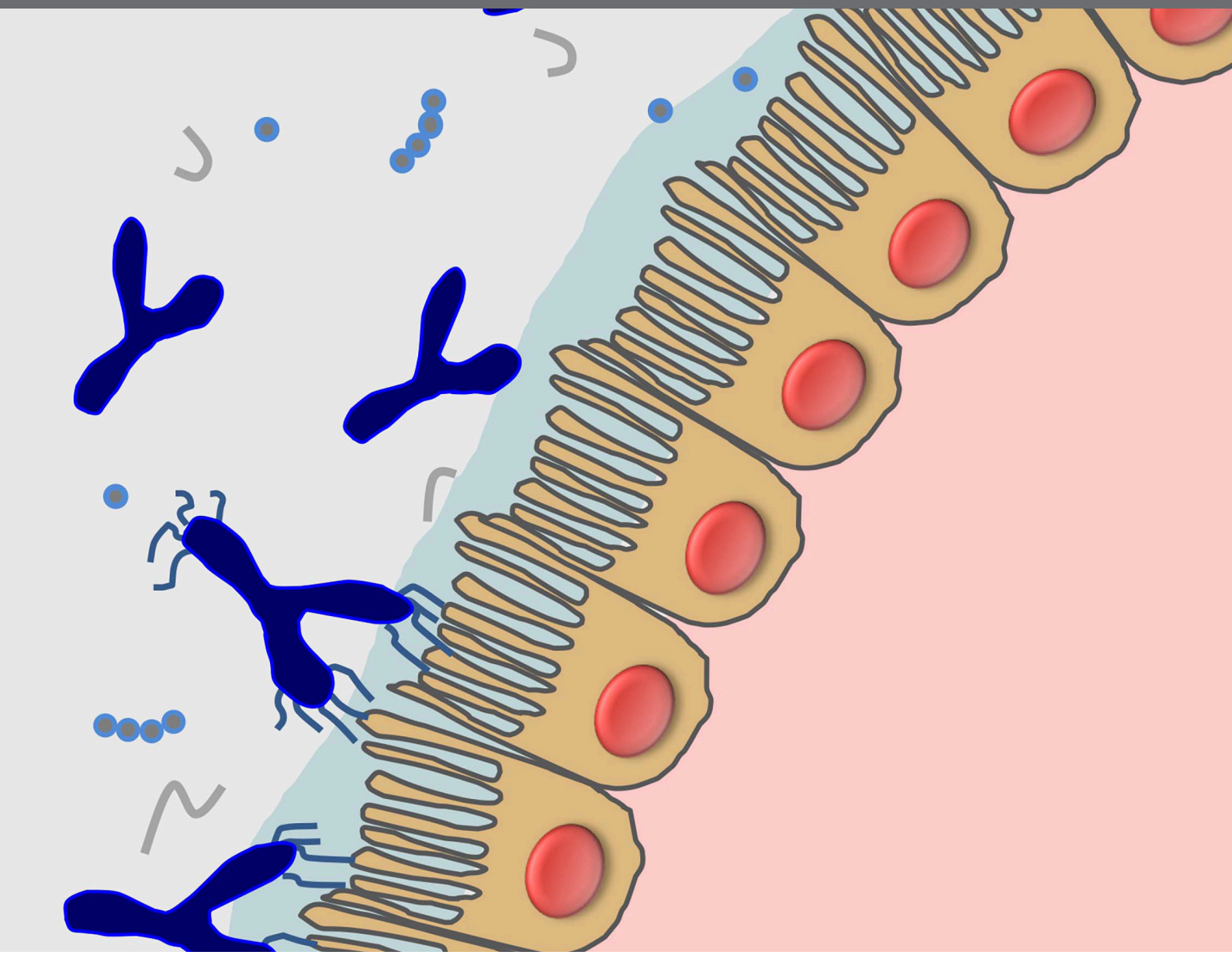
MICROORGANISMS FOR FUNCTIONAL FOOD, 2nd Edition

EDITED BY: Fabio Minervini and Maria De Angelis

PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-147-6
DOI 10.3389/978-2-88963-147-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

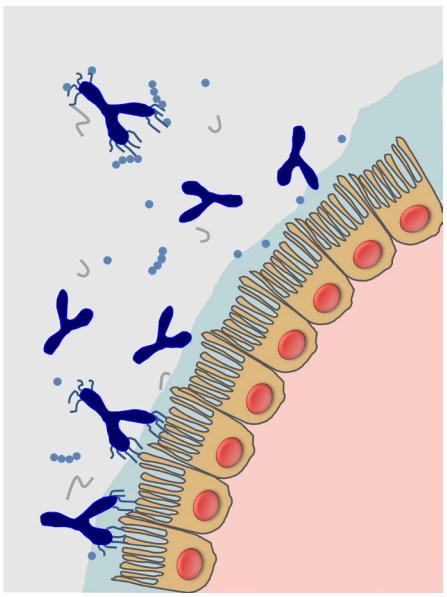
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MICROORGANISMS FOR FUNCTIONAL FOOD, 2nd Edition

Topic Editors:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy
Maria De Angelis, Università degli Studi di Bari Aldo Moro, Italy



Schematic representation of the main effects produced by Bifidobacterium bifidum in the human gut.
Figure taken from: Turroni F, Duranti S, Bottacini F, Guglielmetti S, Van Sinderen D and Ventura M (2014) Bifidobacterium bifidum as an example of a specialized human gut commensal. Front. Microbiol. 5:437. doi: 10.3389/fmicb.2014.00437

Nowadays, most of Western consumers are aware that a targeted diet could be an important tool for fighting ageing and diseases such as hypertension, diabetes, and obesity. Microorganisms may be exploited for setting up novel foods that, beside their nutritional value, may positively impact on consumers' health. Some microorganisms may benefit host when ingested as viable cells carried by food and beverages. This falls in the intriguing field of probiotics, recently brought forward by the numerous probiotic claims rejected by the European Food Safety Authority. This research topic includes research articles and reviews/perspectives that (i) contribute to understand the mechanism underlying the health effects of probiotic microorganisms; (ii) show

integrated approaches for selecting new probiotics; (iii) report about non-dairy food items as novel carriers of probiotics; and (iv) deal with biologically active compounds from microorganisms.

