larauche, M., Duboc, H., Earle, K. A., et al. (2013). Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. *Gastroenterology* 144, 967–977. doi: 10.1053/j.gastro.2013.01.047

Katoh, K., Misawa, K., Kuma, K. I., and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. *Nucleic Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436

Kim, S., Covington, A., and Pamer, E. G. (2017). The intestinal microbiota: antibiotics, colonization resistance, and enteric pathogens. *Immunol. Rev.* 279, 90–105. doi: 10.1111/imr.12563

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., et al. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U. S. A.* 115, E3463–E3470.

Knight, R., Callewaert, C., Marotz, C., Hyde, E. R., Debelius, J. W., McDonald, D., et al. (2017). The microbiome and human biology. *Annu. Rev. Genomics Hum. Genet.* 18, 65–86.

Korpela, K. (2018). Diet, microbiota, and metabolic health: trade-off between saccharolytic and proteolytic fermentation. *Annu. Rev. Food Sci. Technol.* 9, 65–84. doi: 10.1146/annurev-food-030117-012830

Leonard, W., Zhang, P., Ying, D., and Fang, Z. (2020). Hydroxycinnamic acids on gut microbiota and health. *Compr. Rev. Food Sci. Food Saf.* 20, 710–737. doi: 10.1111/1541-4337.12663

Liang, N., and Kitts, D. D. (2015). Role of chlorogenic acids in controlling oxidative an inflammatory stress conditions. *Nutrients* 8:16. doi: 10.3390/nu8010016

Lobritz, M. A., Belenky, P., Porter, C. B. M., Gutierrez, A., Yang, J. H., Schwarz, E. G., et al. (2015). Antibiotic efficacy is linked to bacterial cellular respiration. *Proc. Natl. Acad. Sci.* 112, 8173–8180. doi: 10.1073/pnas.1509743112

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/aem.01996-06

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/aem.71.12.8228-8235.2005

Manchón, N., Mateo-Vivaracho, L., Darrigo, M., García-Lafuente, A., Guillamón, E., Villares, A., et al. (2013). Distribution patterns of polyphenols and alkaloids in instant coffee, soft and energy drinks, and tea. *Czech J. Food Sci.* 31, 483–500. doi: 10.17221/443/2012-cjfs

Müller, M., Canfora, E. E., and Blaak, E. E. (2018). Gastrointestinal transit time, glucose homeostasis and metabolic health: modulation by dietary fibers. *Nutrients* 28:275. doi: 10.3390/nu10030275

Nair, A., and Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. *J. Basic Clin. Pharm.* 7, 27–31. doi: 10.4103/0976-0105.177703

Nakayama, T., and Oishi, K. (2013). Influence of coffee (*Coffea arabica*) and galacto-oligosaccharide consumption on intestinal microbiota and the host responses. *FEMS Microbiol. Lett.* 343, 161–168. doi: 10.1111/1574-6968.12142

Olajuyigbe, O. O., Adeoye-Isijola, M. O., Okon, V., Adedayo, O., and Coopoosamy, R. M. (2017). In vitro pharmacological interaction of caffeine and first-line antibiotics is antagonistic against clinically important bacterial pathogens. *Acta Biochim. Pol.* 64, 255–263.

Preidis, G. A., and Versalovic, J. (2009). Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology* 136, 2015–2031. doi: 10.1053/j.gastro.2009.01.072

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596.

Rezende, E. S. V., Lima, G. C., and Naves, M. M. V. (2021). Dietary fibers as beneficial microbiota modulators: a proposal classification by prebiotic categories. *Nutrition* 89:111217. doi: 10.1016/j.nut.2021.111217

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., and Gasbarrini, A. (2017). Proteobacteria: a common factor in human diseases. *Biomed Res. Int.* 2017:9351507.

Scoffone, V. C., Chiarelli, L. R., Trespidi, G., Mentasti, M., Riccardi, G., and Buroni, S. (2017). Burkholderia cenocepacia infections in cystic fibrosis patients: drug resistance and therapeutic approaches. *Front. Microbiol.* 8:1592.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., Dori-Bachash, M., Bashiardes, S., et al. (2018). Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell* 174, 1406–1423.e16.

Teekachunhatean, S., Tosri, N., Rojanasthien, N., Srichairatanakool, S., and Sangdee, C. (2013). Pharmacokinetics of caffeine following a single administration of coffee enema versus oral coffee consumption in healthy male subjects. *ISRN Pharmacol.* 2013:147238.

Thompson, L. R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Locey, K. J., et al. (2017). A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* 551, 457–463.

Wiegand, I., Hilpert, K., and Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* 3, 163–175. doi: 10.1038/nprot.2007.521

Win, Y. Y., Singh, M., Sadiq, M. B., and Anal, A. K. (2019). Isolation and identification of caffeine-degrading bacteria from coffee plantation area. *Food Biotechnol.* 33, 109–124. doi: 10.1080/08905436.2019.1570854

Zarrinpar, A., Chaix, A., Xu, Z. Z., Chang, M. W., Marotz, C. A., and Saghatelian, A. (2018). Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. *Nat. Commun.* 9:2872.

Conflict of Interest: AB was employed by the company TarMeta Biosciences Inc. This company had no financial stake or involvement in the project.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 13 August 2021
doi: 10.3389/fmicb.2021.626144

Delivery Mode Affects Intestinal Microbial Composition and the Development of Intestinal Epithelial Cells

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 04 November 2020
Accepted: 06 July 2021
Published: 13 August 2021

Citation:
Lyu L, Zhou X, Zhang M, Liu L, Niu H, Zhang J, Chen S, Gong P, Jiang S, Pan J, Li Y, Han X, Cheng D and Zhang L (2021) Delivery Mode Affects Intestinal Microbial Composition and the Development of Intestinal Epithelial Cells. Front. Microbiol. 12:626144. doi: 10.3389/fmicb.2021.626144

Background: The infant's intestine contains diverse microbiota, which play an important role in an infant's health.

Objective: This study aimed to analyze the different intestinal microbiota and their function in two delivery modes [vaginal delivery and cesarean section (C-section)] and to investigate the proprieties of bacteria associated with vaginal delivery on the development of intestinal epithelial cells in rat pups.

Materials and Methods: We evaluated the intestinal microbial diversity of the stool samples of 51 infants of subjects who underwent vaginal delivery and C-section by sequencing the V4 regions of the 16S rRNA gene and predicted the function of the microbiotas. The infant stool microbiota in the vaginal delivery group was associated with the digestive system and cell growth and death, whereas that of the C-section group was associated with membrane transport. Then, we isolated the strains based on function prediction.

Results: A total of 95 strains were isolated in the vaginal delivery group. *Bifidobacterium bifidum* FL-228.1 (FL-228.1) was screened and selected owing to its good surface hydrophobicity, bacterial survivability in the simulated gastrointestinal condition and adhesion ability to the IEC-6 cell line as well as owing to the development of intestinal epithelial cells. Furthermore, *in vivo* experiments revealed that FL-228.1 exhibited favorable effects on the development of intestinal epithelial cells in rat pups.

Conclusion: The results of this study indicate an apparent difference in the bacterial composition of the stool samples collected from infants of the two delivery modes. By analyzing and screening the bacteria in infant stool samples, we found that one strain, i.e., *B bifidum* FL-228.1, exhibited favorable effects on the development of intestinal epithelial cells.

Keywords: delivery modes, intestinal microbiota, bacterial screening, infant intestinal epithelial cells, cell proliferation

INTRODUCTION

The human intestinal microbiota comprises trillions of microbes and contains millions of functional genes that are mutually beneficial to the host (Friedrich, 2013). After birth, an infant's intestine begins to be colonized by various microorganisms, including *Enterobacteria*, *Enterococci*, *Staphylococci*, *Bifidobacteria,* and *Lactobacilli* (Nagpal et al., 2017). These microbiota colonized on the intestine play an important role in infant growth and affect the child's overall health status (Maldonadolobón et al., 2015).

Research has shown that the diversity and composition of the intestinal microbiota in early infants are greatly influenced by external factors (Madan et al., 2016b). Furthermore, many studies have reported that delivery mode is an important factor affecting the composition of the intestinal microbiota throughout the neonatal period and into infancy (Shao et al., 2019). During vaginal delivery, the microorganisms in the vagina are transmitted to the infants vertically. Therefore, Actinobacteria and Bacteroidetes are abundant at the phylum level, whereas *Bifidobacterium* is the dominant species at the genus level (Lundgren et al., 2018). Compared with vaginal delivery infants, the intestinal microbiota of cesarean section (C-section) infants predominantly originate from the mother's skin as well as from microorganisms in the environment. Therefore, the relative abundance of Firmicutes and a few microbes from the skin, such as *Staphylococcus* and *Clostridium*, are higher than that of other bacteria (Jakobsson et al., 2014).

Studies in infants have indicated that C-section infants tend to have lower number of anaerobes (e.g., Bacteroidetes) and a less diverse microbiota than vaginal delivery infants (Chong et al., 2018). Further, the composition of microbiota in vaginal delivery infants is relatively more stable than that of C-section infants until 2 months of life (Reyman et al., 2019). Analysis of the intestinal microbiota of infants has revealed that there are differences in the composition of the intestinal microbiota of C-section and vaginal delivery infants (Shao et al., 2019). Interestingly, although there are apparent differences in stool microbiological composition of C-section and vaginal delivery infants after birth, a study has shown that C-section subjects gradually progress toward harboring microbiota reassembling natural birth infants by week 8 of life, which is maintained till week 24 (Hill et al., 2017).

Previous studies have compared the bacterial composition of stool samples of C-section and vaginal delivery infants (Madan et al., 2016b). Epidemiological evidence has revealed that C-section affects the initialization of intestinal microbiota colonization in infants, which in turn affects the formation and maturation of the intestinal immune system (Planer et al., 2016). A study by Salam (Salam et al., 2006) reported that C-section damages intestinal immunity by increasing the risk of gastrointestinal infections and allergies. However, to the best of our knowledge, no study has screened and isolated bacteria based on these two delivery modes for functional exploration. As a result, this study aimed to analyze the different intestinal microbiota and their function in the two delivery modes as well as to investigate the proprieties of bacteria associated with vaginal delivery on the development of intestinal epithelial cells in rat pups.

MATERIALS AND METHODS

Participants

Fifty-one healthy families were recruited from the Third Affiliated Hospital of Heilongjiang University of Chinese Medicine (China) and Harbin Children's Hospital (Harbin, China). The Institutional Review Board of Harbin Children's Hospital approved this study; written informed consent was obtained from all the mothers. Prior to sampling, we surveyed the mothers via a questionnaire to collect clinical data. Infant stool samples were collected at the hospital or during a home visit in a period of 30 days. They were promptly transported to the laboratory and stored at −80°C until further analysis (Bäckhed et al., 2015).

Isolation of Different Strains

One gram of the stool sample was diluted with 9 mL of phosphate-buffered saline (PBS buffer). The semi-selective medium method was used to screen the strains, as shown in **Table 1**. The plates were anaerobically incubated at 37°C for 72 h and then left in the air for 4 h. Single, blue colonies were isolated and subcultured for further purification and experiments. Each selected strain was subjected to microscopic examination and gram staining. The gram-positive strains were selected for further research (Riaz Rajoka et al., 2017).

Surface Hydrophobicity

The surface hydrophobicity of the selected strains was determined using a previously described method with some modifications (Xu et al., 2018). Briefly, the bacteria were incubated at 37°C for 72 h, centrifuged for 3 min at 6000 r/min and then resuspended in 3 mL of sterile PBS buffer. The absorbance of the suspension was measured at 600 nm (A_0). Then, the suspension was mixed with 1 mL of ethyl acetate and placed at room temperature for 10 min. The two-phase system was mixed via swirl oscillation for 2 min. Then, the two-phase system was reconstructed by standing for 20 min. The aqueous phase was carefully removed, and the absorbance was measured at 600 nm (A_1). The reference strain *Lactobacillus rhamnosus* LGG (ATCC 53103) was used as a control. Surface hydrophobicity was calculated according to the following formula (Pan et al., 2006):

$$H\% = (1 - A_1/A_0) \times 100$$

TABLE 1 | Semi-selective medium.

Putatively common strains	Additive	Semi-selective medium (Martín et al., 2009; Lau et al., 2016)
Lactobacillus	Nalidixic acid and X-gal	MRS-Cys/LBS-Cys
Bifidobacterium	Nalidixic acid and X-gal	MRS-Cys/LBS-Cys

Bacterial Survivability in a Simulated Gastrointestinal Condition

The isolates were incubated in a simulated gastric fluid of PBS buffer supplemented with pepsin (0.5%) and adjusted to pH 2.5 with 0.1 M hydrochloric acid. After incubation at 37°C for 1.5 h, the viable isolates were evaluated via plate count on MRS agar. The simulated intestinal juice tolerance of the isolates that survived for 2 h in the simulated intestinal juice was determined using PBS buffer containing 0.3% (w/v) bile salt and trypsin (1 mg/mL); the pH was adjusted to 8.0. Following a 2-h incubation at 37°C, the simulated intestinal juice was assessed by measuring the survived isolates using MRS agar medium (Xu et al., 2018). The reference strain *Lactobacillus rhamnosus* LGG (ATCC 53103) was used as a control.

Bacterial Adhesion Ability to the IEC-6 Cell Line

IEC-6 (National Collection of Authenticated Cell Cultures) were incubated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. For adhesion assays, IEC-6 cells were seeded into 24-well tissue culture plates at a concentration of 2.5×10^5 cells/well and incubated at 37°C for 24 h in 5% CO_2 and 95% air. Then, the bacteria were added into the corresponding wells. Following 4-h incubation, IEC-6 cells were washed with PBS buffer to remove the bacterial suspensions, followed by lysis of non-adherent bacteria with 0.1% Triton X-100 solution. The adherent bacteria were serially diluted and spread on MRS agar plates. The plates were incubated in an anaerobic environment at 37°C for 72 h to calculate the number of adherent bacteria. The reference strain *Lactobacillus rhamnosus* LGG (ATCC 53103) was used as a control. The adhesion percentage of the bacteria was calculated according to the following formula (Oh and Jung, 2015):

Adhesion ability rate (%) = [adhered cell number (log CFU/mL)/initial cell number (log CFU/mL)] × 100%

Development of Intestinal Epithelial Cells *in vitro*

A concentration of 2.5×10^5 cells/mL was inoculated into a 24 well plate; the culture medium was changed after overnight culturing at 37°C. Then, bacteria were added into the corresponding wells at the ratio of 1:100. The bacteria and IEC-6 cells were cultured together for 4 h, followed by washing IEC-6 cells with PBS buffer. The blood cell counting plate was used to count the number of IEC-6 cells in each well. The reference strain *Lactobacillus rhamnosus* LGG (ATCC 53103) was used as a control. The experiment was repeated three times.

TABLE 2 | Intervention treatment.

Groups	Intervention treatment
Blank control group	Normal lactation and intragastric administration of PBS
Positive control group	Normal lactation and intragastric administration of LGG PBS suspension (10^9 CFU/mL)
Experimental group	Normal lactation and intragastric administration of the FL-228.1 strain in a PBS suspension (10^9 CFU/mL)

Identification of the Different Strains

The 16S rRNA gene sequencing method was used to identify the isolated strains. Briefly, genomic DNA was extracted using the E.Z.N.A. bacterial DNA kit (Omega Bio-Tek, Norcross, GA). The 16S rRNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Meng et al., 2018). The amplicons were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). Sequence similarity analysis was performed by comparing the sequences with those in GenBank via the BLAST search program of the National Centre for Biotechnology Information.[1]

Establishment of the Intrauterine Growth Retardation (IUGR) Animal Model

Pregnant female Sprague Dawley rats were provided by Charles River Laboratory Animal Technology Co., Ltd. (Beijing, China). Pregnant rats were fed separately. The IUGR animal model was established via maternal nutritional restriction. Pregnant rats were randomly divided into two groups: Normal diet group and food restriction group. The rats in the normal diet group were allowed *ad libitum* access to food and water. From the 3rd day of pregnancy to the day of delivery, the rats in food restriction group were provided with 50% of food. Both groups received adequate food and water on the day of delivery. Compared with normal neonatal rats, IUGR neonatal rats were successfully modeled when the weight of neonatal rats was less than two standard deviations in the food restriction group. Following successful

[1]http://www.ncbi.nlm.nih.gov

TABLE 3 | Basic information of participants.

Characteristics	Delivery modes			
	C-section		Vaginal	
	Median or No.	Interquartile ranges or percentage	Median or No.	Interquartile ranges or percentage
Mother's age (y)	31	22–38	29	23–35
Father's age (y)	33	25–43	29	24–37
Mother's pregnancy weight (kg)	74.2	57–96	69.75	60.6–92.5
Gestation age (d)	277	266–284	279	266–288
Birth weight (g)	3725	3,000–4,800	3400	2,700–3,900
Birth length (cm)	52	49–55.6	50	49–51
Infant sex				
Male	18	69.23%	14	56.00%
Female	8	30.77%	11	44.00%
Feeding patterns				
Exclusive breastfeeding	16	61.54%	12	48.00%
Mixed feeding	10	38.46%	13	52.00%

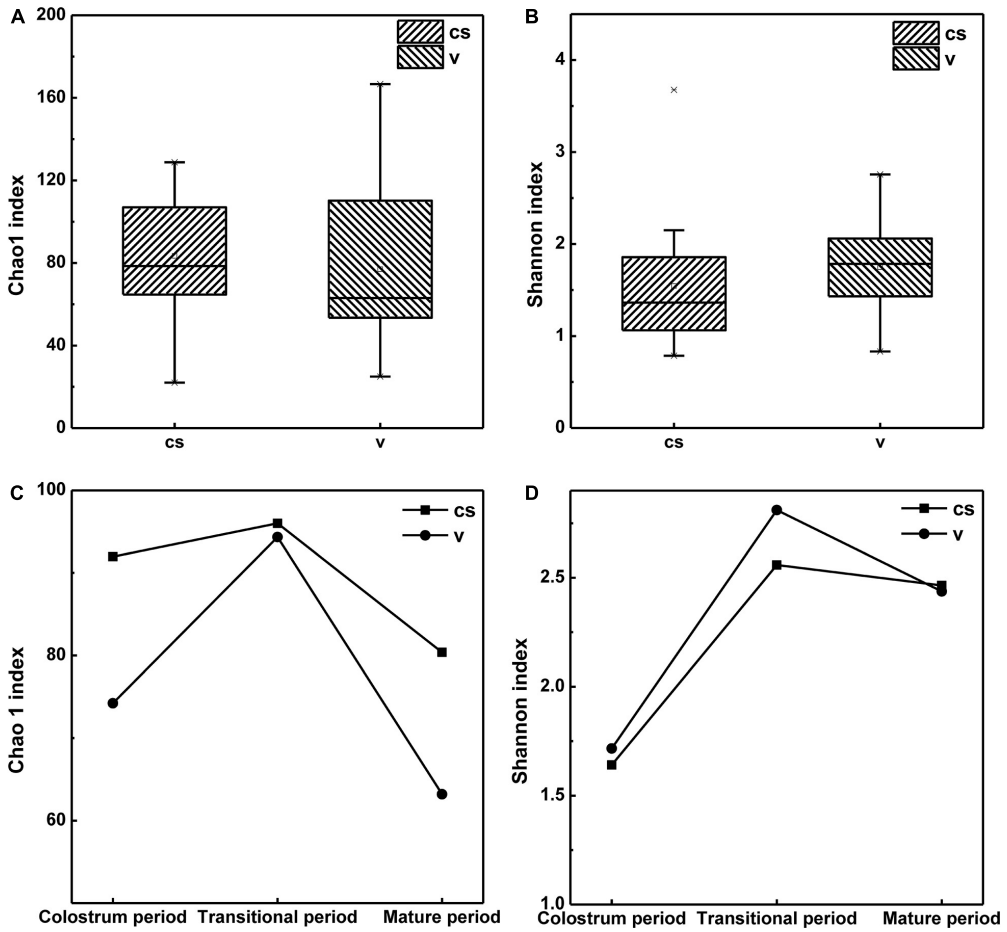

FIGURE 1 | Alpha diversity of the stool microbiota of infant born via different delivery modes. **(A)** The Chao1 index of infant stool. **(B)** The Shannon index of infant stool. **(C)** Changes in Chao1 index in the stool samples of infants in different stages. **(D)** Changes in Shannon index in the stool samples of infants in different stages.

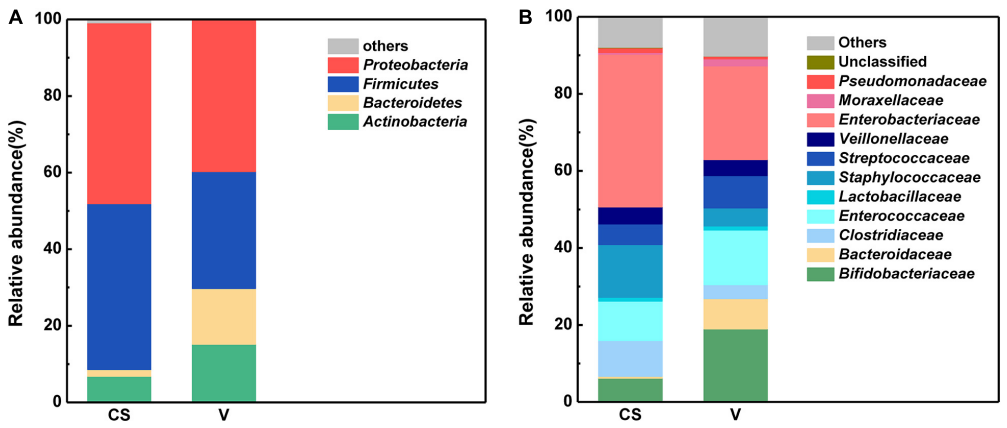

FIGURE 2 | Characteristics of the infant stool microbiota between the two different delivery modes at the phylum and family levels. **(A)** Phylum level characteristics of infant stool microbiota between the two different delivery modes. **(B)** Family level characteristics of infant stool microbiota between the two different delivery modes.

modeling, the models were grouped into the following groups, as shown in **Table 2**: Blank control group, positive control group and experimental group. Neonatal rats were naturally fed for 3 days. Then, the intervention experiment was conducted. The bacteria were supplemented to pups via oral gavage using a special syringe (0.6 mm diameter). Rat pups were anaesthetized

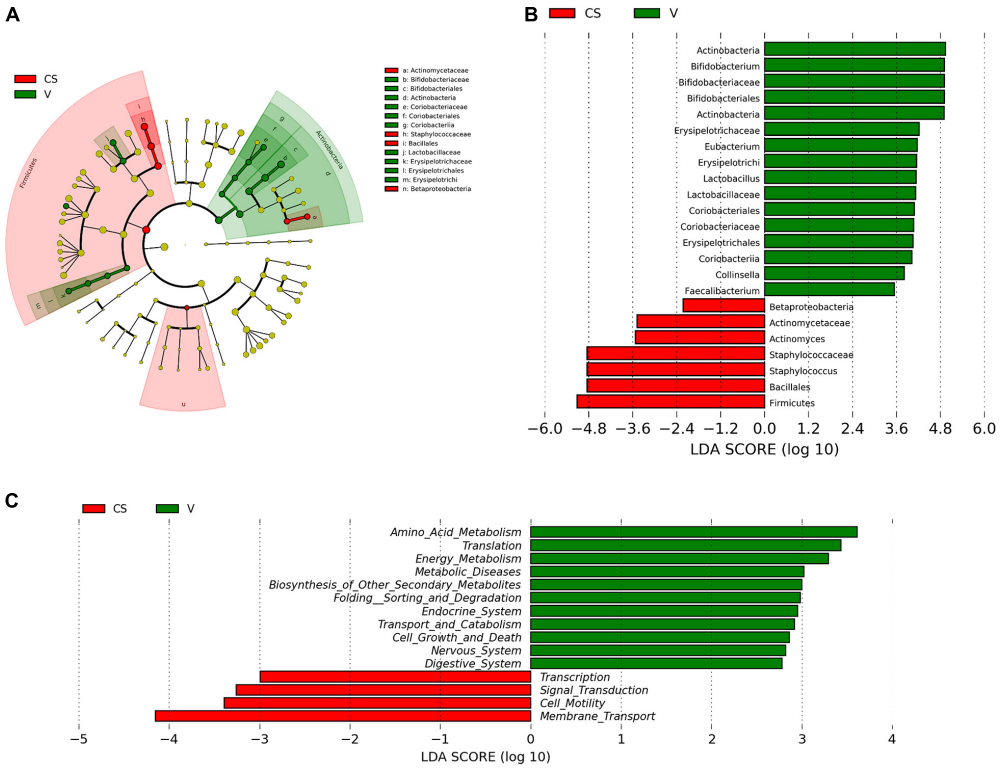

FIGURE 3 | Comparison of the different microbiota in the stool samples of infants born via different delivery modes. **(A)** LEfSe analysis of the different microbiota in stool samples of vaginal delivery and C-section infants. Microbial communities in the samples of the vaginal delivery infants via their LDA value statistics are shown in green; C-section samples with a negative LDA score are shown in red. LDA value is absolute. **(B)** Cladogram derived from LEfSe analysis of the metagenomic sequences from the stool samples of C-section and vaginal delivery infants. Green areas indicate a significant effect on the microbial groups in the vaginal delivery group. Red areas indicate a significant effect on the microbial groups in the C-section group. LDA values were obtained via linear regression analysis. **(C)** Analysis of KEGG differential pathways via LEfSe.

with isoflurane and sacrificed via cervical dislocation on the 10th day (6 pups/group) (Ding et al., 2016).