Publisher's note: In this 2nd edition, the following article has been updated: Tyagi AK and Prasad S (2015) Commentary: Probiotic and technological properties of Lactobacillus spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. Front. Microbiol. 6:433. doi: 10.3389/fmicb.2015.00433

Citation: Minervini, F., De Angelis, M., eds. (2019). Microorganisms for Functional Food, 2nd Edition. Lausanne: Frontiers Media. doi: 10.3389/978-2-88963-147-6

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 08 March 2016
doi: 10.3389/fmicb.2016.00298

Editorial: Microorganisms for Functional Food

Fabio Minervini and Maria De Angelis*

Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Bari, Italy

Keywords: probiotic, fermentation, Lactobacilli, gut microbiome, health claim

The Editorial on the Research Topic

Microorganisms for Functional Food

Notwithstanding the European Food Safety Authority (EFSA) did not approve any health claims for probiotic foods [including terms such as "probiotic," "active bacteria"; European Commission (EC), 2007], most of the research studies in the field of microorganisms used for obtaining functional food deal with (putatively) probiotic bacteria. Probiotics may positively affect human/animal health in several ways, such as by inhibiting gut pathogenic microorganisms (Delgado et al.), modulating immune response, lowering concentration of cholesterol in blood (Damodharan et al.), and exerting antioxidant activity. The mechanisms underlying the health effects of probiotic bacteria raise great interest among researchers. Modulation of the host gut microbiome represents an intriguing mechanism and is the subject of two research articles (Yang et al.; Senan et al.) published in this Research Topic. In detail, reutericyclin, a broad spectrum antimicrobial compound produced by *Lactobacillus reuteri* during feed fermentation, increased the abundance of *Dialister* and *Mitsuokella*, two *Firmicutes* genera that are gut commensals in weanling pigs (Yang et al.). Senan et al. reported that differences in gut microbiome composition (especially for *Lactobacillus*, *Clostridium*, *Eubacterium*, *Blautia*, *Shigella*, *Escherichia*, *Burkholderia*, and *Campylobacter*) affected the response of geriatric individuals to Lassi, a fermented milk containing a cholesterol-lowering strain of *Lactobacillus helveticus*.

Evaluation assays of probiotic potential are traditionally classified in two groups: "*in vitro*" and "*in vivo*." The use of omics approaches is going to flank the traditional assays, thus probably speeding up research progress in the field of probiotics in the near future. For instance, sequencing of genomes will allow to rapidly detect and discard candidate probiotic microorganisms possessing genes coding for antibiotic resistance or virulence factors (Papadimitriou et al.). In this regard, an excellent example is provided in this Research Topic by Balzaretti et al. which performed comparative genome analysis of four strains of *Lactobacillus paracasei* in order to select the two best probiotic strains for oral usage. Anyway, *in vivo* approaches will keep on being unreplaceable, given the variability of host response to probiotics as affected by genotype, age, diet, variations in human environmental exposure, and composition of gut microbiome (Turnbaugh et al., 2009; Senan et al.).

Besides the understanding of the mechanisms underlying probiotic activities and studies dealing with interactions between probiotic microorganisms and gut microbiome, other future perspectives about microorganisms for functional food are: (i) novel putative probiotic bacteria, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* (Varankovich et al.);

OPEN ACCESS

Edited by:
Giovanna Suzzi,
Università degli Studi di Teramo, Italy

Reviewed by:
Maria Schirone,
University of Teramo, Italy

****Correspondence:***
Fabio Minervini
fabio.minervini@uniba.it

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

Received: *09 February 2016*
Accepted: *23 February 2016*
Published: *08 March 2016*

Citation:
Minervini F and De Angelis M (2016) Editorial: Microorganisms for Functional Food. Front. Microbiol. 7:298. doi: 10.3389/fmicb.2016.00298

(ii) non-dairy food items (e.g., table olives) as carriers of probiotic bacteria (Rodríguez-Gómez et al.; Arroyo-López et al.); (iii) health effects of microbial metabolites (Yu et al.; Garrote et al.); and (iv) probiotic interventions on livestock indirectly benefiting human health (Yang et al.).

AUTHOR CONTRIBUTIONS

FM resumed some of the contributions published in this research topic and wrote the Editorial. MD settled the structure of and reviewed the Editorial.

REFERENCES

European Commission (EC) (2007). *Guidance on the Implementation of Regulation n 1924/2006 on Nutrition and Health Claims Made on Foods Conclusions of the Standing Committee on the Food Chain and Animal Health.* Brussels. Available online at: http://ec.europa.eu/food/food/labellingnutrition/claims/guidance_claim_14-12-07.pdf

Turnbaugh, P. J., Ridaura, K., Faith, J. J., Rey, F. E., Knight, R., and Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomics analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* 1:6ra14. doi:10.1126/scitranslmed.3000322

Conflict of Interest Statement: 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 ARTICLE
published: 14 January 2015
doi: 10.3389/fmicb.2014.00766

Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis

Susana Delgado *, Analy M. O. Leite†, Patricia Ruas-Madiedo and Baltasar Mayo

Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (IPLA), Consejo Superior de Investigaciones Científicas (CSIC), Villaviciosa, Spain

Edited by:
Maria De Angelis, University of Bari Aldo Moro, Italy

Reviewed by:
Luca Cocolin, Univeristy of Turin, Italy
Amit Kumar Tyagi, The University of Texas MD Anderson Cancer Center, USA

****Correspondence:***
Susana Delgado, Instituto de Productos Lácteos de Asturias (IPLA), Paseo del Río Linares s/n, 33300 Villaviciosa, Spain
e-mail: sdelgado@ipla.csic.es

†Present address:
Analy M. O. Leite, Departamento de Microbiologia Geral, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

This work characterizes a set of lactobacilli strains isolated from the stomach of healthy humans that might serve as probiotic cultures. Ten different strains were recognized by rep-PCR and PFGE fingerprinting among 19 isolates from gastric biopsies and stomach juice samples. These strains belonged to five species, *Lactobacillus gasseri* (3), *Lactobacillus reuteri* (2), *Lactobacillus vaginalis* (2), *Lactobacillus fermentum* (2) and *Lactobacillus casei* (1). All ten strains were subjected to a series of *in vitro* tests to assess their functional and technological properties, including acid resistance, bile tolerance, adhesion to epithelial gastric cells, production of antimicrobial compounds, inhibition of *Helicobacter pylori*, antioxidative activity, antibiotic resistance, carbohydrate fermentation, glycosidic activities, and ability to grow in milk. As expected, given their origin, all strains showed good resistance to low pH (3.0), with small reductions in counts after 90 min exposition to this pH. Species- and strain-specific differences were detected in terms of the production of antimicrobials, antagonistic effects toward *H. pylori*, antioxidative activity and adhesion to gastric epithelial cells. None of the strains showed atypical resistance to a series of 16 antibiotics of clinical and veterinary importance. Two *L. reuteri* strains were deemed as the most appropriate candidates to be used as potential probiotics against microbial gastric disorders; these showed good survival under gastrointestinal conditions reproduced *in vitro*, along with strong anti-*Helicobacter* and antioxidative activities. The two *L. reuteri* strains further displayed appropriated technological traits for their inclusion as adjunct functional cultures in fermented dairy products.

Keywords: stomach microbiota, gastric lactobacilli, specific probiotics, functional characterization, antioxidative activity, anti-*Helicobacter* activity, fermentation capability

INTRODUCTION

Over recent decades, culture-independent techniques have revealed the stomach to be home to a well-adapted, niche-specific microbial community (Bik et al., 2006; Andersson et al., 2008; Delgado et al., 2013). Gastric microbial communities are of potential probiotic use in the treatment of several diseases, but only a few studies have attempted to cultivate their members (Adamson et al., 1999; Li et al., 2009; Delgado et al., 2013). The isolation and characterization of stomach originated strains could provide novel probiotic candidates with enhanced capacities to counteract gastric pathogens such as *Helicobacter pylori* (Cui et al., 2010). This Gram-negative, microaerophilic microorganism infects over 50% of the population worldwide (Bruce and Maaroos, 2008). Indeed, *H. pylori* is the most important aetiological agent in chronic gastritis, peptic ulcers and gastric cancer (Peek and Blaser, 2002). The eradication (efficiency 80–90%) of *H. pylori* is possible using a combination of antibiotics and antacids. However, side-effects are common. As an alternative or complementary therapy, or indeed as a preventive strategy, use might be made of probiotics for the management of this infection (Malfertheiner et al., 2007). To date, studies have focused on conventional probiotic cultures from different origins. Among these, *Lactobacillus reuteri* having anti *H. pylori* action is one promising approach (Francavilla et al., 2008).

The aim of the present work was to examine *in vitro* the functional and technological characteristics of *Lactobacillus* isolates recovered from the stomach mucosa and gastric juice of healthy individuals in a previous study (Delgado et al., 2013) for their evaluation as probiotics in functional products against *H. pylori* infection. Gastric candidate probiotic strains with potential application in the prevention and treatment of gastric disorders and dysbiosis were then selected.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

Nineteen *Lactobacillus* isolates belonging to five different species were cultured from gastric biopsies and stomach juice samples provided by healthy subjects (see Delgado et al., 2013). Unless otherwise indicated, all isolates were cultured in de Man, Rogosa and Sharpe (MRS; Merck, Darmstadt, Germany) medium

supplemented with 0.25% cysteine (Merck) (MRSC). Incubations proceeded at 37°C for 24 h in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK) containing an anoxic atmosphere (10% H_2, 10% CO_2, 80% N_2).

TYPING OF LACTOBACILLI

The isolates were genotyped by repetitive extragenic palindromic (rep)-PCR and by pulsed-field gel electrophoresis (PFGE) fingerprinting. For rep-PCR, total DNA from the isolates was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's recommendations. Amplifications were performed using the primer BoxA2-R (5′-ACGTGGTTTGAAGAGATTTTCG-3′), as reported by Koeuth et al. (1995). For PFGE, genomic DNA was isolated and digested at 30°C for 4 h in agarose plugs containing 20 U of the restriction endonuclease SmaI (Boehringer Mannheim, Mannheim, Germany). Electrophoresis was performed in 1% FastLane agarose gels (FMC, Philadelphia, PA, USA) in 0.5X TBE (Tris-borate-EDTA) for 20 h at 14°C and at 6 V/cm, using a CHEF-DRII apparatus (Bio-Rad, Richmond, CA, USA). Pulse times ranged from 0.1 to 2 s for 4 h, from 2 to 5 s for 12 h, and from 5 to 10 s for 4 h. Low-range and bacteriophage lambda ladder PFGE markers (both from New England BioLabs, Ipswich, MA, USA) were included in the gels.

GASTROINTESTINAL TOLERANCE

Viability at low pH

The ability of the isolates to survive under acidic conditions was assessed by exposing the cells to an acidic solution. HCl was added to cell suspensions ($\approx 10^8$ cells/ml) in a sterile saline solution (0.9%) to achieve pHs ranging from 2.0 to 6.5 (pH 7.0 was used as a control). The cells were then incubated at 37°C for 90 min. After incubation the pH of the medium was recorded again and the viability of the strains assessed by plate counting on MRSC.

Resistance to bile

The tolerance of the strains to bovine bile (Ox-gall, Sigma-Aldrich) was assayed following the procedure of Charteris et al. (1998). Briefly, individual colonies from MRSC plates were suspended in 2 ml of sterile saline solution until a density corresponding to McFarland standard 1 was obtained. Aliquots of this suspension (10 μl) were spotted onto bile-containing agar plates. The concentration of bile assayed ranged from 0.25 to 4% (w/v).

PRODUCTION OF ANTIMICROBIAL COMPOUNDS

Anti-Helicobacter pylori activity

H. pylori DSM 10242 was routinely grown in solid or liquid brain heart infusion medium (BHI; Oxoid, Basingstoke, Hampshire, UK), supplemented with either 5% (w/v) horse blood (Oxoid) or 10% (w/v) fetal bovine serum (Oxoid), respectively. Inoculated cultures were then incubated at 37°C for 3–7 days under the microaerophilic conditions generated by the CampyGen system (Oxoid). Antagonistic activity of lactobacilli against *H. pylori* was assessed by a broth inhibition assay after growing of the test strains in Elliker broth (Scharlau, Barcelona, Spain), since MRS medium inhibits the growth of this pathogen (Ryan et al., 2008b). Inhibition assays were performed in 96-well, "U"-bottom polystyrene microtitre plates. Supplemented BHI was inoculated at 1.5 (v/v) with a concentrated *H. pylori* culture ($OD_{600\ nm}$ 1.0), and the cell suspension was dispensed into the wells of a microtitre plate. Then, 45 μl of the supernatant of each individual lactobacilli strain were added to the wells. As a negative control, aliquots of non-inoculated Elliker medium were also tested. The multi-well plates were then incubated for 3 days under the same conditions as above, and the $OD_{600\ nm}$ recorded using a microplate spectrophotometer (Benchmark; Bio-Rad Laboratories, Hercules, CA, USA). Assays were also performed with pH-neutralized supernatants (pH 6.6). All experiments were repeated twice using independent cultures; all supernatants were further assayed in triplicate.