Intestinal Morphological Characteristics of Rat Pups

The intestinal morphology of rat pups was assessed via the villus length to determine whether the intestinal structure was affected by bacterial supplementation using the hematoxylin-eosin -staining. Following dehydration, the fixed intestinal villus tissues were embedded in paraffin, sliced (5 μm), patched in warm water at 42°C and copied at 42°C for 8 h. The hematoxylin–eosin-stained paraffin sections were observed under a Zeiss Axio Imager microscope (Désir-Vigné et al., 2018).

Effect of Strains on Intestinal Cell Development

The small and large intestinal tissues of the nuclear development marker Ki-67 were collected via immunostaining on the 10th day to detect the contribution of bacteria toward the changes in intestinal structures. The immunohistochemical method was used to detect the expression of the cell development activity indicator in the intestinal mucosa. Parallel tissue slices of the cells were taken. Rabbit anti-Ki-67 monoclonal antibody was incubated in a wet box at room temperature. HRP-labeled anti-rabbit IgG was incubated at room temperature for 1 h. The positive result of DAB staining was brown at the antigen location (Bhinder et al., 2017).

Illumina Sequencing and Statistical Analyses

Total genomic DNA was extracted from infant stool samples using the E.Z.N.A. bacterial DNA kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. The V4 region of the 16S rRNA gene was sequenced on the HiSeq 2500 sequencer, which was performed by BGI Tech Solutions Co., Ltd. (Shenzhen, China). The microbiome data were then analyzed using the Mothur software (V1.31.2). All samples were analyzed at the operational taxonomic unit (OTU) level. An OTU was defined as a group of bacteria with more than 97% similarity. Differences in OTUs were analyzed in every sample via α-diversity (chao1 and Shannon indices) using the R package. The bacterial diversity of infant stool samples was analyzed using the R package (ade4). Community composition analysis between the groups was performed using distance matrices (Adonis, R vegan package) (Pragman et al., 2018). All linear discriminant analysis effect size (LEfSe) taxonomic information was detected using the

Metaphlan 2 program, with the cladogram derived from LEfSe analysis (Liu et al., 2016). The Illumina sequences were submitted to the NCBI Sequence Read Archive database with an accession number of PRJNA683130. The 16S rRNA sequence of FL-228.1 was submitted to the GeneBank database with an accession number of MT071594.

All experiment results were analyzed with SPSS software (version 22). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Questionnaire and Subject Information

Fifty-one healthy infants were recruited; their basic information is summarized in **Table 3**. Among the infants recruited, there were 32 boys and 19 girls. In total, 25 (49%) infants were born via vaginal delivery and 26 (51%) were born via C-section.

Biodiversity of the Intestinal Microbiota of Infant Born via Different Delivery Modes

Within-sample (alpha) diversity of the infant stool samples was assessed using two indices, namely, the Shannon and Chao1 indices. As shown in **Figure 1A**, for all samples, the microbiota of C-section infants exhibited higher Chao1 index than those of vaginal delivery infants; however, the difference was not significant. This indicated that the infants in the vaginal delivery group had a higher number of bacterial species than the infants in the C-section group. Meanwhile, the microbiota in the stool samples of C-section infants showed a non-significantly lower Shannon index than those in stool samples of vaginal delivery infants (**Supplementary Table 1**). This result suggested that vaginal delivery had higher evenness in infant stool (**Figure 1B**).

All samples were grouped according to infants' age: 1–7 days (colostrum period), 8–14 days (transitional period) and 15–30 days (mature period). The results also indicated that after birth, the bacterial species in the infant stool had increased from the 1st day to the 14th day after birth and then declined until the 30th day. Shannon index also showed a similar trend, indicating that the evenness of the intestinal microbiota was richest during the transitional period and then decreased during the maturation period. Both Chao1 and Shannon indices showed the highest values during the transitional period, whether in the stool samples of C-section infants or in those of vaginal delivery infants (**Figures 1C,D**). This meant that there were more species and bacterial homogeneity in the transitional period than in the other periods both in the C-section and vaginal delivery modes.

Bacterial Components in the Infant's Intestine Between the Two Delivery Modes

The relative abundance of the different bacteria in the infants born via the two delivery modes is depicted in **Figure 2**. Proteobacteria and Firmicutes were the dominant phyla observed in these two delivery modes (**Figure 2A**). Further, the number of Proteobacteria and Firmicutes was higher in the C-section group than in the vaginal delivery group. In contrast, Bacteroidetes *(Bacteroidaceae)* and Actinobacteria *(Bifidobacteriaceae)* occupied only a small percentage compared with the above two phyla. At the family level (**Figure 2B**), *Enterobacteriaceae* exhibited the highest relative abundance in the C-section group, followed by *Staphylococcaceae and Lactobacillaceae*. In contrast, *Enterobacteriaceae*, *Bifidobacteriaceae,* and *Lactobacillaceae* were dominant in the vaginal delivery group, and other families only occupied a small percentage.

LEfSe and cladistics analyses were performed to investigate the differences in the community composition between the two different delivery modes. At the phylum level, Actinobacteria and Firmicutes were dominant in both groups. At the family level, there were five different families, with enrichment of *Bifidobacteriaceae*, *Erysipelotrichaceae*, *Lactobacillaceae*, *Coribacteriaceae,* and *Coribacteriaceae* in the vaginal delivery group and that of *Actinomycetaceae* and *Staphylococcaceae* in the C-section group (**Figures 3A,B**). There were seven different genera between the two groups. *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Collinsella,* and *Faecalibacterium* exhibited a relatively high abundance in the vaginal delivery group, whereas *Actinomyces* and *Staphylococcus* were more abundant in the C-section group (**Figures 3A,B**).

Function Prediction of Infant's Intestinal Microbiota Between the Two Delivery Modes

LEfSe analysis was performed to analyze the different functions between the two delivery modes. The abscissa is the log value of the value obtained via linear regression analysis (LDA), as depicted in **Figure 3C**. According to function prediction of the microbiota (**Figure 3C**), it could be concluded that the infant stool microbiota in the C-section group was related to membrane transport, cell motility, signal transduction and transcription. In contrast, the infant stool microbiota in the vaginal delivery group was associated with the digestive system.

Strain Isolation

Semi-selective medium was used to isolate the target strains from 25 vaginal-delivered infant stool samples. A total of 95 strains were isolated from 25 vaginal-delivered infant stool samples. Among them, 40 putative *Lactobacillus* and *Bifidobacterium* strains were screened via microscopic examination and colony morphology analysis.

Surface Hydrophobicity of the Isolates

The hydrophobicity method was used to screen the strains, as shown in **Figure 4A**. The surface hydrophobicity of the 40 putatively differential strains ranged from 28.31 to 63.47%, as shown in **Figure 4A**. Among them, 15 strains were screened because they had higher hydrophobicity percentages than the reference strain LGG (52.26%); in particular, the strain named FL-228.1 exhibited the highest hydrophobicity (63.47%). On average, *Lactobacillus* (51.62%) showed better hydrophobicity than *Bifidobacterium* (47.61%).

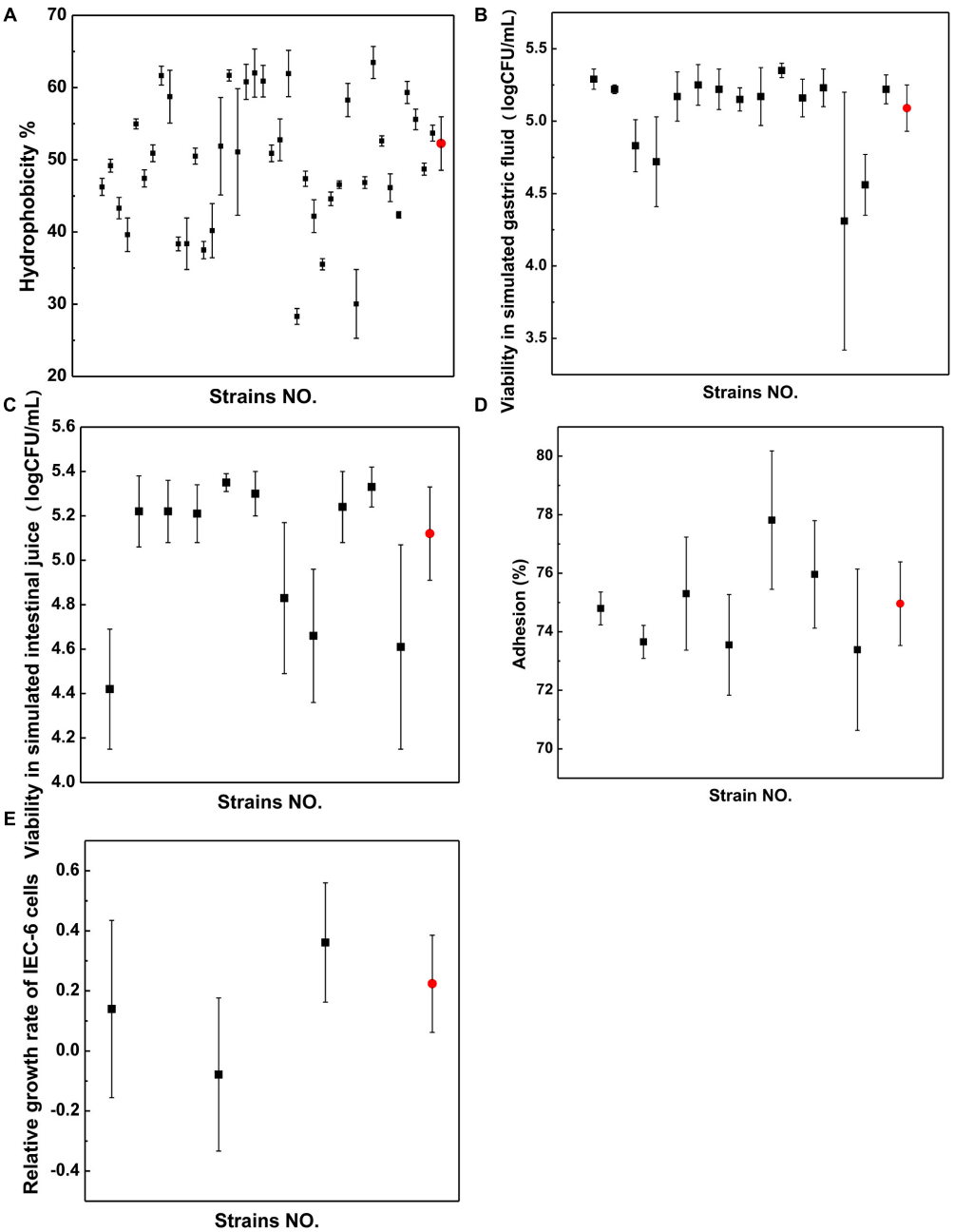

FIGURE 4 | Properties of strains. **(A)** Surface hydrophobicity. **(B)** Viability in simulated gastric fluid. **(C)** Viability in simulated intestinal juice. **(D)** Adhesion ability. **(E)** Relative growth rate. Black dots represent strains from infant stool samples and red dots represent those from LGG.

Gastrointestinal Tract Viability of the Isolates

The survival rates of 11 strains showed higher resistance to acidic conditions than LGG and were screened following exposure to simulated gastric fluid from the 15 strains, as shown in **Figure 4B**. The survival rates of seven strains were screened following exposure to simulated intestinal juice from 11 strains, as shown in **Figure 4C**. The results indicated that compared with LGG, the seven strains showed higher resistance to the simulated intestinal juice condition. The isolate FL-228.1 showed the highest resistance. A total of seven strains were screened for surface hydrophobicity and gastrointestinal tolerance indicator.

Adhesion Ability of the Different Strains to IEC-6 Cells

Through gastrointestinal fluid simulation and hydrophobicity experiments, seven bacterial strains were screened for further cell adhesion tests. Compared with LGG, three isolates from the seven

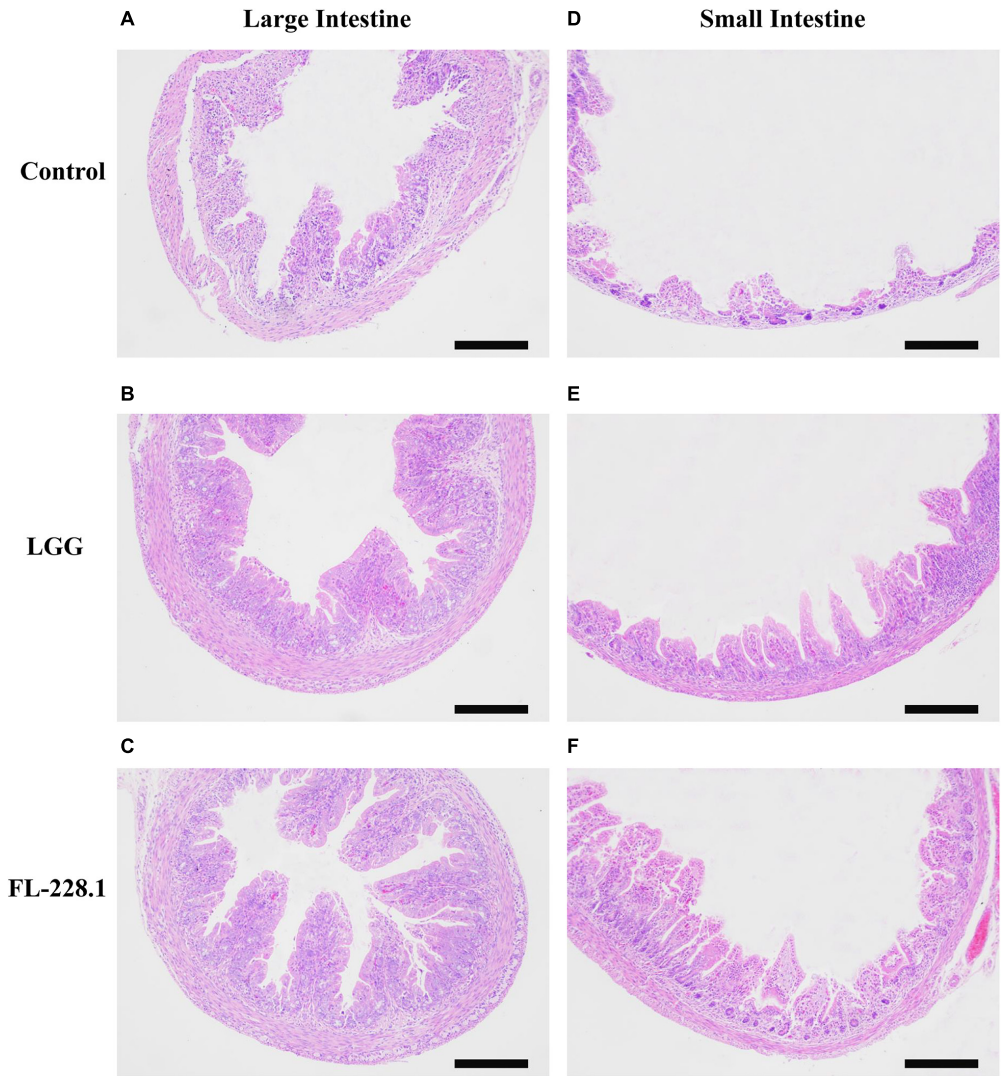

FIGURE 5 | The intestinal morphological characteristics of the different groups based on hematoxylin–eosin (H&E) staining (all scale bar, 100 μm). **(A)** Morphology of the large intestine of the pups in the blank control group. **(B)** Morphology of the large intestine of the pups fed with the LGG strain. **(C)** Morphology of the large intestine of the pups fed with the FL-228.1 strain. **(D)** Morphology of the small intestine of the pups in the blank control group. **(E)** Morphology of the small intestine of the pups fed with the LGG strain. **(F)** Morphology of the small intestine of the pups fed with the FL-228.1 strain.

strains, namely, FL-215, FL-216.9 and FL-228.1, showed higher adhesion abilities to IEC-6 cells. Further, among the three isolates, FL-228.1 showed the strongest adhesion ability to IEC-6 cells, as shown in **Figure 4D**. The specific results of the adhesion ability of the different strains are shown in **Supplementary Table 2**.

Effect of the Isolates on the Development of Intestinal Epithelial Cells *in vitro*

Through cell adhesion tests, three bacterial strains were screened for promoting the development of intestinal epithelial cells. The *in vitro* study revealed that FL-215 and FL-228.1 promoted the development of intestinal epithelial cells and that FL-216.9 could not function in promoting the development of intestinal epithelial cells. Further, FL-228.1 showed improvements compared with the LGG group in terms of the relative growth rate of IEC-6 cells (**Figure 4E**).

Effect of *B. bifidum* FL-228.1 on the Intestinal Morphological Characteristics of Rat Pups

The morphological characteristics of the different parts of the rat pup's intestine after intervention with the FL-228.1 strain for 10 days are depicted in **Figure 5**. Compared with the blank control group and LGG, the pups fed with FL-228.1 exhibited longer large intestine villi (**Figures 5A–C**). Furthermore, FL-228.1 exhibited longer small intestine villi in the offspring compared with blank control group and LGG (**Figures 5D–F**).

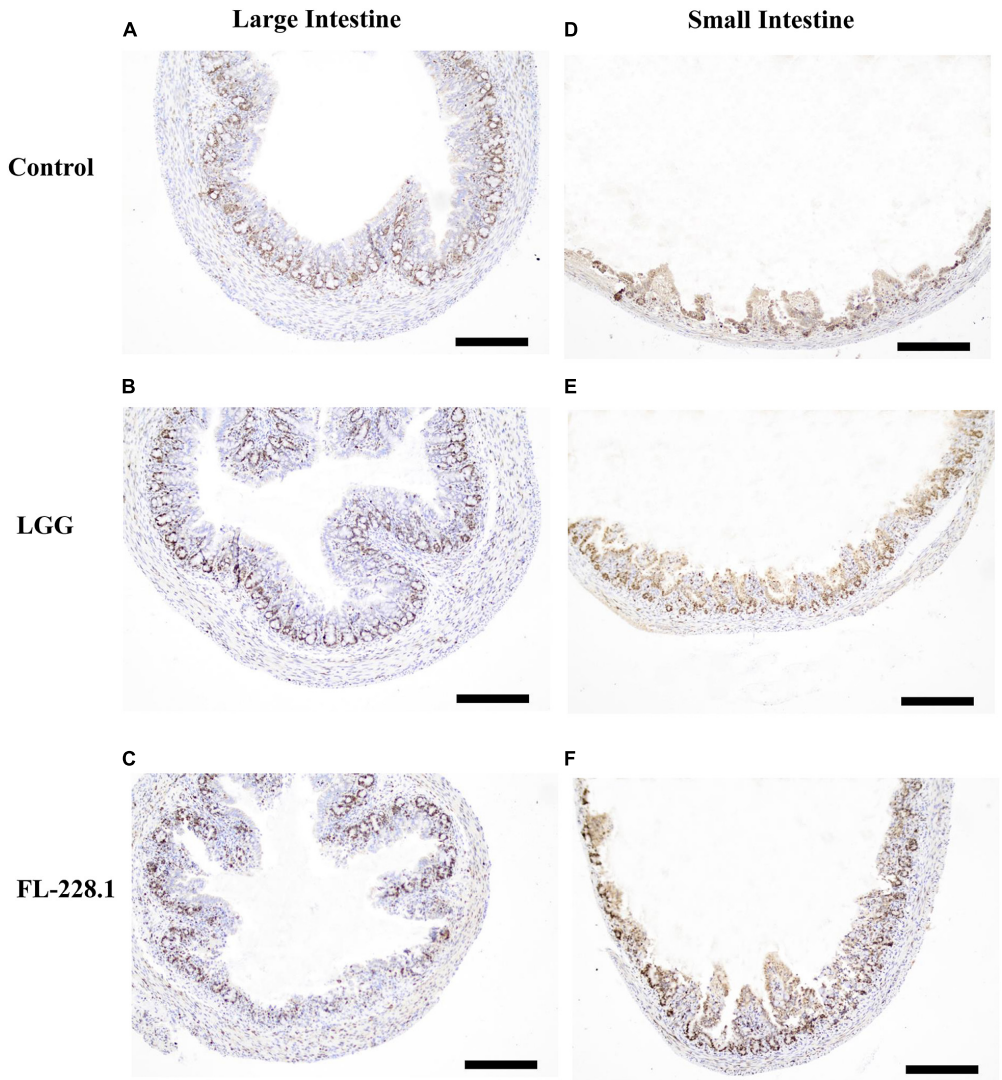

FIGURE 6 | Intestinal cell development in the different groups based on immunostaining for Ki67 (all scale bar, 100 μm). **(A)** Morphology of the large intestine of the pups in the blank control group. **(B)** Morphology of the large intestine of the pups fed with the LGG strain. **(C)** Morphology of the large intestine of the pups fed with the FL-228.1 strain. **(D)** Morphology of the small intestine of the pups in the blank control group. **(E)** Morphology of the small intestine of the pups fed with the LGG strain. **(F)** Morphology of the small intestine of the pups fed with the FL-228.1 strain.

Therefore, FL-228.1 promoted the development of small and large intestinal villi in the offspring.

Effect of *B. bifidum* FL-228.1 on Intestinal Cell Development in the Rat Pups

Following intervention with the FL-228.1 strain for 10 days, intestinal cell development in the rat pups is depicted in **Figures 6**, **7**. Compared with the blank and LGG groups, the mean densities of the large intestine and small intestine of the rats fed with FL-228.1 were the highest. Furthermore, for the large intestine, the mean density of the FL-228.1-fed pups was significantly higher than that of LGG-fed or blank groups ($P < 0.05$). The mean density of the LGG-fed pups was significantly higher than that of the control group ($P < 0.05$) (**Figure 7A**). Moreover, in the small intestine, the mean density of the FL-228.1-fed pups was significantly higher than that of LGG-fed and blank groups ($P < 0.05$) (**Figure 7B**). Therefore, FL-228.1 can promote intestinal cell development in the small and large intestines of the offspring.