Bacteriocins

Bacteriocin production was consecutively examined by an agar spot test and a well-diffusion assay as previously described (Delgado et al., 2007), using two well-recognized bacteriocin-susceptible strains (*Lactobacillus sakei* CECT 906 and *Lactococcus lactis* subsp. *lactis* IL1403) as indicators.

Hydrogen peroxide (H_2O_2)

H_2O_2 production was tested following the procedure described by Song et al. (1999). MRSC agar plates supplemented with 0.25 mg/ml of tetramethylbenzidine (TMB, Sigma-Aldrich) and 0.01 mg/ml of horseradish peroxidase (HRP, Sigma-Aldrich) were inoculated with the strains and incubated at 37°C under both aerobic and anaerobic conditions. The presence of any blue pigment in the H_2O_2–producing colonies was recorded after 2 days. *Lactobacillus jensenii* CECT 4306 (Martín and Suárez, 2010) was used as positive control.

Reuterin

The presence of the gene coding for the large subunit of glycerol dehydratase, which is essential in the production of reuterin (3-hydroxypropionaldehyde) (Claisse and Lonvaud-Funel, 2001), was checked for in the *Lactobacillus reuteri* strains via PCR. *L. reuteri* CECT 925, a reuterin-producing strain (Martín et al., 2005), was used as positive control.

ADHESION TO AN EPITHELIAL GASTRIC CELL LINE

The adhesion of the strains to the gastric mucosa was assessed *in vitro* using the gastric cell line AGS (ECACC number 89090402, Sigma-Aldrich), which is derived from a human gastric adenocarcinoma. The latter cells were cultured as per routine in Ham's F12 medium (LabClinics, Barcelona, Spain) supplemented with 2 mM of L-glutamine (PAA Laboratories GmbH, Paschina, Austria), 10% fetal bovine serum, plus 50 μg/ml of penicillin, 50 μg/ml of streptomycin, 50 μg/ml of gentamicin, and 1.25 μg/ml amphotericin B (Sigma-Aldrich). Monolayers of the cells were prepared in 24-well tissue culture plates (Becton Dickinson, New Jersey, USA) at a concentration of 10^5 cells/ml. After reaching confluence and differentiating (3 + 1 days), the strains were added at a ratio 1:1. For this, bacterial cultures were harvested by centrifugation, washed with Dulbecco's phosphate-buffered saline solution (Sigma-Aldrich), and suspended in Ham's F12 medium without

antibiotics. After 1 h of co-incubation at 37°C in a 5% CO_2 atmosphere, the monolayers were washed 3 times with a phosphate-buffered saline solution to remove any non-attached bacteria. The monolayers were then disrupted with a 0.25% trypsin-EDTA solution (Sigma-Aldrich), and the attached bacteria enumerated by standard dilution and plate counting on MRSC. Experiments were carried out in triplicate and each strain tested in duplicate. The adhesion capacity of the strains was compared to that of the recognized probiotic strain *Lactobacillus rhamnosus* ATCC 53103 (strain GG). The results were expressed as the percentage of adhered bacteria with respect to the initial number of bacteria added.

ANTIOXIDATIVE ACTIVITY

The total antioxidative activity (TAA) of the gastric lactobacilli was assessed using the linolenic acid (LA) test, which evaluates the ability to inhibit lipid peroxidation. Bacterial cultures (24 h) were centrifuged, washed twice in saline solution, and suspended in the same solution to an $OD_{600\ nm}$ of 1.0. Intact cells in the saline solution were examined, as were lysates obtained using a cell disruptor (Constant Systems, Daventry, UK). Reactions were performed according to the procedure described by Kullisaar et al. (2002), using 45 µl samples (whole cells or lysate). The absorbance at 534 nm was measured using a UV-Vis Spectrophotometer (Hitachi High-Technologies, Tokyo, Japan). Intact cells and cell lysates were assayed in triplicate. The results were expressed as the percentage of LA oxidation inhibition.

ANTIBIOTIC RESISTANCE

The resistance/susceptibility profiles of the different strains to 16 antibiotics were determined by microdilution using VetMIC™ plates for lactic acid bacteria (LAB) (National Veterinary Institute of Sweden, Uppsala, Sweden). The strains were grown in LSM (Klare et al., 2005) agar plates and then suspended in 2 ml of sterile saline solution to obtain a density corresponding to McFarland standard 1. The suspension was further diluted 1:1000 with LSM, and 100 µl of this inoculum were added to each well. The minimum inhibitory concentration (MIC) was defined as the lowest antibiotic concentration at which no visual growth was observed after incubation at 37°C for 48 h.

TECHNOLOGICAL TRAITS

Fermentation capabilities

The carbohydrate fermentation profile of the strains was initially determined using a miniaturized commercial system (PhenePlate, Stockholm, Sweden), following the manufacturer's instructions. Growth in the presence of different substrates (lactose, maltose, trehalose, melibiose and raffinose [all from Sigma-Aldrich]) was further evaluated by recording the $OD_{600\ nm}$ at 24 and 48 h. The strains were inoculated (1% v/v) into a basal fermentation medium (MRS without glucose) supplemented with 2% (w/v) of the carbohydrate under test. All strains had been previously adapted overnight in the corresponding fermentation broth.