DISCUSSION

Effect of Different Delivery Modes on the Composition of Infant Intestinal Microbiota

The 16S rRNA gene Illumina HiSeq platform was used to analyze stool samples from healthy infants. Although our study was

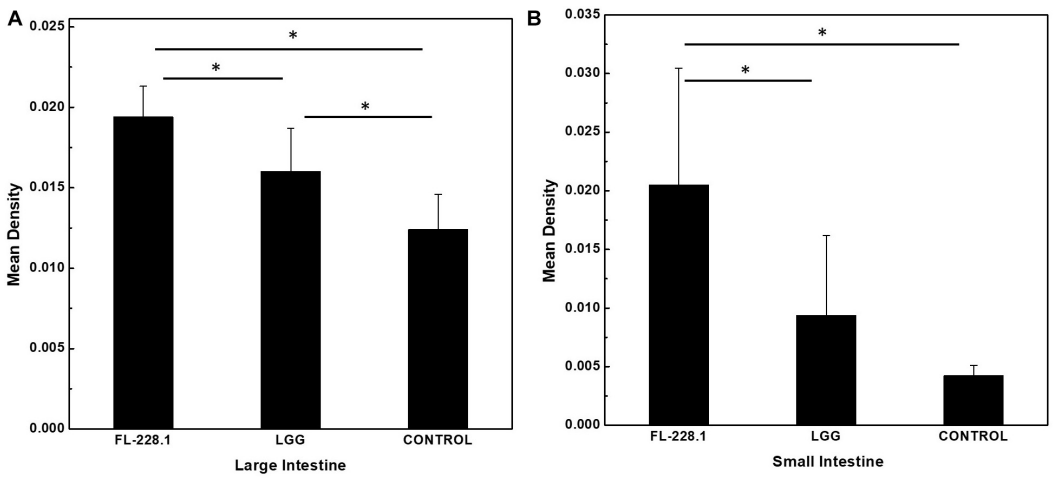

FIGURE 7 | The mean density of the small and large intestines of the different groups. **(A)** The mean density of the large intestine of the different groups. **(B)** The mean density of the small intestine of the different groups. *$P < 0.05$.

limited by sequencing depth and length, to our knowledge, our study was the first to investigate the microbial diversity of infant stool samples from two delivery methods from subjects living in northeast China.

We observed some associations among stool microbiota, community composition and modes of delivery (**Figure 2**). At the phylum level, the most abundant bacterial phyla were Firmicutes and Proteobacteria in these two delivery modes. This result is consistent with that of a recent investigation in another cohort (Wampach et al., 2018). Moreover, the most apparent differences at the family level were the relative abundances of *Bifidobacteriaceae*, which were dominant in the vaginal delivery group. This result was generally consistent with that of previous studies, which found a greater relative abundance of *Bifidobacteriaceae* in the stool samples of vaginal delivery infants (Shao et al., 2019). In contrast, the relative abundance of *Clostridiaceae* and *Staphylococcaceae* was higher in the stool samples of C-section infants presented by mother's skin and the hospital environment (Madan et al., 2016a; Tamburini et al., 2016; Shi et al., 2018). Moreover, samples from vaginal delivery infants were enriched with *Bacteroidaceae*; this result is consistent with that of a recent investigation in another cohort (Wampach et al., 2018).

There were apparent differences in infant intestinal microbiota in the two different delivery modes. According to the results of biodiversity analysis (**Figures 3A,B**), we found that *Bifidobacterium* and *Lactobacillus* were the different intestinal microbiota in the vaginal delivery and C-section groups. Meanwhile, according to the results of LEfSe analysis and function prediction, it was speculated that different bacterial groups lead to different functions. The vaginal delivery group was associated with the digestive system and cell growth and death. Function prediction could provide guidance for subsequent bacterial isolation and functional verification. Therefore, we analyzed the potential efficacies of these two genera, which are anaerobic and gram-positive. Meanwhile, many studies have confirmed that *Bifidobacterium* and *Lactobacillus* have certain effects on intestinal development (Mi et al., 2017; O'Connell Motherway et al., 2019; Zhou et al., 2020). Therefore, we analyzed only anaerobic and gram-positive bacterial strains and used *Bifidobacterium* and *Lactobacillus* as our target strains.

Bacterial Strains Promote Intestinal Development

Bifidobacterium and *Lactobacillus* had important effects on intestinal development (Blanton et al., 2016; Mi et al., 2017; O'Connell Motherway et al., 2019; Zhou et al., 2020). The tight adhesion (Tad) pili of *Bifidobacterium* is an important colonizing factor that promotes the development of intestinal epithelial cells (O'Connell Motherway et al., 2019). It was confirmed that the development of intestinal epithelial cells significantly increased 5 days after *Bifidobacterium* ingestion in mice. Furthermore, the Tad pili of *Bifidobacterium* promoted the intestinal mucosal growth of neonates by producing specific extracellular protein scaffolds, thereby promoting intestinal maturation of early neonates (O'Connell Motherway et al., 2019). This result is consistent with our results as FL-228.1 exhibited greater ability to develop in the intestine than the LGG and blank control group. Another study demonstrated that *Bif. longum* can enhance intestinal development and mucosal repair, promote lysozyme production and ameliorate dysbiosis of the microbiota in WAS rats by upregulating the stem niche factors WNT3A and TGF-β, which are secreted by Paneth cells (Zhou et al., 2020). Mi et al. (2017) studied the effects of *B. infantis* in attenuating the severity of chemotherapy-induced intestinal mucositis. They demonstrated that the *B. infantis* group showed higher intestinal villus height and deeper crypt depth than the chemotherapy group. It has been shown that LGG can promote intestinal epithelial homeostasis through specific signaling pathways. Researchers have also found that two proteins secreted from LGG, i.e., p75 (75 Dalton) and P40 (40 Dalton), promote the

growth of the human and mouse colonic epithelial cells. At the same time, it promotes the growth of mouse colon cells *in vitro*. The current study screened one *B. bifidum* FL-228.1 strain that exhibited a higher ability to promote intestinal development than LGG. Overall, the results of this study are consistent with those of previous studies. However, previous studies have only evaluated the function of the intestinal microbiota and the mechanism involved in promoting the development of intestinal epithelial cells. This study was more specific and might be beneficial to the study of probiotic effects in the future. Further research should also focus on specific probiotic functions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional (HRYLL201607). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LZL: writing-original draft preparation, formal analysis, and investigation. XZ, MZ, LL, SJ, JP, and YL: samples collections. PG: reviewing and editing. HN, JZ, and SC: investigation and data curation. XH: project administration. LZ and DC: funding acquisition, supervision, conceptualization, writing–review, and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by National Key R&D Program of China (2018YFC1604302) and the Heilongjiang Province Key Science and Technology Plan (GA16B201-2).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.626144/full#supplementary-material

REFERENCES

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17, 690–703.

Bhinder, G., Allaire, J. M., Garcia, C., Lau, J. T., Chan, J. M., Ryz, N. R., et al. (2017). Milk Fat globule membrane supplementation in formula modulates the neonatal gut microbiome and normalizes intestinal development. *Sci. Rep.* 7:45274.

Blanton, L. V., Charbonneau, M. R., Salih, T., Barratt, M. J., Venkatesh, S., Ilkaveya, O., et al. (2016). Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science* 351:10.1126/science.aad3311 aad3311.

Chong, C. Y. L., Bloomfield, F. H., and O'sullivan, J. M. (2018). Factors affecting gastrointestinal microbiome development in neonates. *Nutrients* 10:274. doi: 10.3390/nu10030274

Désir-Vigné, A., Haure-Mirande, V., De Coppet, P., Darmaun, D., Le Dréan, G., and Segain, J.-P. (2018). Perinatal supplementation of 4-phenylbutyrate and glutamine attenuates endoplasmic reticulum stress and improves colonic epithelial barrier function in rats born with intrauterine growth restriction. *J. Nutr. Biochem.* 55, 104–112. doi: 10.1016/j.jnutbio.2017.12.007

Ding, Y. X., Shi, Y., Han, W.-J., and Cui, H. (2016). Regulation of glucocorticoid-related genes and receptors/regulatory enzyme expression in intrauterine growth restriction filial rats. *Life Sci.* 150, 61–66. doi: 10.1016/j.lfs.2016.02.079

Friedrich, M. J. (2013). Genomes of microbes inhabiting the body offer clues to human health and disease. *JAMA* 309, 1447–1449. doi: 10.1001/jama.2013.2824

Hill, C. J., Lynch, D. B., Murphy, K., Ulaszewska, M., Jeffery, I. B., O'Shea, C. A., et al. (2017). Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome* 5:4.

Jakobsson, H. E., Abrahamsson, T. R., Jenmalm, M. C., Harris, K., Quince, C., Jernberg, C., et al. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section. *Gut* 63, 559–566. doi: 10.1136/gutjnl-2012-303249

Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P., et al. (2016). Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Med.* 8:72.

Liu, W., Zhang, J., Wu, C., Cai, S., Huang, W., Chen, J., et al. (2016). Unique features of ethnic mongolian gut microbiome revealed by metagenomic analysis. *Sci. Rep.* 6:34826.

Lundgren, S. N., Madan, J. C., Emond, J. A., Morrison, H. G., Christensen, B. C., Karagas, M. R., et al. (2018). Maternal diet during pregnancy is related with the infant stool microbiome in a delivery mode-dependent manner. *Microbiome* 6:109.

Madan, J. C., Hoen, A. G., Lundgren, S. N., Farzan, S. F., Cottingham, K. L., Morrison, H. G., et al. (2016a). Association of cesarean delivery and formula supplementation with the intestinal microbiome of 6-week-old infants. *JAMA Pediatr.* 170, 212–219. doi: 10.1001/jamapediatrics.2015.3732

Madan, J. C., Hoen, A. G., Lundgren, S. N., Farzan, S. F., Cottingham, K. L., Morrison, H. G., et al. (2016b). Effects of Cesarean delivery and formula supplementation on the intestinal microbiome of six-week old infants. *JAMA Pediatr.* 170, 212–219. doi: 10.1001/jamapediatrics.2015.3732

Maldonadolobón, J. A., Gilcampos, M., Maldonado, J., Lópezhuertas, E., Floresrojas, K., Valero, A. D., et al. (2015). Long-term safety of early consumption of Lactobacillus fermentum CECT5716: a 3-year follow-up of a randomized controlled trial. *Pharmacol. Res.* 95-96, 12–19. doi: 10.1016/j.phrs.2015.01.006

Martín, R., Jiménez, E., Heilig, H., Fernández, L., Marín, M. L., Zoetendal, E. G., et al. (2009). Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. *Appl. Environ. Microbiol.* 75, 965–969. doi: 10.1128/aem.02063-08

Meng, Z., Zhang, L., Xin, L., Lin, K., Yi, H., and Han, X. (2018). Technological characterization of *Lactobacillus* in semihard artisanal goat cheeses from different Mediterranean areas for potential use as nonstarter lactic acid bacteria. *J. Dairy Sci.* 101, 2887–2896. doi: 10.3168/jds.2017-14003

Mi, H., Dong, Y., Zhang, B., Wang, H., Peter, C. C. K., Gao, P., et al. (2017). Bifidobacterium Infantis ameliorates chemotherapy-induced intestinal mucositis via regulating T cell immunity in colorectal cancer rats. *Cell. Physiol. Biochem.* 42, 2330–2341. doi: 10.1159/000480005

Nagpal, R., Tsuji, H., Takahashi, T., Nomoto, K., Kawashima, K., Nagata, S., et al. (2017). Ontogenesis of the gut microbiota composition in healthy, full-term, vaginally born and breast-fed infants over the first 3 years of life: a quantitative bird's-eye view. *Front. Microbiol.* 8:1388. doi: 10.3389/fmicb.2017.01388

O'Connell Motherway, M., Houston, A., O'callaghan, G., Reunanen, J., O'brien, F., O'driscoll, T., et al. (2019). A Bifidobacterial pilus-associated protein promotes colonic epithelial proliferation. *Mol. Microbiol.* 111, 287–301. doi: 10.1111/mmi.14155

Oh, Y. J., and Jung, D. S. (2015). Evaluation of probiotic properties of Lactobacillus and Pediococcus strains isolated from Omegisool, a traditionally fermented millet alcoholic beverage in Korea. *LWT Food Sci. Technol.* 63, 437–444. doi: 10.1016/j.lwt.2015.03.005

Pan, W.-H., Li, P.-L., and Liu, Z. (2006). The correlation between surface hydrophobicity and adherence of Bifidobacterium strains from centenarians' faeces. *Anaerobe* 12, 148–152. doi: 10.1016/j.anaerobe.2006.03.001

Planer, J. D., Peng, Y., Kau, A. L., Blanton, L. V., Ndao, I. M., Tarr, P. I., et al. (2016). Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. *Nature* 534, 263–266. doi: 10.1038/nature17940

Pragman, A. A., Lyu, T., Baller, J. A., Gould, T. J., Kelly, R. F., Reilly, C. S., et al. (2018). The lung tissue microbiota of mild and moderate chronic obstructive pulmonary disease. *Microbiome* 6:7.

Reyman, M., Van Houten, M. A., Van Baarle, D., Bosch, A. A. T. M., Man, W. H., Chu, M. L. J. N., et al. (2019). Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. *Nat. Commun.* 10:4997.

Riaz Rajoka, M. S., Mehwish, H. M., Siddiq, M., Haobin, Z., Zhu, J., Yan, L., et al. (2017). Identification, characterization, and probiotic potential of Lactobacillus rhamnosus isolated from human milk. *LWT Food Sci. Technol.* 84, 271–280. doi: 10.1016/j.lwt.2017.05.055

Salam, M. T., Margolis, H. G., Mcconnell, R., Mcgregor, J. A., Avol, E. L., and Gilliland, F. D. (2006). Mode of delivery is associated with asthma and allergy occurrences in children. *Ann. Epidemiol.* 16, 341–346. doi: 10.1016/j.annepidem.2005.06.054

Shao, Y., Forster, S. C., Tsaliki, E., Vervier, K., Strang, A., Simpson, N., et al. (2019). Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* 574, 117–121. doi: 10.1038/s41586-019-1560-1

Shi, Y.-C., Guo, H., Chen, J., Sun, G., Ren, R.-R., Guo, M.-Z., et al. (2018). Initial meconium microbiome in Chinese neonates delivered naturally or by cesarean section. *Sci. Rep.* 8, 3255–3255.

Tamburini, S., Shen, N., Wu, H. C., and Clemente, J. C. (2016). The microbiome in early life: implications for health outcomes. *Nat. Med.* 22, 713–722. doi: 10.1038/nm.4142

Wampach, L., Heintz-Buschart, A., Fritz, J. V., Ramiro-Garcia, J., Habier, J., Herold, M., et al. (2018). Birth mode is associated with earliest strain-conferred gut microbiome functions and immunostimulatory potential. *Nat. Commun.* 9:5091.

Xu, X., Luo, D., Bao, Y., Liao, X., and Wu, J. (2018). Characterization of diversity and probiotic efficiency of the autochthonous lactic acid bacteria in the fermentation of selected raw fruit and vegetable juices. *Front. Microbiol.* 9:2539. doi: 10.3389/fmicb.2018.02539

Zhou, C., Fang, X., Xu, J., Gao, J., Zhang, L., Zhao, J., et al. (2020). *Bifidobacterium longum* alleviates irritable bowel syndrome-related visceral hypersensitivity and microbiota dysbiosis via Paneth cell regulation. *Gut Microbes* 12:1782156. doi: 10.1080/19490976.2020.1782156

Conflict of Interest: SJ, JP, and YL were employed by Heilongjiang Feihe Dairy Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

MINI REVIEW
published: 07 September 2021
doi: 10.3389/fnut.2021.667188

Probiotics, Prebiotics, and Synbiotics for the Prevention of Necrotizing Enterocolitis

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Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in preterm infants. The exact mechanism by which NEC develops is poorly understood however there is growing evidence to suggest that perturbations in the early-life gut microbiota composition increase the risk for NEC. Modulation of the gut microbiota with probiotics, prebiotics, or in combination (synbiotics) is an area which has attracted intense interest in recent years. In this narrative review, we present an overview of the role of the gut microbiota in the pathogenesis of NEC. We also examine the evidence currently available from randomized controlled trials, observational studies, systematic reviews, and meta-analysis examining the role of probiotics, prebiotics, and synbiotics in reducing the risk of or preventing NEC. Current clinical practice guidelines with recommendations on the routine administration of probiotics to preterm infants for NEC are also explored.

Keywords: microbiome, prebiotic, probiotic, synbiotic, necrotizing enterocolitis

OPEN ACCESS

Edited by:
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Reviewed by:
Maria Guadalupe Vizoso Pinto, CONICET Higher Institute of Biological Research (INSIBIO), Argentina
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *18 February 2021*
Accepted: *09 August 2021*
Published: *07 September 2021*

Citation:
Murphy K, Ross RP, Ryan CA, Dempsey EM and Stanton C (2021) Probiotics, Prebiotics, and Synbiotics for the Prevention of Necrotizing Enterocolitis. Front. Nutr. 8:667188. doi: 10.3389/fnut.2021.667188

INTRODUCTION

The early life gut microbiome is a dynamic community of microorganisms that play an important role in infant health. Factors influencing the development of the infant gut microbiota include mode of delivery (caesarean section vs. vaginal birth), gestational age (premature vs. full-term birth), antibiotic use, mode of feeding (formula vs. breastfeeding), and environmental factors (1, 2). *Bifidobacterium* typically dominate the microbiota in vaginally delivered, breastfed infants. Infants delivered by caesarean section are characterised by reduced *Bacteroides* and *Bifidobacterium* and increased colonization by opportunistic pathogens such as *Enterococcus*, *Enterobacter*, *Clostridium*, and *Klebsiella* species (1–5). Disrupted microbiota acquisition during this critical developmental window may have both short and long-term health implications. Imbalances in the composition of the gut microbiota have been associated with a wide range of diseases including allergic disorders, type 1 diabetes, inflammatory bowel disease, obesity, sepsis, and necrotizing enterocolitis (NEC) (6–10).

With our growing understanding of the role of the microbiome in health and disease, the use of probiotics to promote a healthy microbiome is an active area of research. Probiotics are defined by the FAO/WHO as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (11). Probiotics may positively contribute to host health by modulating immune responses such as inflammation, improving the function of the intestinal mucosal barrier, modulating the expression of host genes, and preventing

colonization by pathogenic bacteria. One of the mechanisms through which probiotics influence a range of health parameters is through the production of bioactive compounds. Vitamins, antimicrobial peptides, conjugated linoleic acid (CLA), exopolysaccharides, gamma aminobutyric acid (GABA), and short-chain fatty acids (SCFAs) are all examples of microbially produced bioactive compounds (12). SCFAs including acetate, propionate, and butyrate are crucial for gut health and can modulate metabolic activity including colonocyte function, gut homeostasis, and the immune system (13). While CLA has immunomodulating properties, reducing the proinflammatory cytokines (14).

Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (15). Prebiotics specifically stimulate the growth of beneficial microbes including bifidobacteria and lactobacilli. Prebiotics are naturally found in human milk (HM), which contains over 200 human milk oligosaccharides (HMOs) (16). HMOs can increase the proportion of HMO-consuming bifidobacteria and *Bacteroides* in breast-fed infants. Infant formula are now often supplemented with prebiotics and probiotics to mimic the functional effects of HMOs and HM bacteria (17). A synbiotic is defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (18). A synbiotic may be classified as complementary or synergistic. In a complementary synbiotic the probiotic and prebiotic provide a health benefit together but are not co-dependent. In synergistic synbiotics, the prebiotic is chosen based on its ability to be selectively utilized by the probiotic.

One particular area that has produced positive results in probiotic intervention studies is the prevention of NEC in preterm infants (19). NEC is a serious acquired disease of the gastrointestinal tract, characterized by acute intestinal necrosis. The incidence rate of NEC is reported as approximately 5–10% of very preterm or very low birth weight (VLBW) infants (20, 21). A 2020 systematic review reported that seven out of 100 VLBW infants in the neonatal intensive care unit (NICU) are likely to develop NEC (22). The mortality rate is reported at 20–30%, and infants who survive NEC have a greater risk of neurodevelopmental delays (23). Preterm infants represent a particularly vulnerable group especially those weighing <1,500 g, VLBW infants, and <1,000 g, extremely low birth weight (ELBW).

INFANT GUT MICROBIOTA AND NECROTIZING ENTEROCOLITIS

The pathogenesis of NEC is complex and the exact etiology remains unknown; however, immaturity of the intestinal barrier and immune system are thought to contribute (24). The intestinal microbiome is also believed to contribute to the pathogenesis of NEC. Experiments using animal models have shown that NEC does not occur in germ-free mice and toll-like receptor targeted knockout mice strongly suggesting that the gut microbiome is critical for NEC development (25–28). Studies using 16S rRNA gene sequencing have reported a reduction in microbial community diversity, decreases in Firmicutes, and an increase in Proteobacteria in the stool of NEC patients (29–31). Proteobacteria contain numerous gram-negative pathogens with high levels of lipopolysaccharide (LPS). TLR4 recognizes LPS and TLR4 activation leads to inhibition of mucosal repair. Breakdown of the gut barrier and translocation of pathogenic bacteria leads to an increased inflammatory response, resulting in NEC (32). Patients with NEC have been reported to have higher levels of LPS in their plasma (25). In addition, intraperitoneal injection of LPS to rats and mice has been demonstrated to induce intestinal injury and shock (33). Several studies have linked colonization by clostridia with NEC and pointed toward a potential deleterious role in the pathogenesis of NEC (34, 35). The exact mechanism is unclear but it is thought that lactose fermentation leading to an overproduction of butyric acid and the presence of toxin genes may play a role (36, 37).

Olm et al. performed metagenomic analysis of faecal samples from premature infants to identify microbial features predictive of NEC (38). Samples collected prior to NEC onset contained significantly higher *Klebsiella*, bacteria encoding fimbriae, and secondary metabolite gene clusters related to bacteriocin production and quorum sensing. Bacterial replication rates were measured from metagenomic data by determining the difference in DNA sequencing coverage between the origin and the terminus of replication. Replication rates, particularly that of Enterobacteriaceae, were significantly higher two days prior to NEC diagnosis. Microbiome analysis of faecal samples may not accurately represent the bacterial communities at the site of injury, the intestinal mucosa. A study by Romano-Keeler et al. examined the microbiome in both NEC tissue and faecal samples in surgical patients with and without NEC (39). The authors reported a tissue-specific overrepresentation of Firmicutes, specifically *Staphylococcus* and *Clostridium* and a lower abundance of *Actinomyces* and *Corynebacterium* in NEC.

EPIPAGE 2, a prospective cohort study in France, assessed nutritional strategies and the gut microbiota as risk factors for NEC (40). Slower rates of progression of enteral feeding and less favorable direct-breastfeeding policies were associated with a higher risk of NEC. An association between *Clostridium neonatale* and *Staphylococcus aureus* with NEC was also noted. Interestingly, no relation between antibiotic treatment and the onset of NEC was observed. This is in contrast to several studies which have reported that early antibiotic use in preterm infants increases the risk of NEC (41–43). Acid-suppressive medications such as histamine-2 receptor antagonists and proton pump inhibitors (PPIs) are routinely used for the treatment of upper gastrointestinal bleeding or gastroesophageal reflux in preterm infants. Exposure to these acid-suppressive medicines has been associated with an increased risk of NEC (44, 45). Changes in gut microbiota composition related to PPI therapy have been well-documented (46, 47). Feeding with HM provides beneficial bacteria and essential prebiotic substances including non-digestible HMOs, immunoprotective IgA, and lactoferrin, and has been reported to reduce the risk of development of NEC (48–50). HM may also protect against NEC through the presence of epidermal growth factor, which attenuates TLR4 signaling via activation of the phosphoinositide 3-K signaling pathway (51).

RANDOMIZED CONTROLLED TRIALS AND OBSERVATIONAL STUDIES

Due to the role of the intestinal microbiome in the pathogenesis of NEC, dietary supplementation with probiotics to modulate the intestinal microbiome has been proposed as a strategy to reduce the risk of NEC and associated morbidity and mortality. An overview of the characteristics of randomized controlled trials (RCTs) evaluating probiotics, prebiotics, and synbiotics for NEC are shown in **Table 1**. Of the thirty-four RCTs evaluating probiotics for NEC, seventeen reported significant beneficial effects, eleven reported no health benefit, and six reported a trend to prevent NEC. The Probiotics in Preterm Infants (PiPs) trial, the largest trial to date of a probiotic intervention, assessed the effectiveness of *Bifidobacterium breve* BBG-001 to reduce NEC, late onset sepsis (LOS), and death in 1,315 preterm infants in the UK (60). The trial did not find a significant reduction in NEC and the authors did not recommend the routine use of probiotics in this population. An important limitation to note of this trial was the high rates of cross-colonization in the placebo group which may have confounded the results. The ProPrems RCT compared daily administration of a probiotic mixture containing *Bifidobacterium infantis*, *Streptococcus thermophilus*, and *Bifidobacterium lactis* with placebo in 1,099 very preterm infants (70). Infants receiving the probiotic mixture had a significantly lower incidence of NEC (stage 2 or greater) compared to control infants. The 2015 ProPre-Save RCT evaluated the efficacy of probiotic alone, prebiotic alone, or combined (synbiotic), on the prevention of NEC in 400 VLBW infants (88). Infants were randomized to either a control group or one of three study groups. The study groups were administered probiotic (*B. lactis*), prebiotic (inulin), or synbiotic (*B. lactis* plus inulin) for up to eight weeks. The probiotic and synbiotic groups had a lower incidence of NEC compared to the prebiotic and control groups. The study groups had reduced mortality, reduced nosocomial sepsis, faster time to reach full enteral feeding, and shorter NICU duration compared to the control group. Another large RCT randomly assigned 750 preterm infants to receive *Lactobacillus reuteri* DSM 17938 or placebo (76). Here, a non-significant 40% decrease in NEC was reported in the probiotic group compared with control group.

A 2016 retrospective multi-center study examined data from 10,890 preterm infants from 44 NICUs in Germany with routine use of a dual strain probiotic (Infloran™, *Lactobacillus acidophilus* and *B. infantis*) (94). Infloran administration significantly reduced the incidence of NEC, mortality after NEC, overall mortality, and nosocomial bloodstream infection. Subgroup analysis in ELBW infants revealed that these effects were even more pronounced in these infants. Gray et al. performed a multi-center cohort study of 78,076 preterm infants from 289 NICUs in the United States from 1997 to 2016 (95). The most commonly administered probiotic was *Lactobacillus* (71%), followed by Ultimate Flora (*Bifidobacterium* and *Lactobacillus*), ABC Dophilus (*Bifidobacterium*, *Lactobacillus*, and *Streptococcus*), and Align (*Bifidobacterium*). Probiotic administration increased over time and was associated with a decrease in the incidence of NEC and death. In contrast to other studies reporting that probiotics reduce *Candida* colonization, an increase in *Candida* infection was observed here. The authors state that confirmatory reports are required to determine if the findings are clinically significant. Probiotic use was not associated with an increase in bloodstream infection or meningitis. Concerning the safety of probiotics in preterm infants, their use has very rarely been associated with deleterious side effects such as bacterial sepsis due to probiotic translocation (61, 96, 97). The cost benefits ratio is very much in favor of probiotics considering the data from the numerous preterm infants who have receive such supplementation (98). In Canada, a 2019 retrospective cohort study evaluated the effect of probiotic administration on extremely preterm infants (<29 weeks gestational age) admitted to NICU (99). 3093 infants were included in the analysis with 652 infants receiving probiotic preparations, either Florababy (*B. breve, Bifidobacterium bifidum, B. infantis, Bifidobacterium longum*, and *Lactobacillus rhamnosus* GG) or Biogaia (*L. reuteri*). Probiotic use was associated with a significant reduction in the rate of NEC and mortality but not in the rate of LOS.

SYSTEMATIC REVIEWS AND META-ANALYSIS

A 2020 Cochrane review of 56 RCTs (n = 10,812) compared probiotic supplementation with placebo in very preterm or VLBW infants (100). This review reported that probiotics may reduce the risk of NEC and probably reduces mortality for very preterm or VLBW infants. The evidence for this was assessed as low certainty due to weaknesses in trial design particularly with regards measures used to blind clinicians and caregivers to the intervention. Small-study bias was also a concern with most of the included trials small in size (median n = 149). Heterogeneity of the probiotic interventions used in RCTs was reported by the authors as the main challenge in applying the findings of the review. Additionally, the authors noted that few trials provided data for extremely preterm or ELBW infants.

A 2020 systematic review and network meta-analysis (NMA) analyzed data from 63 RCTs (n = 15,712) to assess the effectiveness of various single-strain and multi-strain probiotics for the prevention of NEC mortality and morbidity (101). High-certainty evidence indicated that combinations of *Bifidobacterium* and *Lactobacillus* were most effective for the prevention of mortality and stage 2 NEC. Moderate-certainty evidence suggested that *B. lactis, L. rhamnosus*, and *L. reuteri* prevent stage 2 NEC. Moderate-certainty evidence also indicated that *B. lactis* and *L. reuteri* reduced hospital stay. Low-certainty evidence suggested that combinations of *Bacillus* and *Enterococcus*; *Lactobacillus*, *Bifidobacterium,* and *Enterococcus*; and *Bifidobacterium* and *S. thermophilus* may prevent stage 2 NEC. Important limitations as noted by the authors were the lack of available data comparing the effects of different probiotic strains with each other and the lack of strain level information in many of the trials. Most recently in 2021, a NMA of 51 RCTs

TABLE 1 | Characteristics of randomized controlled trials evaluating probiotics, prebiotics, and synbiotics for NEC.

Author	Year	Country of origin	Sample size	Participant details	Details of intervention, probiotic species, and strains	Main finding
Probiotic interventions						
Al-Hosni et al. (52)	2012	USA	101	ELBW	LGG, *B. infantis*	No significant difference in incidence of NEC
Arora et al. (53)	2017	India	150	GA ≤34 wk	*S. boulardii, L. rhamnosus, L. acidophilus, B. longum*	Reduced incidence and severity of NEC
Awad et al. (54)	2010	Egypt	150	PT admitted to NICU	*L. acidophilus*, Living and Killed	Reduced incidence of NEC Killed retained similar benefits to live bacteria
Benor et al. (55)	2014	Israel	49	Mothers of VLBW infants	*L. acidophilus* and *B. lactis*	Reduced incidence of NEC and in stage 2 NEC
Bin-Nun et al. (56)	2005	Israel	145	BW ≤1,500 g	*B. infantis, S. thermophilus*, and *B. bifidus*	Reduced both incidence and severity of NEC
Braga et al. (57)	2011	Brazil	231	VLBW, BW 750–1,500 g	*L. casei, B. breve*	Reduced incidence of NEC (stage ≥2)
Chandrashekar et al. (58)	2018	India	145	GA <34 wk	*L. acidophilus, L. rhamnosus, B. longum*, and *S. boulardii*	Reduced incidence and severity of NEC
Chowdhury et al. (59)	2016	Bangladesh	119	VLBW, GA 28–33 wk	LGG, *L. paracasei, L. casei, L. acidophilus*, *L. lactis, B. bifidum, B. longum, B. infantis*	Reduced incidence of NEC
Costeloe et al. (60)	2016	UK	1310	GA 23–30 wk	*B. breve* BBG-001	No evidence of benefit
Dani et al. (61)	2002	Italy	585	GA <33 wk or BW <1,500 g	LGG	Non-significant reduction in incidence of NEC
Dashti et al. (62)	2014	Iran	136	BW 700–1,800 g	*L. acidophilus, L. rhamnosus, L. bulgaricus, L. casei, S. thermophilus, B. longum, B. breve*	No evidence of benefit
Demirel et al. (63)	2013	Turkey	271	BW ≤1,500 g GA ≤32 wk	*S. boulardii*	No significant difference in incidence of NEC
Dongol-Singh et al. (64)	2017	Nepal	72	Hospitalized PT	*L. rhamnosus* LCR35	Trend toward reduction in incidence of NEC
Fernández-Carrocera et al. (65)	2013	Mexico	150	BW <1,500 g	*L. rhamnosus, L. casei, L. plantarum, L acidophilus, B. infantis, S. thermophilus*	Trend toward reduction in incidence of NEC
Gomez-Rodriguez et al. (66)	2019	USA	90	ELBW and VLBW (700–1,500 g)	*L. acidophilus boucardii* versus mix *of L. acidophilus, L. rhamnosus, L. casei, L. plantarum, B. infantis*, and *S. thermophilus*	No difference between use of single strain or multispecies probiotics on NEC incidence
Hays et al. (67)	2015	France	199	GA 25–31 wk, BW 700–1,600 g	*B. lactis* or *B. longum* or both	Incidence rates of NEC similar in the two groups
Hernández-Enríquez et al. (68)	2016	Mexico	44	GA <34 wk or BW ≤1,550 g	*L. reuteri* DSM *17938*	Reduced incidence of NEC
Hoyos et al. (69)	1999	Colombia	1237	NICU	*L. acidophilus* and *B. infantis*	Reduced incidence of NEC and NEC-associated fatalities
Jacobs et al. (70)	2013	Australia and New Zealand	1099	GA <32 wk, BW <1,500 g	*B. infantis, S. thermophilus, B. lactis*	Reduced incidence of NEC of stage 2 or more
Janvier et al. (71)	2014	USA	611	GA <32 wk, NICU	*B. breve, B. bifidum, B. infantis, B. longum, L. rhamnosus HA-111*	Reduced incidence of NEC
Kaban et al. (72)	2019	Indonesia	94	GA 28–34 wk, BW 1,000–1,800 g	*L. reuteri* DSM 17938	Trend to reduction in incidence of NEC
Lin et al. (73)	2005	Taiwan	367	VLBW	*L. acidophilus, B. infantis* (Infloran®)	Reduced incidence and severity of NEC
Lin et al. (74)	2008	Taiwan	434	VLBW	*B. bifidum, L. acidophilus*	Reduced incidence of NEC

(Continued)

TABLE 1 | Continued

Author	Year	Country of origin	Sample size	Participant details	Details of intervention, probiotic species, and strains	Main finding
Oncel et al. (75)	2014	Turkey	424	BW ≤1,500 g, GA ≤32 wk	*L. reuteri* DSM 17938	No significant difference in incidence of NEC
Rojas et al. (76)	2012	Colombia	750	PT, BW ≤2,000 g	*L. reuteri* DSM 17938	Non-significant decrease in incidence of NEC
Saengtawesin et al. (77)	2014	Thailand	60	BW ≤1,500 g, GA ≤34 wk	*L. acidophilus, B. bifidum* (Infloran®)	No difference in incidence of NEC stage ≥2
Samanta et al. (78)	2009	India	186	Very PT or VLBW	*B. infantis, B. bifidum, B. longum, L. acidophilus*	Reduced morbidity due to NEC
Sari et al. (79)	2011	Turkey	221	BW <1,500 g, GA <33 wk	*L. sporogenes*	No significant difference in incidence of death or NEC
Serce et al. (80)	2013	Turkey	208	GA ≤32 wk, BW ≤1,500 g	*S. boulardii*	Did not decrease the incidence of NEC
Shadkam et al. (81)	2015	Iran	60	GA 28–34 wk, BW 1,000–1,800 g	*L. reuteri* DSM 17938	Reduced incidence of NEC
Shashidhar et al. (82)	2017	India	104	BW ≤1,500 g	*L. acidophilus, L. rhamnosus, B. longum, S. boulardii*	Trend toward lower incidence NEC
Van Niekerk et al. (83)	2015	South Africa	184	GA <34 wk and VLBW (<1,250 g)	*Pro-B2: LGG* and *B. infantis*	Reduced incidence of NEC in VLBW but not in HIV-exposed infants
Wang et al. (84)	2014	China	100	FT in NICU	*L. casei, L. acidophilus, B. subtilis* and *E. faecalis*	No significant difference in the incidence of NEC
Zampieri et al. (85)	2013	Italy	32	BW 600–1,500 g with stage 2 NEC	*L. paracasei* subsp. *paracasei* F-19	Reduced clinical progression of NEC
Prebiotic interventions						
Armanian et al. (86)	2014	Iran	75	BW ≤1,500 g, GA ≤34 wk	scGOS/lcFOS	Reduced incidence of NEC
Dasopoulou et al. (87)	2015	Greece	167	PT admitted to NICU	scGOS/lcFOS	No significant difference in incidence of NEC
Dilli et al. (88)	2015	Turkey	400	VLBW	Inulin	No significant difference in incidence of NEC
Manzoni et al. (89)	2009	Italy	743	VLBW	Lactoferrin	Reduced incidence of ≥stage 2 NEC and of death
Riskin et al. (90)	2010	Israel	28	GA 23–34 wk	Lactulose	Fewer episodes of lower stage NEC
Synbiotic interventions						
Dilli et al. (88)	2013	Turkey	100	Infants with cyanotic congenital heart disease	*B. lactis* plus inulin	Reduced incidence of NEC
Dilli et al. (91)	2015	Turkey	400	VLBW	*B. lactis* plus inulin	Probiotic alone and synbiotic but not prebiotic alone reduced incidence of NEC
Guney-Varal et al. (92)	2017	Turkey	110	GA ≤32 wk and BW ≤1,500 g	*L. rhamnosus, L. casei, L. plantarum, B. animalis* plus FOS and GOS	Reduced the incidence of NEC and mortality rate
Manzoni et al. (89)	2009	Italy	743	VLBW	LGG plus lactoferrin	Reduced incidence of ≥stage 2 NEC and of death
Nandhini et al. (93)	2016	India	220	GA 23–34 wk, BW >1,000 g	PREPRO HS®—*L. acidophilus*, *B. longum*, *L. rhamnosus*, *L. plantaris*, *L. casei*, *L. bulgaricus*, *B. infantis*, and *B. breve* plus FOS	Reduced incidence of NEC, did not reduce severity of NEC

VLBW, very low birth weight; wk, weeks; ELBW, extremely low birth weight; GA, gestational age; BW, birth weight; PT, preterm; FT, full term; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; scGOS, short-chain galacto-oligosaccharides; lcFOS, long-chain fructo-oligosaccharides. Search Strategy: We searched PubMed and CENTRAL in May 2021 with the search terms "premature" or "prematurity" and "probiotic" or "prebiotic" or "synbiotic" and "necrotizing enterocolitis." Publication type: "clinical trial" and "randomized controlled trial." The RCT was excluded if only the abstract was available.

($n = 10{,}664$) examined the role of probiotics in preventing NEC (102). *L. acidophilus* LB was ranked as the best supplementation option for reducing NEC risk in preterm infants. The NMA also included a subgroup analysis of type of feeding; exclusively HM fed vs. formula fed (alone or combination with HM). The administration of *B. lactis* Bb-12/B94 was associated with a reduced risk of NEC stage ≥ 2 in exclusively HM-fed infants and non-exclusively HM-fed infants. The relative size effect favored exclusively HM-fed infants.

Chi et al. also employed a NMA approach based on 45 RCTs ($n = 12{,}320$) to compare probiotic, prebiotic, and synbiotics for premature infants (103). The RCTs included strains of *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Bacillus*, and *Saccharomyces*, alone and in combination. Supplementation with *Bifidobacterium* plus *Lactobacillus* was associated with lower rates of morbidity and mortality in NEC. *Lactobacillus* plus prebiotic was associated with lower rates of NEC morbidity and had the highest probability of having the lowest rate of NEC. *Bifidobacterium* plus prebiotic had the highest probability of having the lowest rate of mortality. The authors found that the efficacy of single strain supplements was limited and recommended the use of synbiotics particularly those including both *Bifidobacterium* and *Lactobacillus*. A limitation of this NMA was the insufficient data available for extremely preterm or ELBW infants. A 2018 meta-analysis used 18 RCTs ($n = 1{,}322$) to evaluate whether prebiotics alone could reduce the incidence of sepsis, NEC, and mortality in preterm infants (104). Participants who received prebiotics showed significant decreases in the incidence of sepsis and mortality; however, there was no significant differences between intervention and control groups in relation to the morbidity rate of NEC.

CLINICAL PRACTICE GUIDELINES

In 2020, the European Society of Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) published a position paper aiming to provide recommendations relating to the use of probiotics in preterm infants (105). A conditional recommendation for the use of *L. rhamnosus* GG ATCC 53103 or the combination of *B. lactis* BB-12, *B. infantis* BB-02, and *S. thermophilus* TH-4 was made. It was advised that strains should be selected based on proven effectiveness and an established safety profile. With regard dosage, similar doses as administered in relevant RCTs were recommended. Due to limitations in currently available data, an optimal start of treatment or total duration was not indicated. The paper highlights that probiotics are typically marketed as nutritional supplements and as a result are loosely regulated. Product safety and quality is therefore of concern especially in a vulnerable population such as preterm infants with immature immune systems. ESPGHAN recommended more stringent controls and that probiotic strains be manufactured according to current Good Manufacturing Practice (cGMP) to ensure strain identity, purity, and viability. The probiotic strains should not include any plasmids containing transferable antibiotic resistance genes and local microbiologists must have the ability to routinely detect probiotic sepsis. The panel also recommended against the use of probiotic strains that produce d-lactate, as their potential risks are uncertain. Also in 2020, the American Gastroenterological Association (AGA) published their clinical practice guidelines on the role of probiotics in the management of gastrointestinal disorders (106). The guidelines conditionally recommended probiotics for the prevention of NEC in preterm infants <37 weeks gestational age and low birth weight. The AGA reported that specific probiotics can prevent mortality and severe NEC (stage 2 or greater), reduce days required to reach full feeds, and decrease the duration of hospitalization. The committee identified significant heterogeneity between studies, variability in the strains studied, and a lack of consistent harms reporting as significant knowledge gaps.

Most recently, a 2021 clinical report by the American Academy of Pediatrics (AAP) recommended against the routine administration of probiotics to preterm infants, particularly those whose birth weight is <1,000 g, for the treatment or prevention of NEC (107). The AAP highlights that probiotic products in the US are classified as dietary supplements and are not subject to approval by the US Food and Drug Administration (FDA). As a result, manufacturers can bypass FDA safety, efficacy, and manufacturing standards. The AAP notes that despite the inconsistent data on their safety and efficacy, probiotics are increasingly given to preterm infants in the US with approximately 10% of extremely low gestational age infants receiving a probiotic preparation while in the NICU. The academy advises that centres using probiotics obtain informed consent from parents after discussing the risks and benefits. They also recommended that centres should conduct surveillance to assess the impact of probiotics on the centres microbiota, which could potentially affect all infants, and should carefully document adverse events, outcomes, and safety.

CONCLUSIONS

There is mounting evidence supporting the use of probiotics to decrease the risk of NEC in preterm infants. Several large RCTs have demonstrated that the relative risk for NEC can be reduced using probiotic formulations. It is important to note that some meta-analyses have reported low to moderate level of certainty about the effects of probiotic supplementation on the risk of NEC and the largest RCT to date found no reduction in NEC incidence following supplementation with a single-strain probiotic. A confounding factor in this RCT was the high rates of cross-colonization found in the placebo group. In addition, not all probiotics used in preventing NEC may be equally effective. Therefore, further carefully designed and conducted large-scale RCTs are necessary to determine optimal strains as well as optimal timing and dosing. Furthermore, detailed information about the study population needs to be included such as type of feeding, antibiotic usage, gender, and ethnicity. Data on the particularly vulnerable extremely preterm infants and ELBW infants is limited and more RCTs focused specifically on these groups are needed. Prebiotic and

synbiotic interventions are scarcely investigated in RCTs to date and further trials evaluating their efficacy are required. There are conflicting recommendations from experts as to the administration of probiotics to preterm infants for NEC. Concerns about the safety and purity of commercially available probiotics appears to be the greatest hurdle to overcome in terms of the widespread implementation of probiotics in NICU. Many probiotic products are sold as dietary supplements and are not produced under strict quality control conditions. Probiotics which are licensed as a drug by national regulatory authorities should be recommended.

AUTHOR CONTRIBUTIONS

KM: conceptualization, original draft preparation, review, and editing. RPR and CS: conceptualization, supervision, review, and editing. CAR and EMD: review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Science Foundation Ireland grant number SFI/12/RC/2273-P2.