Glycosidic activity

Enzyme activities were initially measured using the semi-quantitative API-ZYM system (bioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions. Glycosidic activities in cell-free extracts were confirmed and quantified by enzyme assays using the *p*-nitrophenyl (*p*-NP) derivatives 4-NP-β-D-glucopyranoside, 4-NP-α-D-glucopyranoside, 4-NP-β-D-galactopyranoside and 4-NP-α-D-galactopyranoside (all from Sigma-Aldrich) as substrates. Cells from 20 ml cultures in basal fermentation medium with different inducing carbohydrates were harvested by centrifugation, washed with 100 mM potassium phosphate buffer pH 6.8, and the pellets suspended in 2 ml of the same buffer. Cells were disrupted as above and centrifuged to remove cell debris. The extracts were then assayed for glycosidic activity. Reactions were performed using 800 µl of 40 mM buffer acetate pH 5.5, 100 µl of the different *p*-NP derivatives at 10 mM, and 100 µl of the cell-free extracts. Incubation proceeded for 30 min at 37°C and the reactions were then stopped by adding 1 ml of cold 1 M sodium carbonate. After centrifugation, the absorbance at 410 nm was recorded. The protein content of cell-free extracts was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Activities were expressed as specific activity in mU per mg of protein. One unit of activity (1 U) was defined as the amount of protein that released 1 µmol of *p*-NP per min.

Growth and acidification of milk

Overnight cultures, previously washed in a sterile saline solution, were used to inoculate UHT milk (CAPSA, Siero, Spain) to provide an initial cell concentration of about 10^7 cfu/ml. After incubation at 37°C in a 5% CO_2 atmosphere for 24 and 48 h, bacterial counts were performed on MRSC agar plates. The pH of the inoculated and control milk samples was measured using a Crison pH-meter (Crison Instruments, Barcelona, Spain) and via titration with phenolphthalein according to FIL/IDF Standard 86.

RESULTS

TYPING OF GASTRIC LACTOBACILLI STRAINS

The 19 gastric lactobacilli isolates (9 *Lactobacillus gasseri*, 4 *Lactobacillus reuteri*, 3 *Lactobacillus vaginalis,* 2 *Lactobacillus fermentum* and 1 *Lactobacillus casei*) were successively typed by rep-PCR and PFGE (Supplementary Figure 1). The results of these two techniques agreed well, and 10 different strains were considered based on their distinct electrophoretic profiles provided by both methods. The ten selected strains included three *L. gasseri* strains (LG52, LG102, and LG123) from two subjects, two *L. reuteri* strains (LR32 and LR34) from a single subject, two *L. vaginalis* strains (LV51 and LV121) from different subjects, two genetically unrelated *L. fermentum* strains (LF1 and LF2) from a single subject, and a single strain of *L. casei* (LC71).

FUNCTIONAL PROPERTIES

As a whole, high tolerance to acidity was detected; similar plate counts were returned by control (pH 7) and treated samples after exposure to pH 4.5 and above (data not shown). At pH 3 reductions in counts varied among strains but in less than 1 logarithmic unit in all cases (**Figure 1**). However, at pH 2, reductions compared to controls counts were between 1 and 3 logarithmic units. The *L. gasseri* strains were among the most acid tolerant.

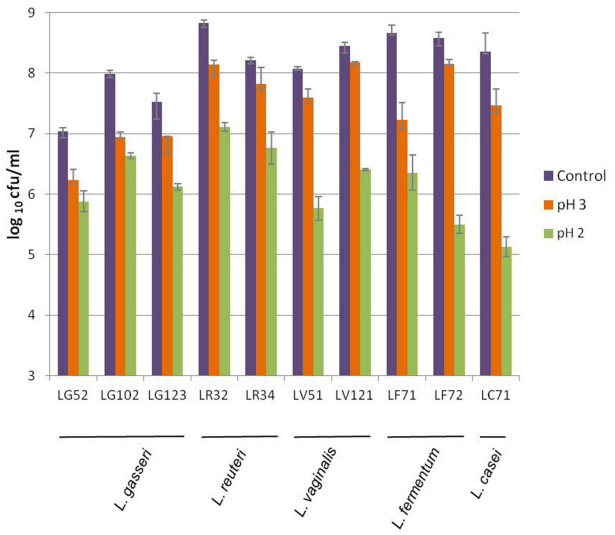


FIGURE 1 | Viability of the gastric *Lactobacillus* strains of this study to acidic conditions after exposure to pH 3 and pH 2 for 90 min, as compared to the control (pH 7.0).

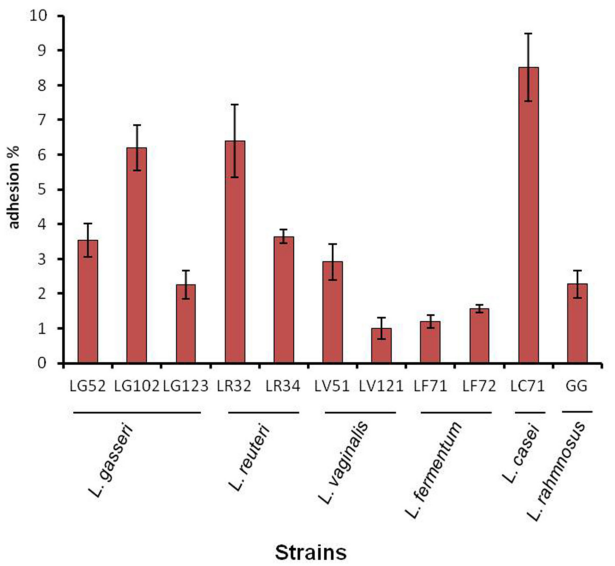


FIGURE 2 | Adhesion ability of gastric lactobacilli to the AGS human epithelial gastric cell line.

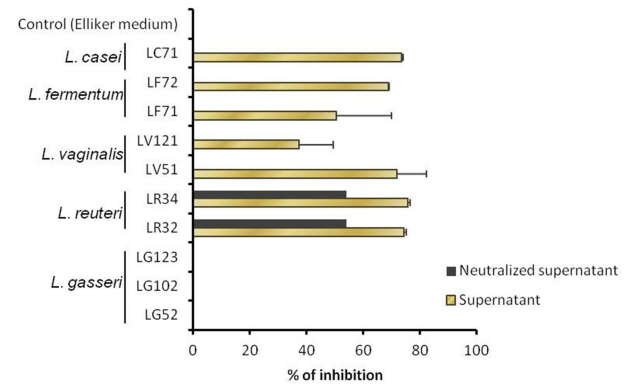


FIGURE 3 | Growth inhibition of *Helicobacter pylori*, as determined in liquid with non-adjusted and pH-neutralized supernatants of the *Lactobacillus* strains grown in Elliker medium.