REFERENCES

1. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA*. (2010) 107:11971–5. doi: 10.1073/pnas.1002601107
2. Gritz EC, Bhandari V. The human neonatal gut microbiome: a brief review. *Front Pediatr*. (2015) 3:17. doi: 10.3389/fped.2015.00017
3. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature*. (2018) 562:583–8. doi: 10.1038/s41586-018-0617-x
4. Hill CJ, Lynch DB, Murphy K, Ulaszewska M, Jeffery IB, O'Shea CA, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET cohort. *Microbiome*. (2017) 5:4. doi: 10.1186/s40168-016-0213-y
5. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature*. (2019) 574:117–21. doi: 10.1038/s41586-019-1560-1
6. Rodriguez JM, Murphy K, Stanton C, Ross RP, Kober OI, Juge N, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis*. (2015) 26:26050. doi: 10.3402/mehd.v26.26050
7. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiol Mol Biol Rev*. (2017) 81:e00036-17. doi: 10.1128/MMBR.00036-17
8. Neu J, Pammi M. Pathogenesis of NEC: impact of an altered intestinal microbiome. *Semin Perinatol*. (2017) 41:29–35. doi: 10.1053/j.semperi.2016.09.015
9. Gurung M, Li Z, You H, Rodrigues R, Jump DB, Morgun A, et al. Role of gut microbiota in type 2 diabetes pathophysiology. *EBioMedicine*. (2020) 51:102590. doi: 10.1016/j.ebiom.2019.11.051
10. Khan I, Ullah N, Zha L, Bai Y, Khan A, Zhao T, et al. Alteration of gut microbiota in Inflammatory Bowel Disease (IBD): cause or consequence? IBD treatment targeting the gut microbiome. *Pathogens*. (2019) 8:126. doi: 10.3390/pathogens8030126
11. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol*. (2014) 11:506–14. doi: 10.1038/nrgastro.2014.66
12. Indira M, Venkateswarulu TC, Abraham Peele K, Nazneen Bobby M, Krupanidhi S. Bioactive molecules of probiotic bacteria and their mechanism of action: a review. *3 Biotech*. (2019) 9:306. doi: 10.1007/s13205-019-1841-2
13. Torres-Fuentes C, Golubeva AV, Zhdanov AV, Wallace S, Arboleya S, Papkovsky DB, et al. Short-chain fatty acids and microbiota metabolites attenuate ghrelin receptor signaling. *FASEB J*. (2019) 33:13546–59. doi: 10.1096/fj.201901433R
14. Kim JH, Kim Y, Kim YJ, Park Y. Conjugated linoleic acid: potential health benefits as a functional food ingredient. *Annu Rev Food Sci Technol*. (2016) 7:221–44. doi: 10.1146/annurev-food-041715-033028
15. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol*. (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75
16. Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci U S A*. (2011) 108(Suppl 1):4653–8. doi: 10.1073/pnas.1000083107
17. Salminen S, Stahl B, Vinderola G, Szajewska H. Infant formula supplemented with biotics: current knowledge and future perspectives. *Nutrients*. (2020) 12:1952. doi: 10.3390/nu12071952
18. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. *Nat Rev Gastroenterol Hepatol*. (2020) 17:687–701. doi: 10.1038/s41575-020-0344-2
19. Deshmukh M, Patole S. Current status of probiotics for preterm infants. *Indian J Pediatr*. (2021) 88:703–8. doi: 10.1007/s12098-021-03736-2
20. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med*. (2011) 364:255–64. doi: 10.1056/NEJMra1005408
21. Rich BS, Dolgin SE. Necrotizing Enterocolitis. *Pediatr Rev*. (2017) 38:552–9. doi: 10.1542/pir.2017-0002
22. Alsaied A, Islam N, Thalib L. Global incidence of necrotizing Enterocolitis: a systematic review and meta-analysis. *BMC Pediatr*. (2020) 20:344. doi: 10.1186/s12887-020-02231-5
23. Hickey M, Georgieff M, Ramel S. Neurodevelopmental outcomes following necrotizing enterocolitis. *Semin Fetal Neonatal Med*. (2018) 23:426–32. doi: 10.1016/j.siny.2018.08.005
24. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. *Nat Rev Gastroenterol Hepatol*. (2016) 13:590–600. doi: 10.1038/nrgastro.2016.119
25. Afrazi A, Sodhi CP, Richardson W, Neal M, Good M, Siggers R, et al. New insights into the pathogenesis and treatment of necrotizing enterocolitis: Toll-like receptors and beyond. *Pediatr Res*. (2011) 69:183–8. doi: 10.1203/PDR.0b013e3182093280
26. Sodhi CP, Neal MD, Siggers R, Sho S, Ma C, Branca MF, et al. Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology*. (2012) 143:708.e5–18.e5. doi: 10.1053/j.gastro.2012.05.053
27. Leaphart CL, Cavallo J, Gribar SC, Cetin S, Li J, Branca MF, et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J Immunol*. (2007) 179:4808–20. doi: 10.4049/jimmunol.179.7.4808
28. Jilling T, Simon D, Lu J, Meng FJ Li D, Schy R, et al. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. *J Immunol*. (2006) 177:3273–82. doi: 10.4049/jimmunol.177.5.3273
29. Neu J. Necrotizing enterocolitis: a multi-omic approach and the role of the microbiome. *Dig Dis Sci*. (2020) 65:789–96. doi: 10.1007/s10620-020-06104-w
30. Denning NL, Prince JM. Neonatal intestinal dysbiosis in necrotizing enterocolitis. *Mol Med*. (2018) 24:4. doi: 10.1186/s10020-018-0002-0
31. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, et al. Intestinal dysbiosis in preterm infants preceding necrotizing

enterocolitis: a systematic review and meta-analysis. *Microbiome*. (2017) 5:31. doi: 10.1186/s40168-017-0248-8
32. Hackam DJ, Sodhi CP. Toll-like receptor-mediated intestinal inflammatory imbalance in the pathogenesis of necrotizing enterocolitis. *Cell Mol Gastroenterol Hepatol*. (2018) 6:229.e1–38.e1. doi: 10.1016/j.jcmgh.2018.04.001
33. Yan X, Managlia E, Tan XD, De Plaen IG. Prenatal inflammation impairs intestinal microvascular development through a TNF-dependent mechanism and predisposes newborn mice to necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol*. (2019) 317:G57–66. doi: 10.1152/ajpgi.00332.2018
34. Butel MJ, Aires J. Editorial Commentary: Neonatal necrotizing enterocolitis: a clostridial disease? *Clin Infect Dis*. (2015) 61:1116–8. doi: 10.1093/cid/civ469
35. Cassir N, Benamar S, La Scola B. *Clostridium butyricum*: from beneficial to a new emerging pathogen. *Clin Microbiol Infect*. (2016) 22:37–45. doi: 10.1016/j.cmi.2015.10.014
36. Cassir N, Benamar S, Khalil JB, Croce O, Saint-Faust M, Jacquot A, et al. *Clostridium butyricum* strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. *Clin Infect Dis*. (2015) 61:1107–15. doi: 10.1093/cid/civ468
37. Waligora-Dupriet AJ, Dugay A, Auzeil N, Huerre M, Butel MJ. Evidence for clostridial implication in necrotizing enterocolitis through bacterial fermentation in a gnotobiotic quail model. *Pediatr Res*. (2005) 58:629–35. doi: 10.1203/01.PDR.0000180538.13142.84
38. Olm MR, Bhattacharya N, Crits-Christoph A, Firek BA, Baker R, Song YS, et al. Necrotizing enterocolitis is preceded by increased gut bacterial replication, Klebsiella, and fimbriae-encoding bacteria. *Sci Adv*. (2019) 5:eaax5727. doi: 10.1126/sciadv.aax5727
39. Romano-Keeler J, Shilts MH, Tovchigrechko A, Wang C, Brucker RM, Moore DJ, et al. Distinct mucosal microbial communities in infants with surgical necrotizing enterocolitis correlate with age and antibiotic exposure. *PLoS ONE*. (2018) 13:e0206366. doi: 10.1371/journal.pone.0206366
40. Roze JC, Ancel PY, Lepage P, Martin-Marchand L, Al Nabhani Z, Delannoy J, et al. Nutritional strategies and gut microbiota composition as risk factors for necrotizing enterocolitis in very-preterm infants. *Am J Clin Nutr*. (2017) 106:821–30. doi: 10.3945/ajcn.117.152967
41. Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu Z, et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. *J Pediatr*. (2014) 165:23–9. doi: 10.1016/j.jpeds.2014.01.010
42. Cotten CM, Taylor S, Stoll B, Goldberg RN, Hansen NI, Sanchez PJ, et al. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. *Pediatrics*. (2009) 123:58–66. doi: 10.1542/peds.2007-3423
43. Alexander VN, Northrup V, Bizzarro MJ. Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis. *J Pediatr*. (2011) 159:392–7. doi: 10.1016/j.jpeds.2011.02.035
44. Guillet R, Stoll BJ, Cotten CM, Gantz M, McDonald S, Poole WK, et al. Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis in very low birth weight infants. *Pediatrics*. (2006) 117:e137–42. doi: 10.1542/peds.2005-1543
45. Patole S. Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis: a case of excessive collateral damage? *Pediatrics*. (2006) 117:531–2. doi: 10.1542/peds.2005-2230
46. Bruno G, Zaccari P, Rocco G, Scalese G, Panetta C, Porowska B, et al. Proton pump inhibitors and dysbiosis: current knowledge and aspects to be clarified. *World J Gastroenterol*. (2019) 25:2706–19. doi: 10.3748/wjg.v25.i22.2706
47. Freedberg DE, Lebwohl B, Abrams JA. The impact of proton pump inhibitors on the human gastrointestinal microbiome. *Clin Lab Med*. (2014) 34:771–85. doi: 10.1016/j.cll.2014.08.008
48. Meinzen-Derr J, Poindexter B, Wrage L, Morrow AL, Stoll B, Donovan EF. Role of human milk in extremely low birth weight infants' risk of necrotizing enterocolitis or death. *J Perinatol*. (2009) 29:57–62. doi: 10.1038/jp.2008.117
49. Quigley M, Embleton ND, McGuire W. Formula versus donor breast milk for feeding preterm or low birth weight infants. *Cochrane Database Syst Rev*. (2019) 7:CD002971. doi: 10.1002/14651858.CD002971.pub3
50. Patel AL, Panagos PG, Silvestri JM. Reducing incidence of necrotizing enterocolitis. *Clin Perinatol*. (2017) 44:683–700. doi: 10.1016/j.clp.2017.05.004
51. Good M, Sodhi CP, Egan CE, Afrazi A, Jia H, Yamaguchi Y, et al. Breast milk protects against the development of necrotizing enterocolitis through inhibition of Toll-like receptor 4 in the intestinal epithelium via activation of the epidermal growth factor receptor. *Mucosal Immunol*. (2015) 8:1166–79. doi: 10.1038/mi.2015.30
52. Al-Hosni M, Duenas M, Hawk M, Stewart LA, Borghese RA, Cahoon M, et al. Probiotics-supplemented feeding in extremely low-birth-weight infants. *J Perinatol*. (2012) 32:253–9. doi: 10.1038/jp.2011.51
53. Arora S, Khurana MS, Saini R. To study the role of probiotics in the prevention of necrotizing enterocolitis in preterm neonates. *Int J Contemp Pediatr*. (2017) 4. doi: 10.18203/2349-3291.ijcp20173787
54. Awad H, Mokhtar H, Imam SS, Gad GI, Hafez H, Aboushady N. Comparison between killed and living probiotic usage versus placebo for the prevention of necrotizing enterocolitis and sepsis in neonates. *Pak J Biol Sci*. (2010) 13:253–62. doi: 10.3923/pjbs.2010.253.262
55. Benor S, Marom R, Ben Tov A, Armoni Domany K, Zaidenberg-Israeli G, Dollberg S. Probiotic supplementation in mothers of very low birth weight infants. *Am J Perinatol*. (2014) 31:497–504. doi: 10.1055/s-0033-1353490
56. Bin-Nun A, Bromiker R, Wilschanski M, Kaplan M, Rudensky B, Caplan M, et al. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. *J Pediatr*. (2005) 147:192–6. doi: 10.1016/j.jpeds.2005.03.054
57. Braga TD, da Silva GA, de Lira PI, de Carvalho Lima M. Efficacy of Bifidobacterium breve and Lactobacillus casei oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. *Am J Clin Nutr*. (2011) 93:81–6. doi: 10.3945/ajcn.2010.29799
58. Chandrashekar GS, Shettigar S, Varghese TC. Role of probiotics in prevention of necrotizing enterocolitis in preterm neonates. *Indian J Child Health*. (2018) 5:112–5.
59. Chowdhury T, Ali MM, Hossain MM, Singh J, Yousuf AN, Yasmin F, et al. Efficacy of probiotics versus placebo in the prevention of necrotizing enterocolitis in preterm very low birth weight infants: a double-blind randomized controlled trial. *J Coll Physicians Surg Pak*. (2016) 26:770–4.
60. Costeloe K, Hardy P, Juszczak E, Wilks M, Millar MR, Probiotics in Preterm Infants Study Collaborative Group. *Bifidobacterium breve* BBG-001 in very preterm infants: a randomised controlled phase 3 trial. *Lancet*. (2016) 387:649–60. doi: 10.1016/S0140-6736(15)01027-2
61. Dani C, Coviello CC, Corsini II, Arena F, Antonelli A, Rossolini GM. Lactobacillus sepsis and probiotic therapy in newborns: two new cases and literature review. *AJP Rep*. (2016) 6:e25–9. doi: 10.1055/s-0035-1566312
62. Dashti AS, Afjeh SA, Basiry A, Shirvani F, Seifi K, Taheri ZM. Prophylactic probiotics for prevention of necrotizing enterocolitis (NEC) in low birth weight neonates. *Arch Pediatr Infect Dis*. (2013) 2:174–9.
63. Demirel G, Erdeve O, Celik IH, Dilmen U. Saccharomyces boulardii for prevention of necrotizing enterocolitis in preterm infants: a randomized, controlled study. *Acta Paediatr*. (2013) 102:e560–5. doi: 10.1111/apa.12416
64. Dongol Singh SS, Klobassa DS, Resch B, Urlesberger B, Shrestha RP. Placebo controlled introduction of prophylactic supplementation of probiotics to decrease the incidence of necrotizing enterocolitis at Dhulikhel Hospital in Nepal. *Kathmandu Univ Med J (KUMJ)*. (2017) 15:319–23.
65. Fernandez-Carrocera LA, Solis-Herrera A, Cabanillas-Ayon M, Gallardo-Sarmiento RB, Garcia-Perez CS, Montano-Rodriguez R, et al. Double-blind, randomised clinical assay to evaluate the efficacy of probiotics in preterm newborns weighing less than 1500 g in the prevention of necrotising enterocolitis. *Arch Dis Child Fetal Neonatal Ed*. (2013) 98:F5–9. doi: 10.1136/archdischild-2011-300435
66. Gomez-Rodriguez G, Amador-Licona N, Daza-Benitez L, Barbosa-Sabanero G, Carballo-Magdaleno D, Aguilar-Padilla R, et al. Single strain versus multispecies probiotic on necrotizing enterocolitis and faecal IgA levels in very low birth weight preterm neonates: A randomized clinical trial. *Pediatr Neonatol*. (2019) 60:564–9. doi: 10.1016/j.pedneo.2019.02.005

67. Hays S, Jacquot A, Gauthier H, Kempf C, Beissel A, Pidoux O, et al. Probiotics and growth in preterm infants: a randomized controlled trial, PREMAPRO study. *Clin Nutr*. (2016) 35:802–11. doi: 10.1016/j.clnu.2015.06.006
68. Hernández-Enríquez NP, Rosas-Sumano AB, Monzoy-Ventre MA, Galicia-Flores L. *Lactobacillus reuteri* DSM 17938 in preventing necrotizing enterocolitis in preterm newborns. Pilot study of efficacy and safety. *Rev Mex Pediatr*. (2016) 83:37–43.
69. Hoyos AB. Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit. *Int J Infect Dis*. (1999) 3:197–202. doi: 10.1016/s1201-9712(99)90024-3
70. Jacobs SE, Tobin JM, Opie GF, Donath S, Tabrizi SN, Pirotta M, et al. Probiotic effects on late-onset sepsis in very preterm infants: a randomized controlled trial. *Pediatrics*. (2013) 132:1055–62. doi: 10.1542/peds.2013-1339
71. Janvier A, Malo J, Barrington KJ. Cohort study of probiotics in a North American neonatal intensive care unit. *J Pediatr*. (2014) 164:980–5. doi: 10.1016/j.jpeds.2013.11.025
72. Kaban RK, Wardhana, Hegar B, Rohsiswatmo R, Handryastuti S, Amelia N, et al. *Lactobacillus reuteri* DSM 17938 improves feeding intolerance in preterm infants. *Pediatr Gastroenterol Hepatol Nutr*. (2019) 22:545–53. doi: 10.5223/pghn.2019.22.6.545
73. Lin HC, Su BH, Chen AC, Lin TW, Tsai CH, Yeh TF, et al. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics*. (2005) 115:1–4. doi: 10.1542/peds.2004-1463
74. Lin HC, Hsu CH, Chen HL, Chung MY, Hsu JF, Lien RI, et al. Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. *Pediatrics*. (2008) 122:693–700. doi: 10.1542/peds.2007-3007
75. Oncel MY, Sari FN, Arayici S, Guzoglu N, Erdeve O, Uras N, et al. Lactobacillus Reuteri for the prevention of necrotising enterocolitis in very low birthweight infants: a randomised controlled trial. *Arch Dis Child Fetal Neonatal Ed*. (2014) 99:F110–5. doi: 10.1136/archdischild-2013-304745
76. Rojas MA, Lozano JM, Rojas MX, Rodriguez VA, Rondon MA, Bastidas JA, et al. Prophylactic probiotics to prevent death and nosocomial infection in preterm infants. *Pediatrics*. (2012) 130:e1113–20. doi: 10.1542/peds.2011-3584
77. Saengtawesin V, Tangpolkaiwalsak R, Kanjanapattankul W. Effect of oral probiotics supplementation in the prevention of necrotizing enterocolitis among very low birth weight preterm infants. *J Med Assoc Thai*. (2014) 97(Suppl 6):S20–5.
78. Samanta M, Sarkar M, Ghosh P, Ghosh J, Sinha M, Chatterjee S. Prophylactic probiotics for prevention of necrotizing enterocolitis in very low birth weight newborns. *J Trop Pediatr*. (2009) 55:128–31. doi: 10.1093/tropej/fmn091
79. Sari FN, Dizdar EA, Oguz S, Erdeve O, Uras N, Dilmen U. Oral probiotics: Lactobacillus sporogenes for prevention of necrotizing enterocolitis in very low-birth weight infants: a randomized, controlled trial. *Eur J Clin Nutr*. (2011) 65:434–9. doi: 10.1038/ejcn.2010.278
80. Serce O, Benzer D, Gursoy T, Karatekin G, Ovali F. Efficacy of Saccharomyces boulardii on necrotizing enterocolitis or sepsis in very low birth weight infants: a randomised controlled trial. *Early Hum Dev*. (2013) 89:1033–6. doi: 10.1016/j.earlhumdev.2013.08.013
81. Shadkam NM, Jalalizadeh F, Nasiriani K. Effects of probiotic lactobacillus reuteri (DSM 17938) on the incidence of necrotizing enterocolitis in very low birth weight premature infants. *Iran J Neonatol (IJN)*. (2015) 6:15–20.
82. Shashidhar A, Suman Rao PN, Nesargi S, Bhat S, Chandrakala BS. Probiotics for promoting feed tolerance in very low birth weight neonates - a randomized controlled trial. *Indian Pediatr*. (2017) 54:363–7. doi: 10.1007/s13312-017-1106-2
83. Van Niekerk E, Nel DG, Blaauw R, Kirsten GF. Probiotics Reduce necrotizing enterocolitis severity in HIV-exposed premature infants. *J Trop Pediatr*. (2015) 61:155–64. doi: 10.1093/tropej/fmv004
84. Wang Y, Gao L, Zhang YH, Shi CS, Ren CM. Efficacy of probiotic therapy in full-term infants with critical illness. *Asia Pac J Clin Nutr*. (2014) 23:575–80. doi: 10.6133/apjcn.2014.23.4.14
85. Zampieri N, Pietrobelli A, Biban P, Soffiati M, Dall'agnola A, Camoglio FS. Lactobacillus paracasei subsp. paracasei F19 in Bell's stage 2 of necrotizing enterocolitis. *Minerva Pediatr*. (2013) 65:353–60.
86. Armanian AM, Sadeghnia A, Hoseinzadeh M, Mirlohi M, Feizi A, Salehimehr N, et al. The Effect of Neutral Oligosaccharides on Reducing the Incidence of Necrotizing Enterocolitis in Preterm infants: A Randomized Clinical Trial. *Int J Prev Med*. (2014) 5:1387–95.
87. Dasopoulou M, Briana DD, Boutsikou T, Karakasidou E, Roma E, Costalos C, et al. Motilin and gastrin secretion and lipid profile in preterm neonates following prebiotics supplementation: a double-blind randomized controlled study. *JPEN J Parenter Enteral Nutr*. (2015) 39:359–68. doi: 10.1177/0148607113510182
88. Dilli D, Aydin B, Fettah ND, Ozyazici E, Beken S, Zenciroglu A, et al. The propre-save study: effects of probiotics and prebiotics alone or combined on necrotizing enterocolitis in very low birth weight infants. *J Pediatr*. (2015) 166:545.e1–51.e1. doi: 10.1016/j.jpeds.2014.12.004
89. Manzoni P, Meyer M, Stolfi I, Rinaldi M, Cattani S, Pugni L, et al. Bovine lactoferrin supplementation for prevention of necrotizing enterocolitis in very-low-birth-weight neonates: a randomized clinical trial. *Early Hum Dev*. (2014) 90(Suppl 1):S60–5. doi: 10.1016/S0378-3782(14)70020-9
90. Riskin A, Hochwald O, Bader D, Srugo I, Naftali G, Kugelman A, et al. The effects of lactulose supplementation to enteral feedings in premature infants: a pilot study. *J Pediatr*. (2010) 156:209–14. doi: 10.1016/j.jpeds.2009.09.006
91. Dilli D, Aydin B, Zenciroglu A, Ozyazici E, Beken S, Okumus N. Treatment outcomes of infants with cyanotic congenital heart disease treated with synbiotics. *Pediatrics*. (2013) 132:e932–8. doi: 10.1542/peds.2013-1262
92. Guney-Varal I, Koksal N, Ozkan H, Bagci O, Dogan P. The effect of early administration of combined multi-strain and multi-species probiotics on gastrointestinal morbidities and mortality in preterm infants: A randomized controlled trial in a tertiary care unit. *Turk J Pediatr*. (2017) 59:13–9. doi: 10.24953/turkjped.2017.01.003
93. Nandhini LP, Biswal N, Adhisivam B, Mandal J, Bhat BV, Mathai B. Synbiotics for decreasing incidence of necrotizing enterocolitis among preterm neonates - a randomized controlled trial. *J Matern Fetal Neonatal Med*. (2016) 29:821–5. doi: 10.3109/14767058.2015.1019854
94. Denkel LA, Schwab F, Garten L, Geffers C, Gastmeier P, Piening B. Protective effect of dual-strain probiotics in preterm infants: a multi-center time series analysis. *PLoS ONE*. (2016) 11:e0158136. doi: 10.1371/journal.pone.0158136
95. Gray KD, Messina JA, Cortina C, Owens T, Fowler M, Foster M, et al. Probiotic use and safety in the neonatal intensive care unit: a matched cohort study. *J Pediatr*. (2020) 222:59.e1–64.e1. doi: 10.1016/j.jpeds.2020.03.051
96. Singh B, Shah PS, Afifi J, Simpson CD, Mitra S, Dow K, et al. Probiotics for preterm infants: a national retrospective cohort study. *J Perinatol*. (2019) 39:533–9. doi: 10.1038/s41372-019-0315-z
97. Esaiassen E, Cavanagh P, Hjerde E, Simonsen GS, Stoen R, Klingenberg C. *Bifidobacterium longum* subspecies infantis bacteremia in 3 extremely preterm infants receiving probiotics. *Emerg Infect Dis*. (2016) 22:1664–6. doi: 10.3201/eid2209.160033
98. Jenke A, Ruf EM, Hoppe T, Heldmann M, Wirth S. *Bifidobacterium septicaemia* in an extremely low-birthweight infant under probiotic therapy. *Arch Dis Child Fetal Neonatal Ed*. (2012) 97:F217–8. doi: 10.1136/archdischild-2011-300838
99. Athalye-Jape G, Patole S. Probiotics for preterm infants - time to end all controversies. *Microb Biotechnol*. (2019) 12:249–53. doi: 10.1111/1751-7915.13357
100. Sharif S, Meader N, Oddie SJ, Rojas-Reyes MX, McGuire W. Probiotics to prevent necrotising enterocolitis in very preterm or very low birth weight infants. *Cochrane Database Syst Rev*. (2020) 10:CD005496. doi: 10.1002/14651858.CD005496.pub5
101. Morgan RL, Preidis GA, Kashyap PC, Weizman AV, Sadeghirad B, McMaster Probiotic P, et al. Probiotics reduce mortality and morbidity in preterm, low-birth-weight infants: a systematic review and network meta-analysis of randomized trials. *Gastroenterology*. (2020) 159:467–80. doi: 10.1053/j.gastro.2020.05.096
102. Beghetti I, Panizza D, Lenzi J, Gori D, Martini S, Corvaglia L, et al. Probiotics for preventing necrotizing enterocolitis in preterm infants: a network meta-analysis. *Nutrients*. (2021) 13:192. doi: 10.3390/nu13010192
103. Chi C, Li C, Buys N, Wang W, Yin C, Sun J. Effects of probiotics in preterm infants: a network meta-analysis. *Pediatrics*. (2021) 147:e20200706. doi: 10.1542/peds.2020-0706