Resistance of the strains to bile varied widely, depending largely on the species. The *L. gasseri* strains were the most susceptible (MICs 0.25–1.5%), followed by those of *L. fermentum* (MIC = 1%). The most resistant strains were those of *L. reuteri*, *L. vaginalis* and *L. casei*, which grew in the presence of 4% bile.

The percentage of adhesion to gastric cells was strain-dependent but <10% for all strains - even for *L. rhamnosus* GG (**Figure 2**). The most adherent strain was *L. casei* LC71 (8.5%) followed by *L. reuteri* LR32 and *L. gasseri* LG102.

Regarding the bacteriocin-mediated antagonism assays, some gastric strains of the species *L. reuteri*, *L. gasseri* and *L. fermentum* were able to slightly inhibit the growth of the indicators in the agar spot test, although clear halos of inhibition using cell-free, neutralized supernatants were only observed for *L. gasseri* LG52 (Supplementary Table 1). The proteinaceous nature of the antimicrobial bacteriocin-like substance produced by this strain was confirmed after treatment of the cell-free supernatants with proteinase K and pronase, both of which eliminated the antibacterial effect.

On the other hand, the *H. pylori* inhibitory test revealed all strains to have some antimicrobial activity against this pathogen, except for those of *L. gasseri* (**Figure 3**). The highest inhibitory values (∼75%) were obtained for the two *L. reuteri* strains. When the supernatants were neutralized, *H. pylori* growth was suppressed only by the pH-adjusted supernatants pertaining to the *L. reuteri* strains, suggesting that the inhibition observed for the other strains was probably due to the production of organic acids. Further, the presence of genes associated with reuterin production (coding for the glycerol dehydratase unit) was revealed by PCR in the two *L. reuteri* strains.

As concerns H_2O_2 production, all *L. gasseri* strains showed slight production under aerobic conditions, while the *L. vaginalis* and *L. reuteri* strains clearly produced this substance under both aerobic and anaerobic conditions (Supplementary Table 1).

Strain-specific differences were also observed with respect to their TAA, as assessed by the LA test (**Table 1**). Comparable results were obtained using either whole cells or cell lysates. Remarkable activity (TAA >20%) was observed for *L. reuteri* LR32 and *L. vaginalis* LV51, and moderate activity (around a 15% reduction of lipid peroxidation) for *L. casei* LC71 and *L. reuteri* LR34. Neither the *L. fermentum* strains, nor *L. gasseri* LG123 and *L. vaginalis* LV121 showed any antioxidative activity under the experimental conditions of this assay.

Atypical antibiotic resistance was not detected for any of the strains (**Table 2**). The MICs of the different antibiotics were always equal to or lower than the microbiological breakpoints defined by the Panel on Additives and Products or Substances

used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA, 2012). The exception was kanamycin, for which a MIC value of 64 μg/ml was observed for the two *L. fermentum* strains; the EFSA's breakpoint established for this species is one dilution lower.

TECHNOLOGICAL TRAITS

Differences were observed between the 10 strains in terms of their utilization of carbohydrates (Supplementary Table 2). All strains fermented maltose, and all but *L. gasseri* LG102 and LG123 fermented sucrose. Lactose was fermented by the single *L. casei*, the two *L. reuteri*, and the two *L. fermentum* strains. The growth capacity of the strains in basal fermentation medium with lactose, maltose, threhalose, melibiose and raffinose was further evaluated by recording the optical density after 24 and 48 h (**Figure 4**). In general, and in agreement with the previous results, the *L. gasseri* strains showed a reduced fermentation capacity compared to the others. Most of the gastric strains grew in the presence of maltose and lactose as the sole carbon source. The ability to grow in trehalose, however, was restricted to a few *Lactobacillus* strains (**Figure 4**). The *L. reuteri, L. vaginalis* and *L. fermentum* strains showed good growth rates in the presence of raffinose and melibiose.

Nineteen enzymatic activities were tested with the API-ZYM system. Moderate inter- and intra-species variability on the substrates utilized was observed (Supplementary Table 3). Some activities were shown by all or most strains and at high levels (Cys-, Val-, and Leu- arylamidase peptidases, and α- and β-galactohydrolase activities). In contrast, other activities (such as those of lipase, trypsin, α-quimotrypsin, α-mamnosidase, and α-fucosidase) were detected at low levels and only rarely. Glycosidic activities were also examined in cell-free extracts after growth of the strains in inducing carbohydrates. These activities may have a prominent role in key probiotic properties, such as utilization of prebiotics, colonization of the gastric epithelium, etc. The results are summarized in **Table 3**. With the exception of

Table 1 | Percentages of total antioxidative activity (TAA) determined by the linolenic acid test in intact cells and lysates of the gastric lactobacilli.

Species	Strain	TAA*	
		Whole cells (%)	Cell extracts (%)
L. gasseri	LG52	5 ± 2†	3 ± 1
	LG102	16 ± 4	3 ± 1
	LG123	0	0
L. reuteri	LR32	22 ± 5	23 ± 7
	LR34	15 ± 3	14 ± 3
L. vaginalis	LV51	32 ± 8	21 ± 4
	LV121	0	0
L. fermentum	LF71	0	0
	LF72	0	0
L. casei	LC71	13 ± 4	15 ± 2

*Following the definition by Hütt et al. (2006), the antioxidative effect was consider significant if the TAA value was >20%.

†Data are expressed as the mean value of three assays ± standard deviation.

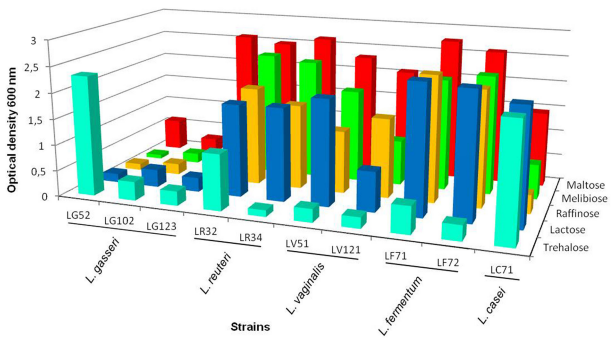


FIGURE 4 | Optical density at 600 nm of the lactobacilli strains grown in basal fermentation medium at 37°C for 48 h using different carbohydrates: maltose, melibiose, raffinose, lactose and trehalose as the carbon source (coefficient of variation <10%).