104. Chi C, Buys N, Li C, Sun J, Yin C. Effects of prebiotics on sepsis, necrotizing enterocolitis, mortality, feeding intolerance, time to full enteral feeding, length of hospital stay, and stool frequency in preterm infants: a meta-analysis. *Eur J Clin Nutr*. (2019) 73:657–70. doi: 10.1038/s41430-018-0377-6
105. van den Akker CHP, van Goudoever JB, Shamir R, Domellof M, Embleton ND, Hojsak I, et al. Probiotics and preterm infants: a position paper by the european society for paediatric gastroenterology hepatology and nutrition committee on nutrition and the european society for paediatric gastroenterology hepatology and nutrition working group for probiotics and prebiotics. *J Pediatr Gastroenterol Nutr*. (2020) 70:664–80. doi: 10.1097/MPG.0000000000002655
106. Su GL, Ko CW, Bercik P, Falck-Ytter Y, Sultan S, Weizman AV, et al. AGA clinical practice guidelines on the role of probiotics in the management of gastrointestinal disorders. *Gastroenterology*. (2020) 159:697–705. doi: 10.1053/j.gastro.2020.05.059
107. Poindexter B, Committee on Fetus and Newborn. Use of probiotics in preterm infants. *Pediatrics*. (2021) 147:e2021051485. doi: 10.1542/peds.2021-051485

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fmicb.2021.657542

Effects of *Clostridium butyricum*, Sodium Butyrate, and Butyric Acid Glycerides on the Reproductive Performance, Egg Quality, Intestinal Health, and Offspring Performance of Yellow-Feathered Breeder Hens

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 23 January 2021
Accepted: 12 August 2021
Published: 16 September 2021

Citation:
Wang Y, Wang Y, Lin X, Gou Z, Fan Q and Jiang S (2021) Effects of Clostridium butyricum, Sodium Butyrate, and Butyric Acid Glycerides on the Reproductive Performance, Egg Quality, Intestinal Health, and Offspring Performance of Yellow-Feathered Breeder Hens. Front. Microbiol. 12:657542. doi: 10.3389/fmicb.2021.657542

Butyrate has been reported to promote the performance and growth of chickens. The specific roles and efficacy of different sources of butyrate remained unclear. Thus, the present study aimed to investigate and compare the effects of *Clostridium butyricum* (CB), sodium butyrate (SB), and butyric acid glycerides (tributyrin, BAG) on the reproductive performance, egg quality, intestinal health, and offspring performance of yellow-feathered breeder hens. A total of 300 Lingnan yellow-feathered breeder hens were assigned to five treatment groups: control (CL), 1×10^8 CFU/kg CB (CBL), 1×10^9 CFU/kg CB (CBH), 500 mg/kg SB, and 300 mg/kg BAG. Results showed that the laying performance and egg quality were increased by CBL, CBH, and BAG. Both CB treatments increased the hatchability of fertilized eggs. Maternal supplementation with both levels of CB significantly elevated the growth performance of offspring. Treatment with CBL, CBH, SB, and BAG all improved the oviduct-related variables and reduced the plasmal antioxidant variables. The CBH, CBL, and BAG treatments also improved the intestinal morphology to different degrees. Jejunal contents of IL-6 were decreased by CBH and BAG, while those of IL-4, IL-6, IL-1β, and IgY were decreased by SB. Transcripts of nutrient transporters in jejunal mucosa were also upregulated by CBH, CBL, and SB treatments and expression of Bcl-2-associated X protein was decreased by CBL, CBH, and BAG. In cecal contents, CBL increased the abundance of Firmicutes and Bacillus, while CBH decreased the abundance of Proteobacteria. Also, the co-occurrence networks of intestinal microbes were regulated by CBH and BAG. In conclusion, dietary inclusion of CB and BAG improved the reproductive parameters, egg quality, and intestinal morphology of breeders. CB also influenced the hatching performance of breeders and growth performance of the offspring, while SB improved the oviduct-related variables. These beneficial effects may result from the regulation of cytokines, nutrient transporters, apoptosis, and gut microbiota; high-level

CB had more obvious impact. Further study is needed to explore and understand the correlation between the altered gut microbiota induced by butyrate and the performance, egg quality, intestinal health, and also offspring performance.

Keywords: reproductive performance, *Clostridium butyricum*, sodium butyrate, butyric acid glycerides, yellow-feathered breeder hens

INTRODUCTION

Butyrate, a short-chain fatty acid (SCFA), is a main end-product of intestinal microbial fermentation of dietary fiber (Xiao et al., 2020). Butyrate is important for the health of animals because it provides nutrition for epithelial cells and helps inhibit pathogens in the gut (Meimandipour et al., 2010).

Clostridium butyricum (CB) is a butyric acid-producing Gram-positive anaerobe and has been used as feed additive in poultry industry. Previous studies in chickens suggested that CB promoted growth performance (Zhang et al., 2011; Yang et al., 2012), improved intestinal morphology (Zhang et al., 2011), altered intestinal microbiota (Meimandipour et al., 2010; Yang et al., 2012), and ameliorated inflammation (Zhang et al., 2016; Takahashi et al., 2018). Similarly, sodium butyrate (SB), functioning as butyric acid in the acidic environment of the proximal digestive tract of the birds (Ahsan et al., 2016), also increased the growth performance (Sikandar et al., 2017), meat quality (Deepa et al., 2017; Gomathi et al., 2018), and anti-inflammatory ability of chickens (Jiang et al., 2015). Butyric acid glycerides (BAG), the tributyl ester compound, is an enriched source with fewer undesirable properties. Previous research indicated that BAG effectively improved the growth performance and lipid metabolism of broilers (Taherpour et al., 2009). In the study of Jahanian and Golshadi (2015), the productive performance of laying hens was found to be enhanced by BAG.

Although CB, SB, and BAG promoted the performance and growth of breeder hens, their specific roles and efficacy remained unclear. The present study, therefore, was designed to investigate and compare the effects of CB, SB, and BAG on the reproductive performance, egg quality, intestinal health, and offspring performance of yellow-feathered breeder hens, breeds of major importance in China. In addition, because of apparent altered function of the small intestine, the relative expression of selected nutrient transporter genes, and other relevant transcripts, was measured.

MATERIALS AND METHODS

Breeder Hens and Management

The experimental protocol was approved by the Animal Care Committee of the Institute of Animal Science, Guangdong Academy of Agriculture Science, Guangzhou, China, with the approval number GAASISA-2019-007. A total of 300 Lingnan yellow-feathered breeder hens of 45 weeks of age with similar BW (3.02 ± 0.01 kg) and laying rate were used.

Breeder hens were randomly allocated into five treatment groups, each with 10 replicates of 6 birds. The trial lasted from 45 to 54 weeks of age. During the experimental period, hens were housed singly in laying cages, received 120 g of feed per bird per day to prevent over-feeding, and had access to fresh water *ad libitum*. Breeder hens received artificial insemination of 25 μl pooled semen per bird every 3 days. Twenty males were used and received the basal diet. At the end of the experiment, eggs were collected and incubated as described below.

One hundred hatched chicks, pooled from each treatment, were divided into five replicates and fed a standard diet for 28 days in pens (stocking density 0.38 m^2/bird). Daylight was eliminated and replaced with 18-h lighting from incandescent bulbs. The temperature of the room was maintained at 32 to 34°C for the first 3 days and then reduced by 2 to 3°C per week to a final temperature of 26°C. Chicks received feed and fresh water *ad libitum*.

Diets

All breeder hens were fed the same basal diet (**Supplementary Table S1**) to which 1×10^8 CFU/kg *Clostridium butyricum* (CBL), 1×10^9 CFU/kg *Clostridium butyricum* (CBH), 500 mg/kg coated sodium butyrate (SB), and 300 mg/kg BAG were added to obtain the treatments. *Clostridium butyricum* (1×10^9 CFU/kg) and coated sodium butyrate (effective content: 40% butyrate) were purchased from Huijia Biological Technology Co., Ltd. (Hangzhou, China). BAG (tributyrin: 45% butyrate) was obtained from Youjiu Biological Technology Co., Ltd. (Shanghai, China). All offspring chicks were fed a common diet (**Supplementary Table S2**).

Production Performance and Egg Quality

Egg production, egg weight, and number of qualified eggs were recorded daily. The unqualified eggs included misshapen eggs, dirty eggs, excessively large or small eggs, broken eggs, cracked eggs, and eggs without a shell (but with intact membrane) according to Duan et al. (2015). The laying rate, daily egg mass, average egg weight, and qualified rate of egg were calculated. At 53 weeks of age, three eggs per replicate were selected to determine the egg quality, including egg shape index, yolk ratio, yolk color, albumen height, Haugh unit (automatic egg analyzer, EMT-5200 Robotmation Co., Ltd., Tokyo, Japan), eggshell thickness (by micrometer), and eggshell strength (eggshell strength tester, FGV-10XY, Orka Food Technology, Ramat HaSharon, Israel).

At 55 weeks of age, 30 eggs from each replicate and 10 replicates from each treatment were selected for hatching.

All eggs were incubated in the same incubator (Bengbu Sanyuan Incubation Equipment Co., Ltd., Anhui, China) at 37.2°C to 38.0°C and 60 to 75% relative humidity. Eggs were turned 12 times/day throughout the incubation period and sprayed with water once daily from the 15th day of incubation until they hatched (Xia et al., 2020). The fertility, hatch of fertile eggs and hatchling weight were recorded and calculated.

At the end of the experiment, one hen from each replicate was selected to measure the weight and length of the oviduct, the weight of ovarian stroma, the number and weight of large yellow follicles (LYF), and the number and weight of small yellow follicles (SYF). The oviduct included the funnel part, enlargement, isthmus, and the uterine. The LYF (>8 mm) and SYF (3–8 mm) were identified by measuring the diameter using the vernier caliper.

The growth performance of the 20 offspring from each replicate was also determined. The average daily gain (ADG) was determined from BW at hatching and day 28. The average daily feed intake was determined from feed consumed by each replicate.

Biochemical Determinations

At the end of the experiment, hens selected at Section "Production Performance and Egg Quality" were weighed and 6 ml heparinized blood was obtained *via* the wing vein. Plasma was obtained by centrifugation at 1,500 × *g* for 10 min. The birds were then killed by cervical dislocation. Liver, ovary and jejunal mucosa (rinsed and scraped with a glass slide) were collected and snap-frozen in liquid N_2, homogenized with ice-cold physiologic saline (1:10, v/v), and centrifuged at 2,000 × *g* for 10 min. The activities of alkaline phosphatase (AKP), total antioxidant capacity (T-AOC), diamine oxidase (DAO), total superoxidase dismutase (T-SOD), and content of uric acid in the plasma and in the supernatants of liver, jejunal mucosa, and ovary were analyzed spectrophotometrically using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Intestinal pH and Morphology

The pH of small intestinal contents was measured with a portable meter equipped with an insertion glass electrode (HI8424, HANA Instrument Science and Technology Co., Ltd., Beijing, China). Duodenum, jejunum, and ileum were collected immediately after slaughter. The intestines were opened with sterile scissors, and pH of the contents was measured by inserting a glass pH electrode probe.

One-centimeter lengths from the medial portions of the jejunum were washed in physiological saline solution and fixed in 10% buffered formalin. Tissue samples were later embedded in paraffin, and a 20-μm sections of each sample were dewaxed, mounted on glass slides, and stained with hematoxylin and eosin. Slides were blindly evaluated microscopically (Eclipse Ti-E, Nikon, Japan). Villus height was measured from the top of the villus to the junction of villus and crypt, and crypt depth was measured as the depth of the invagination between adjacent villi using scanning browsing software (CaseViewer2.2, 3DHISTECH, Hungary) and scanning analysis software (Halo v3.0.311.314, Indica Labs, Albuquerque, NM). For each jejunal sample, the height of five intact villi and the corresponding depths of five crypts were measured and the average value was calculated.

RT-qPCR

Total RNA extraction from the mucosa of jejunum was performed using TRIzol reagent (RNAiso plus 9109, Takara, Tokyo, Japan) and reverse-transcribed with PrimeScript II 1st Strand cDNA Synthesis Kit (6210A, Takara). Real-time PCR was performed with SYBR PremixExTaq II (Takara) and an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA), as described by Satoh et al. (2010). The primers used are provided in **Supplementary Table S3**. Results were normalized to the abundance of β-actin transcripts, and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

Immune Variables

Jejunal mucosal samples were homogenized with ice-cold physiologic saline (1:10, v/v) and centrifuged at 2,000 × *g* for 10 min (Centrifuge 5804R, Eppendorf, Germany). Supernatants were collected, and ELISAs were performed to determine the levels of IL-4, IL-6, IL-1β, TNF-α, IgM, IgA, and IgY using kits (Bio-function Technology Co., Ltd., Beijing, China).

Microbial Analysis

Bacterial genomic DNA was extracted from cecal contents of breeder hens using the TIANamp Stool DNA Kit (Tiangen, Beijing, China). The V3/V4 region of the 16S ribosomal RNA gene was amplified by using the 341F/805R primer pairs, and the sequencing was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Raw sequences were filtered and clustered into operational taxonomic unit (OTU) at 97% similarity by QIIME 2 software.[1] Bacterial OTU representative sequences were assigned to a taxonomic lineage by Ribosomal Database Project classifier based on the Greengenes 13.8 database. Alpha diversity was analyzed by QIIME 2 software. Beta diversity was analyzed and plotted by principal coordinates analysis (PCoA) using the "ggplot2" package of R software. Permutational multivariate analysis of variance (PERMANOVA) was calculated by "vegan" package to determine significant differences in microbial beta diversity among the treatment groups (based on the Bray-Curtis distance matrices). To determine the highly dimensional intestinal microbes and characterize the differences among the treatments, linear discriminant analysis (LDA) effect size (LEfSe) analysis[2] was used. Co-occurrence networks of microbial communities in different treatments were built based on significant correlations (Spearman's $R > 0.6$ and FDR-adjusted $p < 0.05$; Jiao et al., 2016) and were visualized by Gephi software (version 0.9.2). The topological properties of networks were calculated to describe the complex patterns of the interrelationships. All the DNA datasets have been submitted

[1]https://qiime2.org
[2]https://huttenhower.sph.harvard.edu/galaxy/

to the NCBI Sequence Read Archive database under the BioProject ID: PRJNA695347.

Statistical Analysis

Replicate served as the experimental unit. The effects of dietary CB, SB, and BAG were analyzed by one-way ANOVA (SPSS Inc., Chicago, IL). Means were separated by Duncan's multiple range test. Tabulated results are shown as means with SEM derived from the ANOVA error mean square.

RESULTS

Laying Performance of Yellow-Feathered Breeder Hens

As shown in **Table 1**, supplementation with high or low-level CB and BAG increased the laying rate ($p < 0.05$). CBH and BAG also increased the daily egg mass ($p < 0.05$). The feed:egg ratio was decreased by the high and low level of CB and by BAG ($p < 0.05$).

Hatching Performance and Egg Quality of Yellow-Feathered Breeder Hens

According to **Table 2**, CBL, CBH, SB, and BAG had no significant effects on the fertility, hatch of fertile eggs, and hatchling weight, compared to the controls (CL). Birds in the CBL treatment had lower hatchability of fertilized eggs ($p < 0.05$) than those receiving CBH. Significantly, increased ($p < 0.05$) albumen height and eggshell thickness were noted for the CBH treatment and CBL increased the albumen height ($p < 0.05$; **Table 3**). The BAG treatment increased ($p < 0.05$) the yolk color and Haugh unit.

Oviduct-Related Variables of Yellow-Feathered Breeder Hens

Compared with the controls, CBH treatment increased ($p < 0.05$) the weight and length of the oviduct, and number and weight of LYF. Number and weight of LYF also increased ($p < 0.05$) by CBL, SB increased weight of the oviduct, and number and weight of LYF, while BAG treatment increased ($p < 0.05$) the number of LYF (**Table 4**).

Biochemical Variables of Yellow-Feathered Breeder Hens

Treatments with CBH, CBL, and SB all decreased ($p < 0.05$) T-AOC activity and uric acid concentration, CBH also lowered plasmal T-SOD activity ($p < 0.05$), and BAG treatment significantly decreased T-AOC and T-SOD activity (**Table 5**). There were no effects of treatment on the activities of T-AOC and T-SOD in jejunal mucosa, liver, or ovary (**Table 6**).

Intestinal Morphology of Yellow-Feathered Breeder Hens

The pH of duodenal, jejunal, and ileal digesta was not significantly influenced by treatment ($p > 0.05$). Jejunal morphology was improved by CBH, CBL, and BAG. As shown in **Table 6**, CBH increased ($p < 0.05$) the villus height, crypt depth, and the villus:crypt ratio, CBL increased ($p < 0.05$) the villus height and villus:crypt ratio, and BAG increased ($p < 0.05$) the villus height and crypt depth of jejunal mucosa.

TABLE 1 | Laying performance of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
Laying rate, %	50.82^{b}	57.10^{a}	56.24^{a}	54.65^{ab}	58.61^{a}	0.90	0.021
Daily egg mass, g	30.60^{b}	34.75^{a}	33.81^{ab}	32.89^{ab}	34.68^{a}	0.53	0.041
Average egg weight, g	60.90	61.70	60.73	60.71	60.09	0.58	0.841
Qualified rate of egg, %	94.68	97.31	96.27	93.97	96.57	0.45	0.111
Feed/egg	3.50^{a}	3.05^{b}	3.15^{b}	3.24^{ab}	3.06^{b}	0.05	0.039

^{ab}*Means within a row lacking a common superscript differ significantly ($p < 0.05$). CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.*

TABLE 2 | Hatching performance of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
Fertility, %	85.33	80.00	81.33	82.00	86.67	1.76	0.823
Hatch of feritile eggs, %	95.59^{ab}	97.50^{a}	89.53^{b}	90.89^{ab}	96.05^{ab}	1.08	0.029
Chick weight, g	44.14	44.79	43.67	42.91	42.65	0.31	0.061

^{ab}*Means within a row lacking a common superscript differ significantly ($p < 0.05$). CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.*

Immune Factors of Yellow-Feathered Breeder Hens

The contents of cytokines and immunoglobulins in the jejunal mucosa were determined (**Table 7**). The only effect on IL-4 was the reduction ($p < 0.05$) caused by SB treatment. Contents of IL-6 were reduced ($p < 0.05$) by CBH, SB, and BAG. The SB treatment also reduced IL-1β and IgY, the latter quite strikingly, and SB also resulted in lowest contents of IgA.

Jejunal Gene Expression of Yellow-Feathered Breeder Hens

The relative transcript abundance of Na(+)/H(+) exchanger isoform 2 (*NHE2*) was increased ($p < 0.05$) by CBL and SB, that of peptide transporter 1 (*PEPT1*) by CBL and SB, glucose transporter-2 (*GLUT2*) by both CBH and CBL, and apical nutrient transporter SLC6A19 (B^0AT) was increased only by CBH. The apoptosis-related gene Bcl-2-associated X protein (*BAX*) was downregulated ($p < 0.05$) by CBH, CBL, and BAG; the expression of *TNF-α* was not significantly affected by treatment (**Table 8**).

Gut Microbiota Structure of Yellow-Feathered Breeder Hens

The results showed that the CBL treatment decreased the variables of the observed species, Chao1, Shannon and Simpson

TABLE 3 | Egg quality of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
Egg shape index	1.31	1.27	1.32	1.28	1.32	0.01	0.066
Yolk ratio, %	32.68	31.20	31.11	31.99	32.13	0.23	0.206
Yolk color	5.41^{b}	5.27^{b}	5.35^{b}	5.29^{b}	6.11^{a}	0.10	0.033
Albumen height, mm	6.65^{b}	7.33^{a}	7.54^{a}	6.75^{b}	6.80^{b}	0.11	0.043
Haugh unit	70.97^{b}	69.44^{b}	70.10^{b}	70.45^{b}	76.50^{a}	0.82	0.044
Eggshell thickness, mm	0.30^{b}	0.34^{a}	0.31^{b}	0.32^{ab}	0.32^{ab}	0.00	0.001
Eggshell strength, kg/cm^{2}	3.20	3.32	3.29	3.34	3.57	0.06	0.497

^{ab}Means within a row lacking a common superscript differ significantly ($p < 0.05$).
CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.

TABLE 4 | Oviduct-related variables of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
Weight of oviduct/BW, %	0.96^{c}	1.39^{ab}	1.11^{bc}	1.26^{ab}	1.12^{abc}	0.05	0.022
Length of oviduct, cm	34.67^{b}	39.10^{a}	37.67^{ab}	37.70^{ab}	39.00^{ab}	0.73	0.048
Weight of ovarian stroma/BW, %	0.21	0.26	0.26	0.24	0.23	0.01	0.108
LYF number	3.00^{b}	4.10^{a}	4.30^{a}	4.10^{a}	4.30^{a}	0.14	0.034
LYF weight/BW, %	0.82^{b}	1.28^{a}	1.52^{a}	1.50^{a}	1.22^{ab}	0.07	0.022
SYF number	12.11	8.80	11.50	9.10	10.90	0.60	0.230
SYF weight/BW, %	0.07	0.06	0.09	0.05	0.06	0.01	0.165

^{abc}Means within a row lacking a common superscript differ significantly ($p < 0.05$).
BW, body weight; LYF, large yellow follicles, follicles with mean diameter >8 mm; SYF, small yellow follicles, follicles with mean diameter of 3 to 8 mm. CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.

($p < 0.05$) compared to the controls (**Figure 1**). PCoA of intestinal microbiota based on Bray-Curtis distance revealed distinct clusterings of the gut microbiota composition between CL and CBH, and between CL and BAG (**Figure 2**). The differences in the intestinal bacterial compositions between the treatments were also analyzed. The results showed that at the phylum level, CBH and CBL treatments obviously increased the abundance of Firmicutes. Additionally, CBH also decreased the abundance of Proteobacteria. At the family level, CBL increased ($p < 0.05$) the abundance of Bacillaceae over those of the CL and BAG treatments. At the genus level, no significant treatment differences were noticed in the abundance of *Clostridium* (**Figure 3**).

In order to further identify microbial taxa that account for the greatest differences between genders, we performed LDA coupled with effect size measurements (LEfSe). In the total cohort, families with higher abundance in the CL controls included Methanobrevibacter and Ruminiclostridium5. The genus *Shuttleworthia* was higher in the CBH treatment. Bacillaceae, Bacillales, *Bacillus* and Lactobacillaceae, Lactobacillales, and *Lactobacillus* were more abundant in the CBL treatment. The SB treatment induced higher abundances of Barnesiellaceae, ClostridialesvadinBB60, Defluviitaleaceae, *Angelakisella*, Ruminococcaceae, and Mitochondria. Moreover, more abundant Bacteroidales, Bacteroidaceae, Bacteridales, and *Bacteroides* were observed in BAG treatment (**Figure 4**).

To investigate the co-occurrence patterns of intestinal microbes in the groups, three networks were constructed based on the OTU level (**Figure 5** and **Table 9**). Co-occurrence network analysis showed that the microbial networks were roughly at the same edges and nodes among the different treatments. The values of average degree (AD) in breeder hens treated with CBH and BAG were higher than those in the controls; the graph density (GD) and the modularity (MD) values were not altered obviously among groups. Additionally, the negative correlations of the network in the CBH and BAG treatments were more than that of the controls while the positive correlations of the network were less than that of the controls.

Growth Performance of Offspring Broilers

Compared to the control breeders, CBH breeders produced offspring with increases ($p < 0.05$) in the final BW and ADG, and with decreased ($p < 0.05$) feed:gain. The feed:gain of offspring from the CBL treatment was also decreased ($p < 0.05$; **Table 10**).