Table 2 | Minimum inhibitory concentration (MIC) values of 16 antibiotics to the gastric lactobacilli strains.

Species	Strain	Antibiotic* (MIC as μg/ml)														
		GEN	KAN	STP	NEO	TET	ERY	CLI	CHL	AMP	PEN	VAN	VIR	LIN	TRM	CIP	RIF
L. gasseri	LG52	1	32	4	4	4	0.25	1	0.5	0.12	0.06	1	0.5	4	8	64	0.25
	LG102	4	32	4	16	2	0.12	0.5	4	0.5	0.12	1	0.5	2	4	64	1
	LG123	0.5	32	4	4	2	0.016	0.5	1	0.12	0.06	1	0.25	1	16	16	1
L. reuteri	LR32	1	64	16	4	4	0.12	0.03	4	1	0.12	>128	0.5	2	64	64	≤0.12
	LR34	0.5	16	8	0.5	8	0.5	0.12	4	2	0.5	>128	0.5	2	64	32	0.25
L. vaginalis	LV51	1	4	2	0.5	1	0.25	0.03	4	0.12	0.12	>128	0.12	1	0.25	32	≤0.12
	LV121	0.5	8	2	0.5	0.06	0.03	1	4	0.5	0.25	>128	0.12	2	0.25	64	0.25
L. fermentum	LF71	2	64	32	2	8	0.12	0.06	4	0.25	0.25	>128	0.25	2	1	8	1
	LF72	4	64	32	2	8	0.25	0.06	4	0.25	0.5	>128	0.5	2	16	16	0.5
L. casei	LP71	8	64	32	8	1	0.12	0.25	4	1	0.5	>128	1	2	0.5	4	1

*GEN, gentamicin; KAN, kanamycin; STP, streptomycin; NEO, neomycin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; AMP, ampicillin; PEN, penicillin; VAN, vancomycin; VIR, virginiamycin; LIN, linezolid; TRM, trimethoprim; CIP, ciprofloxacin; RIF, rifampicin.

the *L. gasseri* strains (which did not grow in the presence of lactose) all showed high β-galactosidase activity. The *L. vaginalis* strains showed strong α-galactosidase activity, but so too did the *L. reuteri* and *L. fermentum* strains. The *L. casei* strain returned the highest α-glucosidase activity in presence of maltose.

Table 4 shows the strains' ability to grow in and acidify milk. After 24 h of incubation, the cell counts of all the strains increased slightly, but the figures for most (particularly *L. vaginalis* and *L. fermentum*) fell by 48 h. The exceptions were the *L. reuteri* strains, which reached values of around 2×10^8 cfu/ml, and the *L. casei* strain, which showed the highest viable count (1×10^9 cfu/ml) and the lowest pH at both sampling points. As expected, the titratable acidity followed an inverse trend with respect to pH, increasing as the pH decreased. A titratable acidity of around 81% of lactic acid equivalent after 48 h of incubation was recorded for *L. casei* LC71.

DISCUSSION

The acidic environment of the gastric lumen limits the latter's microbial colonization to acidophilic and acid-resistant bacteria. Among these, different LAB species belonging mainly to the genera *Lactobacillus* and *Streptococcus* have been described (Adamson et al., 1999; Ross et al., 2005; Ryan et al., 2008a; Delgado et al., 2013). In this study, members of the genus *Lactobacillus* isolated from the stomach of healthy humans (see Delgado et al., 2013) were studied. Four out of the five *Lactobacillus* species examined (*L. gasseri*, *L. fermentum*, *L. vaginalis* and *L. reuteri*) have also been isolated from human gastric biopsies by other authors (Ryan et al., 2008a), which strongly suggests that they are common inhabitants of the gastric environment. Different strains belonging to the same species in a single individual were detected only occasionally. Based on the typing results, ten different strains were considered; these were characterized for their technological and probiotic properties. In general, they showed good tolerance and survival at low pH, indicating their capacity to survive in the human stomach. However, resistance to low pHs for longer periods of time (and colonization of the gastric epithelium) would be required to ensure persistence in the human stomach. Resistance to acidic conditions is a property of interest for the design and formulation of probiotic cultures. Such an ability would allow not only survival in the upper gastrointestinal tract, but also in fermented products (the most common vehicle for probiotics). The molecular mechanisms involved in such intrinsic resistance are currently unknown. The contribution of urease activity to this resistance however can be ruled out since all the present strains proved to be urease-negative (data not shown).

Since duodenogastric biliary reflux occasionally occurs, strains could also be resistant to bile. In fact, high resistance to bovine bile in lactobacilli strains from the gastric ecosystem has already been reported (Ryan et al., 2008a). Among the strains of this study, the *L. reuteri* proved to be the most resistant to Ox-gall. In contrast, as described for intestinal *L. gasseri* isolates (Delgado et al., 2007), the gastric strains of this species were rather susceptible to bile.

Table 4 | Growth and acidification of UHT milk by gastric lactobacilli strains.

Species	Strains	Cell counts (cfu/ml)*		pH†		Titratable acidity‡	
		24 h	48 h	24 h	48 h	24 h	48 h
L. reuteri	LR32	4.1×10^7	2.1×10^8	6.02	5.75	21.5	24.0
	LR34	3.1×10^7	2.4×10^8	6.09	5.72	22.0	29.0
L. vaginalis	LV51	2.5×10^7	1.6×10^7	6.49	6.45	19.0	20.0
	LV121	1.5×10^7	2.5×10^6	6.50	6.49	19.0	21.0
L. fermentum	LF71	2.0×10^7	9.9×10^6	5.88	5.28	23.5	34.5
	LF72	3.7×10^7	3.1×10^7	5.95	5.53	23.5	33.0
L. casei	LC71	1.2×10^8	1.3×10^9	5.35	4.01	33.5	81.0

**Inoculum* $\approx 1 \times 10^7$ *cfu/ml.*

†pH of the uninoculated milk 6.57.

‡The titratable acidity is expressed as % lactic acid; uninoculated milk 18% lactic acid.

Table 3 | Glycosidic activities of the *Lactobacillus* strains determined in cell-free extracts using *p*-nitrophenyl derivatives.