DISCUSSION

Evidence exists for butyric acid exerting beneficial effects on the laying and hatching performances of hens (Jahanian and Golshadi, 2015; Zhan et al., 2019). In accordance with these findings, in the current study, CB and BAG were also found to increase the laying rate as well as daily egg weight, while decreasing the feed:egg ratio of yellow-feathered breeder hens. Additionally, because butyrate promoted the absorption and utilization of minerals including calcium (Soltan, 2008; Casselbrant et al., 2020), egg quality might be improved by dietary addition of butyric acid. Eggshell strength (Zhan et al., 2019) and yolk color (Wang et al., 2020) were increased by dietary CB. In the present study, parameters of egg quality, such as albumen height and eggshell thickness, were also improved by CB, and yolk color and Haugh unit were elevated by BAG. It was noteworthy that the offspring broilers from breeders fed the CBH diet had a much better growth performance than that in other treatments.

The hen oviduct was of special interest to commercial egg producers because disrupted activity or pathological changes

TABLE 5 | Biochemical variables of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
Plasma							
AKP, U/ml	6.33	6.53	6.34	5.67	5.76	0.20	0.741
T-AOC, U/ml	6.94^{a}	5.10^{b}	5.22^{b}	4.94^{b}	5.82^{b}	0.17	0.003
DAO, U/ml	16.41	19.99	19.52	17.64	16.33	0.91	0.707
T-SOD, U/ml	29.36^{a}	22.57^{b}	24.66^{ab}	24.19^{ab}	21.74^{b}	0.79	0.047
Uric acid, mmol/ml	357.84^{a}	303.48^{b}	302.07^{b}	297.24^{b}	344.62^{ab}	7.80	0.036
Jejunum							
T-SOD, U/mg pro	2086.05^{ab}	1982.63^{ab}	2560.30^{a}	1889.25^{b}	2379.53^{ab}	93.97	0.028
T-AOC, U/mg pro	21.09	23.55	20.06	26.93	16.02	1.51	0.430
Liver							
T-SOD, U/mg pro	1198.71^{b}	1190.07^{b}	1333.98^{a}	1184.43^{b}	1101.78^{b}	36.12	0.018
T-AOC, U/mg pro	3.05	3.79	4.58	3.95	4.03	0.16	0.320
Ovary							
T-SOD, U/mg pro	5086.97	5663.54	4794.13	6008.14	5365.03	248.80	0.633
T-AOC, U/mg pro	1.60	1.48	2.46	2.65	1.88	0.42	0.231
MDA, nmol/mg pro	5.34	4.55	2.73	3.31	3.53	0.43	0.509

^{ab}Means within a row lacking a common superscript differ significantly ($p < 0.05$).
AKP, alkaline phosphatase; T-AOC, total antioxidant capacity; DAO, diamine oxidase; T-SOD, total superoxidase; MDA, malondialdehyde; CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.

TABLE 6 | Intestinal pH and morphology of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
pH							
Duodenum	6.32	6.22	6.33	6.38	6.35	0.03	0.601
Jejunum	6.34	6.31	6.21	6.29	6.31	0.02	0.522
Ileum	6.66	6.72	6.61	6.65	6.55	0.03	0.249
Morphology of jejunum							
Villus height, mm	0.91^{b}	1.24^{a}	1.08^{a}	0.92^{b}	1.11^{a}	0.02	0.026
Crypt depth, mm	0.14^{b}	0.25^{a}	0.17^{b}	0.18^{b}	0.21^{a}	0.01	0.043
Villous crypt ratio	5.35^{b}	7.56^{a}	6.67^{a}	5.08^{b}	5.49^{b}	0.18	0.016

^{ab}Means within a row lacking a common superscript differ significantly ($p < 0.05$).
CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.

TABLE 7 | Jejunal immune factors of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
IL-4 pg/mg pro	9.18^{a}	7.53^{ab}	8.91^{a}	6.53^{b}	7.30^{ab}	0.32	0.068
IL-6 μg/mg pro	2.04^{a}	1.39^{b}	1.69^{ab}	1.36^{b}	1.54^{b}	0.16	0.011
IL-1β ng/mg pro	3.86^{a}	3.80^{a}	3.99^{a}	3.31^{b}	3.94^{a}	0.31	0.020
TNF-α ng/mg pro	9.70	9.28	9.53	7.82	9.83	0.86	0.414
IgM μg/mg pro	10.16	10.63	12.07	9.03	10.49	1.27	0.297
IgA μg/mg pro	12.56^{ab}	12.16^{ab}	14.55^{a}	10.87^{b}	13.45^{ab}	1.09	0.043
IgY μg/mg pro	150.22^{a}	134.93^{ab}	159.60^{a}	117.26^{b}	141.16^{ab}	4.68	0.018

^{ab}Means within a row lacking a common superscript differ significantly ($p < 0.05$).
IL-4, interleukin 4; IL-6, interleukin 6; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; IgM, immunoglobulin M; IgA, immunoglobulin A; IgY, immunoglobulin Y; CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.

directly affected egg quality and ultimately decreased economic profitability (Chousalkar and Roberts, 2008). In the present study, the oviduct-related variables, such as weight of the oviduct, and number and weight of LYF, were improved by the treatment of CB, SB, or BAG. Little was known about the effects of butyrate on oviduct-related variables to serve for comparison with the present results. However, according to Ghosh and Cox (1977), SB induced the secretion of follicle-stimulating hormone (FSH), which stimulated the growth and development of follicles. Thus, butyrate might promote the reproductive ability and egg quality through regulating oviduct development by inducing FSH secretion.

TABLE 8 | Jejunal gene expressions of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
NHE2	0.91^{c}	1.53^{abc}	2.35^{a}	1.95^{ab}	1.39^{bc}	0.28	0.018
PEPT1	1.03^{b}	1.79^{a}	0.70^{b}	2.14^{a}	1.47^{ab}	0.33	<0.001
GLUT2	0.84^{b}	1.72^{a}	1.72^{a}	0.48^{b}	0.45^{b}	0.35	<0.001
B0AT	0.92^{b}	2.39^{a}	1.22^{b}	1.73^{ab}	1.14^{b}	0.37	0.018
BAX	1.30^{a}	0.66^{b}	0.58^{b}	0.87^{ab}	0.64^{b}	0.18	0.036
TNF-α	0.99	1.11	1.32	0.97	0.87	0.19	0.053

^{abc}*Means within a row lacking a common superscript differ significantly ($p < 0.05$). NHE2, Na(+)/H(+) exchanger isoform 2; PEPT1, peptide transporter 1; GLUT 2, glucose transporter-2; B0AT, apical nutrient transporter SLC6A19; BAX, Bcl-2 associated X protein; TNF-α, tumor necrosis factor α; CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.*

It is reported that oxidative stress decreased the hatchability and increased the mortality post-hatch (Nadia et al., 2008). Thus, the elevated hatchability and reproductive performance in the present study prompted us to investigate the oxidative status of breeder hens among the different treatments. It was reported that dietary provision of CB promoted the serum, hepatic, and intestinal antioxidant status of hosts (Liao et al., 2015; Duan et al., 2017; Zhan et al., 2019) and SB also improved antioxidant capacity *in vitro* and *in vivo* (Xing et al., 2016; Honma et al., 2020). It was found here, however, that CB, SB, and BAG had minimal effect on the activities of T-SOD and T-AOC in jejunal mucosa, liver, and ovary; SB did reduce jejunal T-SOD activity. Plasmal activity of T-AOC was decreased by CB, SB, and BAG treatments, and activity of T-SOD was reduced by BAG and CBH. As the performance of yellow-feathered breeder hens in the current study was significantly improved, the decreased antioxidant variables might be due to the decreased expression of reactive oxygen species-generating proteins, rather than the impairment of the antioxidant system. The plasmal content of uric acid was also reduced by treatment with CBH, CBL, and SB. Uric acid was the major final product of nitrogen metabolism in birds and was an endogenous antioxidant (Sauer et al., 2007). In the study of Al-Yasiry et al. (2017), dietary supplementation with *Boswellia serrata* resin, a putative anti-inflammatory agent, improved the performance of broiler chickens and decreased uric acid. Decreased plasmal content of uric acid might suggest greater efficiency of use of absorbed amino acids or reduced turnover of endogenous proteins in broiler chickens (Scanes, 2015; Al-Yasiry et al., 2017). Moreover, oxidative stress played a pivotal role in apoptosis and the elevation of antioxidant status could block or delay apoptosis (Kannan and Jain, 2000). It was found here that the expression of the pro-apoptosis gene *BAX* was downregulated by CBH, CBL, and BAG, but not by SB, suggesting that CB and BAG likely attenuated apoptosis.

Relationships were found between reproductive performance and immune function. For example, TLR4 polymorphisms offered a meaningful tool to judge the reproductive potential (Shimizu et al., 2017). The immunostimulatory property of butyrate in chickens was known (Sunkara et al., 2011). Zhou et al. (2014) discovered that SB suppressed the expression of *IL-1β*, *IL-6*, *IFN-γ*, and *IL-10* in chicken macrophages stimulated by *S. typhimurium* LPS. The present study also showed that SB reduced jejunal contents of IL-4, IL-6, and IL-1β. The CBH treatment decreased jejunal content of IL-6, supporting recent work that CB administration reduced IL-6 level in the intestine of burned mice (Zhang et al., 2020). Immunoglobulins are key immune mediators. Levels of IgM and IgA in jejunal mucosa were not altered by CB, SB, or BAG, but IgY level was reduced by SB. Previous studies have suggested that SB increased IgY to improve immune function. For instance, Makled et al. (2019) found that meat-type chickens receiving a SB diet increased IgY, and Gong et al. (2020) showed that β-carotene, curcumin, allicin, and SB supplementation increased serum IgY in breeder hens. The decreased jejunal IgY of SB-treated breeder hens in the present study may indicate weakened humoral immunity. Altogether, the above findings indicated that CBH, SB, and BAG affected jejunal cytokines and SB might have an adverse effect on humoral immunity of breeder hens.

Interactions between nutrition and immunity were diverse and had profound implications on animal productivity. A supply of nutrients at the appropriate times and amounts was important for the immune system (Humphrey and Klasing, 2004). Nutrient transporters mediated substrate-specific uptake across the plasma membrane. Reports showed that SCFA enhanced the structural and functional adaptation of the mammalian intestine by increasing transcript abundance of *GLUT2* (Tappenden et al., 1997; Tappenden and McBurney, 1998; Mangian and Tappenden, 2009), mediating one of the major pathways of intestinal sugar absorption (George and Edith, 2005). In the present study, both CBH and CBL increased *GLUT2* transcripts in the avian jejunal mucosa. Electroneutral Na absorption in the intestine used NHE isoforms NHE2 and NHE3. Butyrate was reported to stimulate electroneutral Na absorption through NHE2 (Subramanya et al., 2007). In the current study, CBL and SB also upregulated the jejunal expression of *NHE2*. Protein was hydrolyzed to small peptides and amino acids in the small intestine, and dipeptides and tripeptides were transported across enterocytes *via* Pept1 or were catabolized to amino acids by aminopeptidase N (Wang et al., 2020). Dalmasso et al. (2008) reported that butyrate enhanced the expression and activity of Pept 1 in mice. Liu et al. (2017) found that SB supplementation induced *Pept1* expression in juvenile grass carp. Consistent with these previous studies, the present results showed that CBH and SB upregulated the expression of *Pept1*. B^0AT is a Na^+-dependent neutral amino acid transporter (Wang et al., 2020). The present authors were unaware of any exploration of the effect of butyrate on the expression of B^0AT, and it was found here that B^0AT transcripts were induced by CBL. Hence, the above findings demonstrated that CB and SB were likely able to increase capacity for the absorption of nutrients.

Gut microbiota affected the parameters of fitness, such as survival, phenotypic plasticity, and reproductive performance of animals (Zilber-Rosenberg and Rosenberg, 2008). Dietary butyrate functioned as a bactericidal agent because it can lower the pH in the crop and gizzard and proximal intestine, to control harmful bacteria, such as *Salmonella* spps., *Escherichia coli*, and *Campylobacter jejuni* (Van Deun et al., 2008). Hens provided with CB had reduced cecal *E. coli* and increased

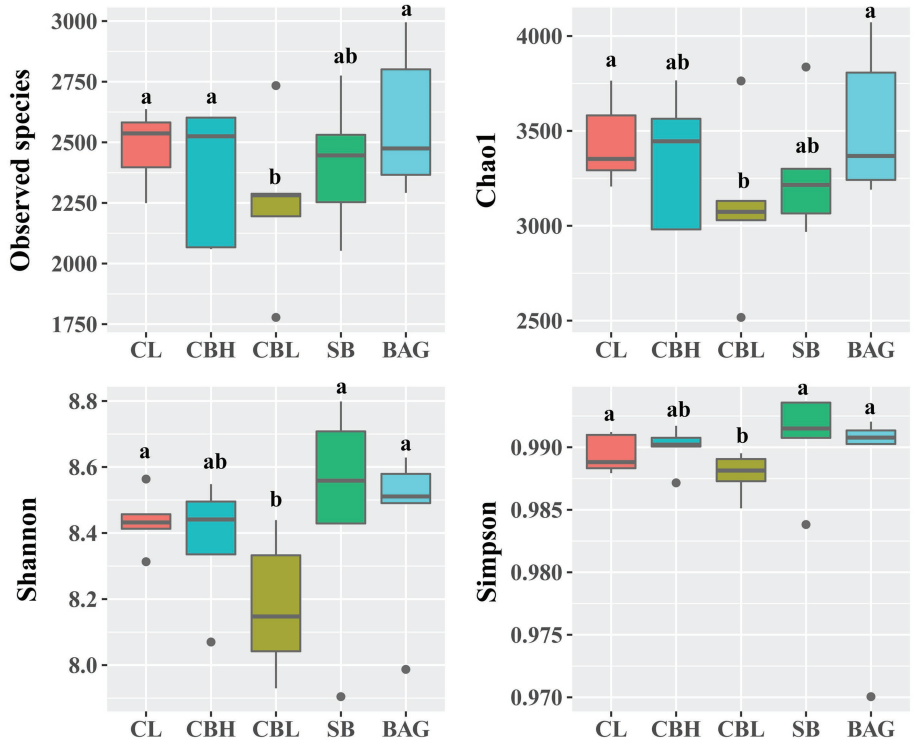

FIGURE 1 | Alpha diversity analysis of intestinal microbiota in different treatments.

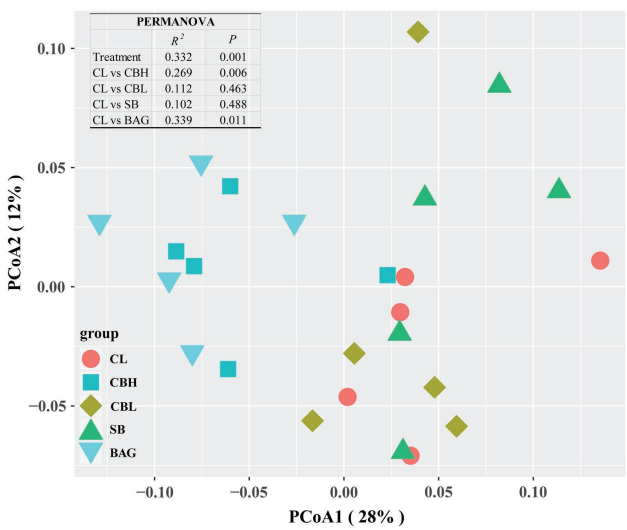

FIGURE 2 | Principal coordinates analysis (PCoA) of microbial communities among treatment groups based on Bray-Curtis distance.

Bifidobacterium (Zhan et al., 2019). Czerwiński et al. (2012) found that dietary SB increased total bacterial numbers and *Lactobacillus* counts in ileal and cecal contents of chickens. Supplementation of laying hens reduced total bacteria and *ileal E. coli* numbers (Jahanian and Golshadi, 2015). In the present study, the cecal microbiota structure of the yellow-feathered breeder hens was examined. Compared with the controls, breeder hens given CBH and CBL had more Firmicutes at the phylum level. As CB belonged to Bacillaceae family and *Clostridium* genus, the relative abundance of Bacillaceae and *Clostridium* was further determined. The present results suggested that CBL increased the presence of Bacillaceae, though abundance of *Clostridium* was unaffected by CB, SB, and BAG treatments. Increased Firmicutes and Bacillaceae in CB treatments might simply stem from the dietary addition of CB. *Firmicutes* bacteria provide a good index of the state of the intestine. For example, Bacillaceae, *Lactobacillus*, and *Lactococcus* could suppress the production of inflammatory cytokines and pathogen-induced disruption of intestine function (Li et al., 2009; Zheng et al., 2017; Dowdell et al., 2020). Dietary CB supplementation of young pigs promoted growth performance and increased the *Bacillus* level (Chen et al., 2018). It is likely, therefore, that elevated abundance of Firmicutes and Bacillaceae induced by CB might promote the growth and health of yellow-feathered breeder hens. Additionally, in agreement with the study of Duan et al. (2018), the abundance of Proteobacteria in the shrimp *Litopenaeus vannamei* was reduced by diets containing 2.5 and 5.0×10^9 CFU/kg, and CBH here, in breeder hens, also decreased the abundance of Proteobacteria. Thus, the altered population structure of the gut microbiota caused by dietary CB might contribute to the improved performance of yellow-feathered breeder hens.

LDA and LEfSe analysis showed increased *Shuttleworthia*, *Lactobacillus*, Barnesiellaceae, and *Bacteroides* in CBH, CBL,

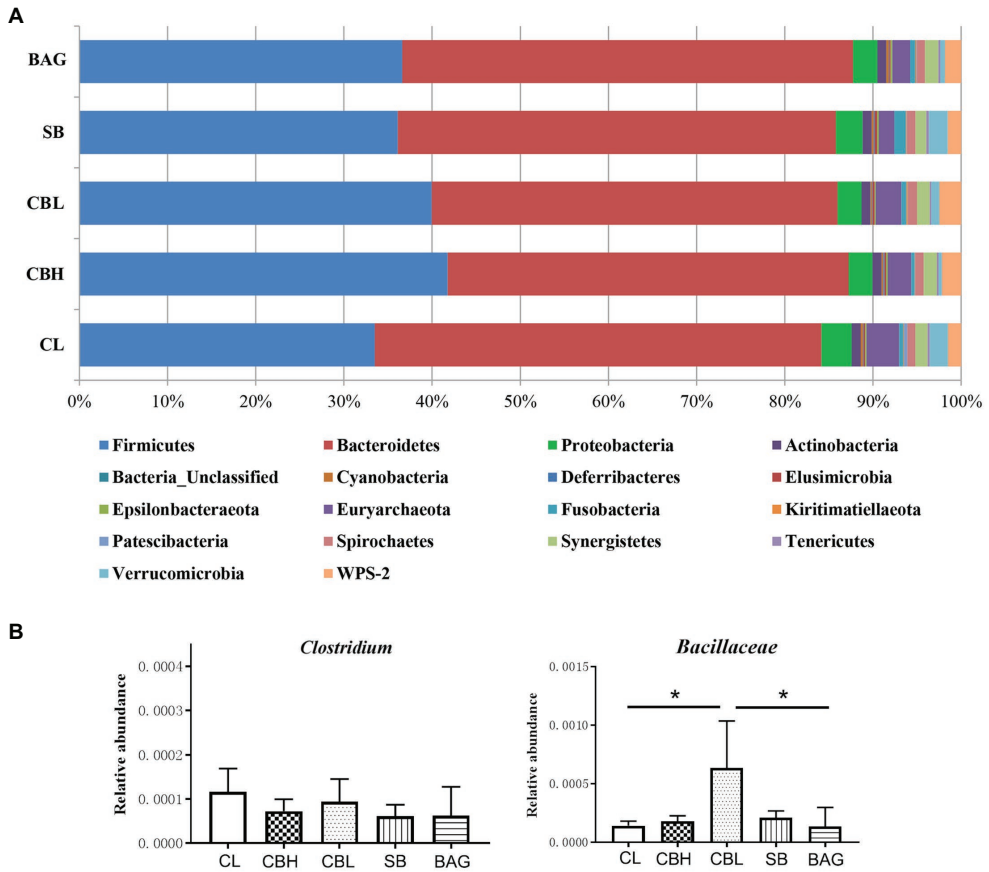

FIGURE 3 | Relative abundance of gut microbiota. **(A)** Relative abundance of gut microbiota at phylum level. **(B)** Relative abundance of *Clostridium* and *Bacillaceae*.

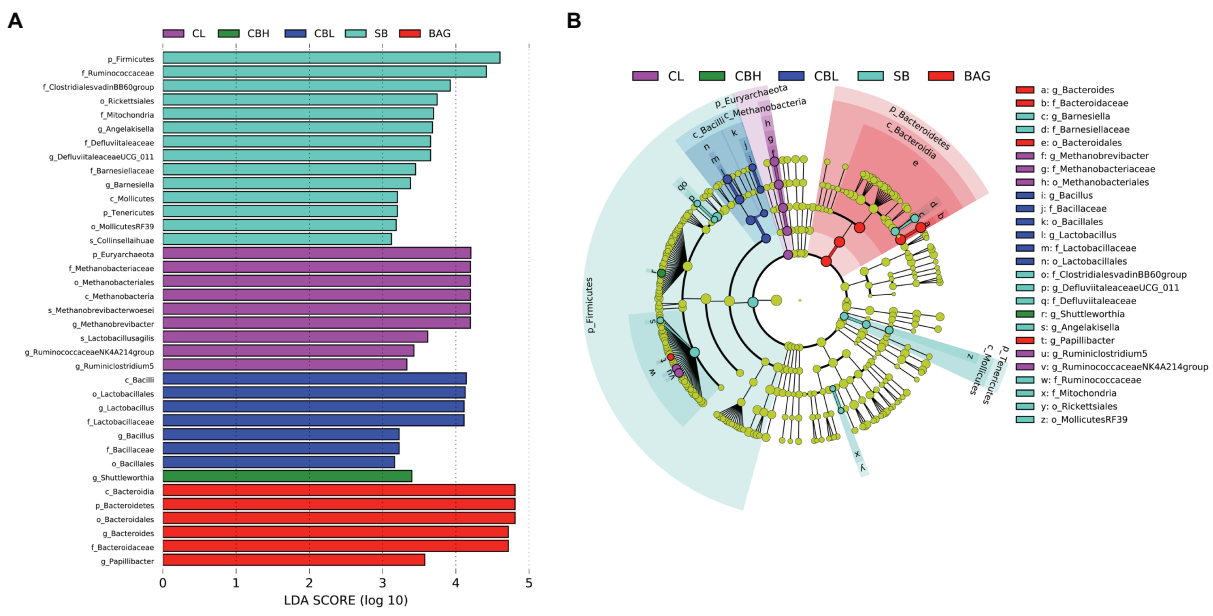

FIGURE 4 | Linear discriminant analysis (LDA) effect size (LEfSe) analysis (LDA > 3.0, $p < 0.05$) of intestinal microbes. The prefixes "p_," "c_," "o_," "f_," "g_," and "s_" represent the annotated level of phylum, class, order, family, genus, and species. **(A)** LDA and **(B)** LEfSe.

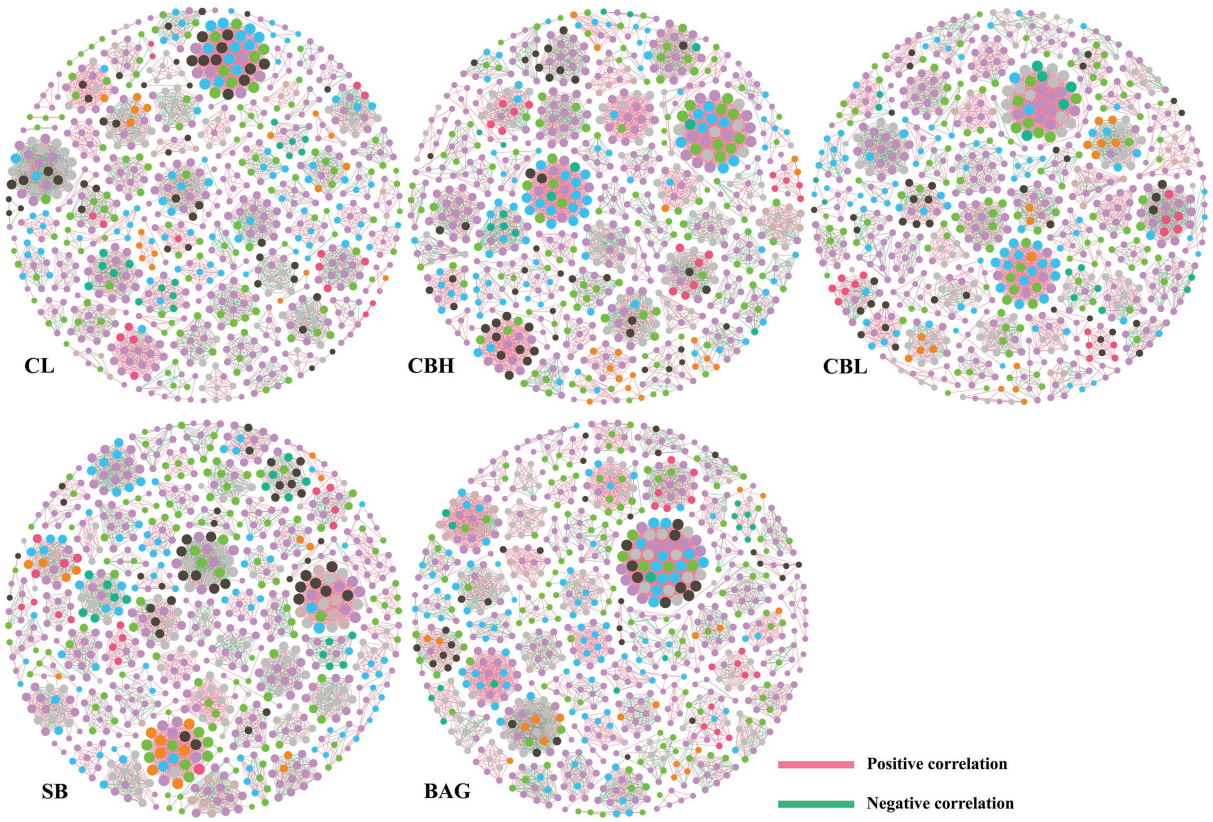

FIGURE 5 | Co-occurrence networks of microbial communities at OTU level. A connection stands for a very strong (Spearman's $R > 0.6$) and significant (FDR-adjusted $p < 0.05$) correlation. The size of each node is proportional to the relative abundance; the thickness of each connection between two nodes (edge) is proportional to the value of Spearman's correlation coefficients. Pink lines represent significant positive correlations, and green lines denote negative correlations.

TABLE 9 | Topological properties of co-occurrence network.

	CL	CBH	CBL	SB	BAG
Nodes	763	756	768	766	786
Edges	4,221	4,552	4,259	4,160	4,919
AD	11.064	12.042	11.091	10.862	12.517
GD	0.015	0.016	0.014	0.014	0.016
MD	0.955	0.954	0.957	0.968	0.93
Positive correlation	69.30%	75.00%	69.00%	69.00%	78.00%
Negative correlation	30.70%	24.00%	30.00%	30.00%	21.00%

AD, average degree; GD, graph density; and MD, modularity.

SB, and BAG treatments, respectively. *Shuttleworthia* had a relationship with carbohydrate and lipid metabolic pathways and thus contributed to weight gain and growth performance in broiler chickens (Lee et al., 2017). *Lactobacillus* generally improved the gastrointestinal tract environment and protected the gut from pathogens (Dicks and Botes, 2010). Moreover, although the roles of Barnesiellaceae were not well known, research suggested that the Behçet's disease and the use of antibiotic led to decreased abundance of Barnesiellaceae (van der Houwen et al., 2020). Additionally, meta-analysis revealed that lower level of *Bacteroides* was associated with intestinal inflammation (Zhou and Zhi, 2016). Finally, co-occurrence patterns of intestinal microbes were employed to investigate the microbial interactions. In the present study, the positive correlation of the microbial networks in the CBH and BAG treatments was more than those in the controls, while the negative correlation of the microbial networks in the CBH and BAG groups was less than those in controls, which could be interpreted as a reduction in competitive relationships within intestinal microbes (Fan et al., 2018).

CONCLUSION

In conclusion, the supplementation with CB and BAG in the diets of breeder hens increased the reproductive performance and improved the egg quality and intestinal morphology. CB treatment also promoted the hatching performance of breeder hens and growth performance of offspring. These effects might result from the regulation of cytokines, nutrient transporters, apoptosis, and profiles of gut microbiota. The beneficial effects described were more obvious with CBH treatment than the CBL treatment, as CBH induced the higher hatchability, eggshell thickness, jejunal crypt depth, and B^0AT expression. Although SB had no beneficial effect on reproductive performance, egg

TABLE 10 | Offspring performance of yellow-feathered breeder hens.

	CL	CBH	CBL	SB	BAG	SEM	Value of *p*
Final body weight, g	700.08[b]	745.80[a]	722.00[ab]	702.50[b]	713.00[ab]	6.09	0.037
Average daily gain, g	44.98[b]	45.65[a]	44.37[ab]	43.85[b]	44.78[ab]	0.22	0.037
Total daily feed intake, g	1259.48	1278.10	1242.30	1227.80	1253.90	15.37	0.155
Feed/gain	1.81[a]	1.71[b]	1.72[b]	1.75[ab]	1.76[ab]	0.01	0.019

[ab]*Mean values within a row with no common superscript differ significantly ($p < 0.05$).*

CL, control; CBL, low-level Clostridium butyricum; CBH, high-level Clostridium butyricum; SB, sodium butyrate; BAG, butyric acid glycerides; and SEM, standard error.

quality, or intestinal morphology, it did impact the oviduct-related variables, nutrient transporters, and immune factors, but the decreased IgY might indicate an adverse influence of SB in breeder hens.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of the Institute of Animal Science, Guangdong Academy of Agriculture Science, Guangzhou, China, with the approval number of GAASISA-2019-007.

AUTHOR CONTRIBUTIONS

YiW and YaW designed the experiments. QF and XL performed animal husbandry. ZG and SJ analyzed 16S rRNA data. YiW did the animal experiments. YaW and SJ wrote the final article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by the China Agriculture Research System of MOF and MARA (CARS-41), National Natural Science Foundation of China (3180131530), Foundation from Guangdong Province (2021A1515012412 and 2021A1515010830), the Science and Technology Program of Guangdong Academy of Agricultural Sciences (202106TD, R2019PY-QF008 and R2018QD-076), China and the Key Realm R&D Program of Guangdong Province (2020B0202090004).

ACKNOWLEDGMENTS

W. Bruce Currie (Emeritus Professor, Cornell University) made suggestions on presentation. The authors thank all workers of the study for their participation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.657542/full#supplementary-material

REFERENCES

Ahsan, U., Cengiz, O., Raza, I., Kuter, E., Chacher, M. F. A., Iqbal, Z., et al. (2016). Sodium butyrate in chicken nutrition: the dynamics of performance, gut microbiota, gut morphology, and immunity. *World Poult. Sci. J.* 72, 265–275. doi: 10.1017/S0043933916000210

Al-Yasiry, A. R. M., Kiczorowska, B., Samolinska, W., Kowalczuk-Vasilevand, E., and Kowalczyk-Pecka, D. (2017). The effect of Boswellia serrata resin diet supplementation on production, hematological, biochemical and immunological parameters in broiler chickens. *Animal* 11, 1890–1898. doi: 10.1017/S1751731117000817

Casselbrant, A., Fndriks, L., and Wallenius, V. (2020). Glycocholic acid and butyrate synergistically increase vitamin D-induced calcium uptake in Caco-2 intestinal epithelial cell monolayers. *Bone Rep.* 13:100294. doi: 10.1016/j.bonr.2020.100294

Chen, L., Li, S., Zhang, J., Li, W., Jiang, X., Zhao, X., et al. (2018). Effects of dietary *Clostridium butyricum* supplementation on growth performance, intestinal development, and immune response of weaned piglets challenged with lipopolysaccharide. *J. Anim. Sci. Biotechnol.* 9:62. doi: 10.1186/s40104-018-0275-8

Chousalkar, K. K., and Roberts, J. R. (2008). Ultrastructural changes in the oviduct of the laying hen during the laying cycle. *Cell Tissue Res.* 332, 349–358. doi: 10.1007/s00441-007-0567-3

Czerwiński, J., Højberg, O., Smulikowska, S., Engberg, R. M., and Mieczkowska, A. (2012). Effects of sodium butyrate and salinomycin upon intestinal microbiota, mucosal morphology and performance of broiler chickens. *Arch. Anim. Nutr.* 66, 102–116. doi: 10.1080/1745039X.2012.663668

Dalmasso, G., Nguyen, H. T. T., Yan, Y., Charrier-Hisamuddin, L., Sitaraman, S. V., and Merlin, D. (2008). Butyrate transcriptionally enhances peptide transporter PepT1 expression and activity. *PLoS One* 3:e2476. doi: 10.1371/journal.pone.0002476

Deepa, K., Purushothaman, M. R., Vasanthakumar, P., and Sivakumar, K. (2017). Serum biochemical parameters and meat quality influenced due to supplementation of sodium butyrate in broiler chicken. *Int. J. Livest. Res.* 7, 108–116. doi: 10.5455/ijlr.20170610051212

Dicks, L. M. T., and Botes, M. (2010). Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Benef. Microbes* 1, 11–29. doi: 10.3920/BM2009.0012

Dowdell, P., Chankhamhaengdecha, S., Panbangred, W., Janvilisri, T., and Aroonnual, A. (2020). Probiotic activity of *Enterococcus faecium* and *Lactococcus lactis* isolated from Thai fermented sausages and their protective effect against *Clostridium difficile*. *Probiotics Antimicrob. Proteins* 12, 641–648. doi: 10.1007/s12602-019-09536-7

Duan, X., Li, F., Mou, S., Feng, J., Liu, P., and Xu, L. (2015). Effects of dietary L-arginine on laying performance and anti-oxidant capacity of broiler breeder

hens, eggs, and offspring during the late laying period. *Poult. Sci.* 94, 2938–2943. doi: 10.3382/ps/pev283

Duan, Y., Wang, Y., Dong, H., Ding, X., Liu, Q., Li, H., et al. (2018). Changes in the intestine microbial, digestive, and immune-related genes of *Litopenaeus vannamei* in response to dietary probiotic *Clostridium butyricum* supplementation. *Front. Microbiol.* 9:2191. doi: 10.3389/fmicb.2018.02191

Duan, Y., Zhang, Y., Dong, H., Wang, Y., and Zhang, J. (2017). Effect of the dietary probiotic *Clostridium butyricum* on growth, intestine antioxidant capacity and resistance to high temperature stress in kuruma shrimp *Marsupenaeus japonicus*. *J. Therm. Biol.* 66, 93–100. doi: 10.1016/j.jtherbio.2017.04.004

Fan, K. K., Weisenhorn, P., Gilbert, J. A., Shi, Y., Bai, Y., and Chu, H. Y. (2018). Soil pH correlates with the co-occurrence and assemblage process of diazotrophic communities in rhizosphere and bulk soils of wheat fields. *Soil Biol. Biochem.* 121, 185–192. doi: 10.1016/j.soilbio.2018.03.017

George, L. K., and Edith, B. L. (2005). Apical GLUT2: a major pathway of intestinal sugar absorption. *Diabetes* 54, 3056–3062. doi: 10.2337/diabetes.54.10.3056

Ghosh, N. K., and Cox, R. P. (1977). Induction of human follicle-stimulating hormone in HeLa cells by sodium butyrate. *Nature* 267, 435–437. doi: 10.1038/267435a0

Gomathi, G., Senthilkumar, S., Natarajan, A., Amurtha, R., and Purushothaman, M. R. (2018). Effect of dietary supplementation of cinnamon oil and sodium butyrate on carcass characteristics and meat quality of broiler chicken. *Vet. World* 11, 959–964. doi: 10.14202/vetworld.2018.959-964

Gong, H., Lang, W., Lan, H., Fan, Y., Wang, T., Chu, Q., et al. (2020). Effects of laying breeder hens dietary β-carotene, curcumin, allicin, and sodium butyrate supplementation on the jejunal microbiota and immune response of their offspring chicks. *Poult. Sci.* 99, 3807–3816. doi: 10.1016/j.psj.2020.03.065

Honma, K., Oshima, K., Takami, S., and Goda, T. (2020). Regulation of hepatic genes related to lipid metabolism and antioxidant enzymes by sodium butyrate supplementation. *Metab. Open* 7:100043. doi: 10.1016/j.metop.2020.100043

Humphrey, B. D., and Klasing, K. C. (2004). Modulation of nutrient metabolism and homeostasis by the immune system. *World Poult. Sci. J.* 60, 90–100. doi: 10.1079/WPS20037

Jahanian, R., and Golshadi, M. (2015). Effect of dietary supplementation of butyric acid glycerides on performance, immunological responses, ileal microflora, and nutrient digestibility in laying hens fed different basal diets. *Livest. Sci.* 178, 228–236. doi: 10.1016/j.livsci.2015.05.038

Jiang, Y., Zhang, W., Gao, F., and Zhou, G. (2015). Effect of sodium butyrate on intestinal inflammatory response to lipopolysaccharide in broiler chickens. *Can. J. Anim. Sci.* 95, 389–395. doi: 10.4141/cjas-2014-183

Jiao, S., Liu, Z. S., Lin, Y. B., Yang, J., Chen, W. M., and Wei, G. H. (2016). Bacterial communities in oil contaminated soils: biogeography and co-occurrence patterns. *Soil Biol. Biochem.* 9, 64–73. doi: 10.1016/j.soilbio.2016.04.005

Kannan, K., and Jain, S. K. (2000). Oxidative stress and apoptosis. *Pathophysiology* 7, 153–163. doi: 10.1016/S0928-4680(00)00053-5

Lee, K. C., Kil, D. Y., and Sul, W. J. (2017). Cecal microbiome divergence of broiler chickens by sex and body weight. *J. Microbiol.* 55, 939–945. doi: 10.1007/s12275-017-7202-0

Li, J., Tan, B., and Mai, K. (2009). Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). *Aquaculture* 291, 35–40. doi: 10.1016/j.aquaculture.2009.03.005

Liao, X., Wu, R., Ma, G., Zhao, L., Zheng, Z., and Zhang, R. (2015). Effects of *Clostridium butyricum* on antioxidant properties, meat quality and fatty acid composition of broiler birds. *Lipids Health Dis.* 14:36. doi: 10.1186/s12944-015-0035-0

Liu, M., Guo, W., Wu, F., Qu, Q., Tan, Q., and Gong, W. (2017). Dietary supplementation of sodium butyrate may benefit growth performance and intestinal function in juvenile grass carp (*Ctenopharyngodon idellus*). *Aquac. Res.* 48, 4102–4111. doi: 10.1111/are.13230

Makled, M. N., Abouelezz, K. F. M., Gad-Elkareem, A. E. G., and Sayed, A. M. (2019). Comparative influence of dietary probiotic, yoghurt, and sodium butyrate on growth performance, intestinal microbiota, blood hematology, and immune response of meat-type chickens. *Trop. Anim. Health Prod.* 51, 2333–2342. doi: 10.1007/s11250-019-01945-8

Mangian, H. F., and Tappenden, K. A. (2009). Butyrate increases GLUT2 mRNA abundance by initiating transcription in Caco2-BBe cells. *J. Parenter. Enteral. Nutr.* 33, 607–617. doi: 10.1177/0148607109336599

Meimandipour, A., Shuhaimi, M., Soleimani, A. F., Azhar, K. S., Hair-Bejo, M., Kabeir, B. M., et al. (2010). Selected microbial groups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of *Lactobacillus*. *Poult. Sci.* 89, 470–476. doi: 10.3382/ps.2009-00495

Nadia, R., Hassan, R. A., Qota, E. M., and Fayek, H. M. (2008). Effect of natural antioxidant on oxidative stability of eggs and productive and reproductive performance of laying hens. *Int. J. Poult. Sci.* 7, 134–150. doi: 10.3923/ijps.2008.134.150

Satoh, T., Takeuchi, O., Vandenbon, A., Yasuda, K., Kumagai, Y., Miyake, T., et al. (2010). The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat. Immunol.* 11, 936–944. doi: 10.1038/ni.1920

Sauer, J., Richter, K. K., and Pool-Zobel, B. L. (2007). Physiological concentrations of butyrate favorably modulate genes of oxidative and metabolic stress in primary human colon cells. *J. Nutr. Biochem.* 18, 736–745. doi: 10.1016/j.jnutbio.2006.12.012

Scanes, C. G. ed. (2015). *Sturkie's Avian Physiology*, *6th Edn*. London, UK: Academic Press.

Shimizu, T., Kawasaki, Y., Aoki, Y., Magata, F., Kawashima, C., and Miyamoto, A. (2017). Effect of single nucleotide polymorphisms of toll-like receptor 4 (TLR 4) on reproductive performance and immune function in dairy cows. *Biochem. Genet.* 55, 212–222. doi: 10.1007/s10528-017-9790-0

Sikandar, A., Zaneb, H., Younus, M., Masood, S., Aslam, A., Khattak, F., et al. (2017). Effect of sodium butyrate on performance, immune status, microarchitecture of small intestinal mucosa and lymphoid organs in broiler chickens. *Asian Australas. J. Anim. Sci.* 30, 690–699. doi: 10.5713/ajas.16.0824

Soltan, M. A. (2008). Effect of dietary organic acid supplementation on egg production, egg quality and some blood serum parameters in laying hens. *Int. J. Poult. Sci.* 7, 613–621. doi: 10.3923/ijps.2008.613.621

Subramanya, S. B., Rajendran, V. M., Srinivasan, P., Nanda Kumar, N. S., and Binder, H. J. (2007). Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 293, G857–G863. doi: 10.1152/ajpgi.00462.2006

Sunkara, L. T., Achanta, M., Schreiber, N. B., Bomminein, Y. R., Dai, G., Jiang, W., et al. (2011). Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. *PLoS One* 6:e27225. doi: 10.1371/journal.pone.0027225

Taherpour, K., Moravej, H., Shivazad, M., Adibmoradi, M., and Yakhchali, B. (2009). Effects of dietary probiotic, prebiotic and butyric acid glycerides on performance and serum composition in broiler chickens. *Afr. J. Biotechnol.* 8, 2329–2334.

Takahashi, M., Mccartney, E., Knox, A., Francesch, M., Oka, K., Wada, K., et al. (2018). Effects of the butyric acid-producing strain *Clostridium butyricum* MIYAIRI 588 on broiler and piglet zootechnical performance and prevention of necrotic enteritis. *Anim. Sci. J.* 89, 895–905. doi: 10.1111/asj.13006

Tappenden, K. A., and McBurney, M. I. (1998). Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. *Dig. Dis. Sci.* 43, 1526–1536. doi: 10.1023/A:1018819032620

Tappenden, K. A., Thomson, A. B., Wild, G. E., and McBurney, M. I. (1997). Short chain fatty acid-supplemented total parenteral nutrition enhances functional adaptation to intestinal resection in rats. *Gastroenterology* 112, 792–802. doi: 10.1053/gast.1997.v112.pm9041241

van der Houwen, T. B., van Laar, J. A. M., Kappen, J. H., van Hagen, P. M., de Zoete, M. R., van Muijlwijk, G. H., et al. (2020). Behçet's disease under microbiotic surveillance? A combined analysis of two cohorts of Behçet's disease patients. *Front. Immunol.* 11:1192. doi: 10.3389/fimmu.2020.01192

Van Deun, K., Haesebrouck, F., Van Immerseel, F., Ducatelle, R., and Pasmans, F. (2008). Short chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers. *Avian Pathol.* 37, 379–383. doi: 10.1080/03079450802216603

Wang, W., Wang, J., Zhang, H., Wu, S., and Qi, G. (2020). Effects of *Clostridium butyricum* on production performance and intestinal absorption function of laying hens in the late phase of production. *Anim. Feed Sci. Technol.* 264:114476. doi: 10.1016/j.anifeedsci.2020.114476

Xia, W. G., Chen, W., Abouelezz, K., Ruan, D., and Mohammed, K. (2020). The effects of dietary Se on productive and reproductive performance, tibial quality, and antioxidant capacity in laying duck breeders. *Poult. Sci.* 99, 3971–3978. doi: 10.1016/j.psj.2020.04.006

Xiao, S., Jiang, S., Qian, D., and Duan, J. (2020). Modulation of microbially derived short-chain fatty acids on intestinal homeostasis, metabolism, and neuropsychiatric disorder. *Appl. Microbiol. Biotechnol.* 104, 589–601. doi: 10.1007/s00253-019-10312-4

Xing, X., Jiang, Z., Tang, X., Wang, P., Li, Y., Sun, Y., et al. (2016). Sodium butyrate protects against oxidative stress in HepG2 cells through modulating Nrf2 pathway and mitochondrial function. *J. Physiol. Biochem.* 73, 405–414. doi: 10.1007/s13105-017-0568-y

Yang, C., Cao, G., Ferket, P. R., Liu, T., Zhou, L., Zhang, L., et al. (2012). Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. *Poult. Sci.* 91, 2121–2129. doi: 10.3382/ps.2011-02131

Zhan, H., Dong, X., Li, L., Zheng, Y., Gong, Y., and Zou, X. (2019). Effects of dietary supplementation with *Clostridium butyricum* on laying performance, egg quality, serum parameters, and cecal microflora of laying hens in the late phase of production. *Poult. Sci.* 98, 896–903. doi: 10.3382/ps/pey436

Zhang, B., Yang, X., Guo, Y., and Long, F. (2011). Effects of dietary lipids and *Clostridium butyricum* on the performance and the digestive tract of broiler chickens. *Arch. Anim. Nutr.* 65, 329–339. doi: 10.1080/1745039X.2011.568274

Zhang, L., Zhang, L., Zhan, X., Zeng, X., Zhou, L., Gao, G., et al. (2016). Effects of dietary supplementation of probiotic, *Clostridium butyricum*, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with *Escherichia coli* K88. *J. Anim. Sci. Biotechnol.* 7:3. doi: 10.1186/s40104-016-0061-4

Zhang, D., Zhou, C., Fang, Z., Zhang, H., Yang, J., Tao, K., et al. (2020). Remodeling gut microbiota by *Clostridium butyricum* (*C. butyricum*) attenuates intestinal injury in burned mice. *Burns* 46, 1373–1380. doi: 10.1016/j.burns.2020.01.007

Zheng, X. T., Duan, Y. F., Dong, H. B., and Zhang, J. S. (2017). Effects of dietary *Lactobacillus plantarum* in different treatments on growth performance and immune gene expression of white shrimp *Litopenaeus vannamei* under normal condition and stress of acute low salinity. *Fish Shellfish Immunol.* 62, 195–201. doi: 10.1016/j.fsi.2017.01.015

Zhou, Z. Y., Packialakshmi, B., Makkar, S. K., Dridi, S., and Rath, N. C. (2014). Effect of butyrate on immune response of a chicken macrophage cell line. *Vet. Immunol. Immunopathol.* 162, 24–32. doi: 10.1016/j.vetimm.2014.09.002

Zhou, Y., and Zhi, F. (2016). Lower level of Bacteroides in the gut microbiota is associated with inflammatory bowel disease: a meta-analysis. *Biomed Res. Int.* 2016:5828959. doi: 10.1155/2016/5828959

Zilber-Rosenberg, I., and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev.* 32, 723–735. doi: 10.1111/j.1574-6976.2008.00123.x

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