Species	Strain	Activity (mU*/mg protein)				
		α-glucosidase (maltose)	α-glucosidase (trehalose)	β-galactosidase (lactose)	α-galactosidase (melibiose)	α-galactosidase (raffinose)
L. gasseri	LG52	–	36	–	–	–
	LG102	–	–	–	–	–
	LG123	<0.5	–	–	–	–
L. reuteri	LR32	<0.5	<0.5	865	63	8
	LR34	<0.5	–	574	6	24
L. vaginalis	LV51	12	–	551	1368	109
	LV121	<0.5	–	248	1170	106
L. fermentum	LF71	<0.5	–	145	795	12
	LF72	<0.5	–	318	2	96
L. casei	LC71	33	<0.5	86	–	–

**The enzymatic Unit was defined as the amount of protein that releases 1 μmol of p-NP per min.*

–OD 600 nm less than 0.7; activity was, therefore, not determined.

The strains were shown to adhere to gastric epithelial cells just as well, or even better than, the well-recognized adherent strain *L. rhamnosus* GG (Tuomola and Salminen, 1998). However, comparison of the results with those reported on the literature is difficult given the use of different cell lines (mostly colorectal Caco-2 and HT-29) and different cell-to-bacteria ratios employed.

Probiotics may be useful in the treatment of gastric dysbiosis, such as those caused by *H. pylori* infections. In fact, several studies have reported an inhibitory effect of probiotic lactobacilli on colonization and development of this pathogen (Johnson-Henry et al., 2004; Sykora et al., 2005; Francavilla et al., 2008). *H. pylori* colonizes the epithelium of the stomach and duodenum, occasionally invading the cells. Colonization seems to reduce systemic and cellular antioxidative defenses (Hütt et al., 2009). The antioxidative potential of the strains was therefore tested, a property that has already been reported for certain lactobacilli strains (Kullisaar et al., 2002). According to Hütt et al. (2006), total antioxidative (TAA) values >20% are considered noteworthy. In the present work, two strains, *L. reuteri* LR32 and *L. vaginalis* LV51, met this criterion. This protective property may be useful as a defense mechanism for the gastric mucosa, preserving the tissue from oxidant-induced damage.

The gastric lactobacilli were also screened for their antimicrobial activities against Gram-positive and Gram-negative bacteria. The production of bacteriocin-like inhibitory substances varies widely among LAB species and strains (de Vuyst and Leroy, 2007). After successive solid and liquid medium assays, a single *L. gasseri* strain (LG52) was consistently shown to be bacteriocin producer. However, whether LG52 produces an active bacteriocin under gastric conditions remains yet to be investigated. Another potential source of inhibitory effects is H_2O_2, which in certain environments might be more important than the production of organic acids. The stomach is a microaerobic environment and the production of H_2O_2 might be greater under such conditions than in anaerobiosis. This prompted us to evaluate the production of H_2O_2 under aerobic and anaerobic conditions. The two *L. reuteri* strains and *L. vaginalis* LV51 were shown to produce this compound under both conditions. These strains must have H_2O_2 detoxification mechanisms that allow them to protect themselves from its toxic effects. Superoxide dismutases, peroxidases and dehydrogenases are all able to degrade H_2O_2,and have been described in lactobacilli species (Kullisaar et al., 2002; Hütt et al., 2006; Martín and Suárez, 2010). These enzymes contribute toward the antioxidative cell defense system. In the present work, the strains with the largest H_2O_2 production capacity were those with the greatest antioxidative activity.

The inhibition of *H. pylori* by the two *L. reuteri* strains was probably due to the production of reuterin; certainly, a gene essential for its production was detected. Reuterin is a potent antimicrobial agent that inhibits both Gram-positive and Gram-negative bacteria. *L. reuteri* also produces other potent antimicrobial compounds, such as reutericin 6 and reutericyclin, but these have no effects on Gram-negative bacteria (Gänzle, 2004). The inhibition of *H. pylori* by lactobacilli has already been reported (Sgouras et al., 2004; Hütt et al., 2006; López-Brea et al., 2008; Ryan et al., 2008b). However, most authors attribute the observed inhibitory effects to live metabolizing cells. In contrast, the present work provides evidence of the inhibitory effect of *L. reuteri* culture supernatants against *H. pylori*. Wherever this inhibitory activity comes from, the potential use of these human stomach-derived *L. reuteri* strains as probiotics for protecting against *H. pylori* infection should be considered.

While *L. gasseri* seems to be more prevalent in the gastric ecosystem than *L. reuteri*, the strains of the latter species displayed more probiotic-relevant properties and/or higher activity levels. The antimicrobial—and especially anti-*H. pylori*—activity of the *L. reuteri* strains, together with their antioxidative effects, might allow them to protect the gastric mucosa from infection and damage. The *L. reuteri* strains also showed some technological traits that would allow their inclusion in fermented dairy products. Strains of other species further showed desirable traits to be recommended as probiotic candidates. As an example, *L. vaginalis* LV51 showed the strongest α-galactosidase activity. This would be highly desirable in soy-derived products to hydrolyse the α-galactosides (mainly raffinose and stachyose) capable of causing gastrointestinal discomfort and flatulence (LeBlanc et al., 2008).

CONCLUSIONS

In summary, this work reports a genotypic, technological and probiotic description and characterization of a group of lactobacilli from the human stomach. *In vitro*, some of the gastric strains (particularly the *L. reuteri* strains) showed a vast array of desirable properties to be considered as promising probiotic candidates. Additionally, they showed appropriate technological traits to be included in dairy or other fermented functional foods as adjunct cultures. The efficacy of such probiotics for the treatment and/or prevention of gastric microbial dysbiosis should be carefully evaluated *in vivo* through controlled clinical trials.

AUTHOR CONTRIBUTIONS

Susana Delgado and Baltasar Mayo contributed with the conception and design of the study. Susana Delgado and Analy M. O. Leite were involved in the experimental determinations. Patricia Ruas-Madiedo was in charge of the adhesion experiments and in the maintenance of the gastric cell line. Susana Delgado and Baltasar Mayo interpreted the data and drafted the manuscript. Patricia Ruas-Madiedo performed a critical revision of the manuscript. All authors approved the final version of the article.

ACKNOWLEDGMENTS

This research was funded by projects from the Spanish Ministry of Economy and Competitiveness and Innovation (Ref. AGL2011-24300) and FICYT (Ref. IB08-005). Susana Delgado was supported by a contract under Juan de la Cierva program (Ref. JCI-2008-02391). The technical assistance of Elena Fernández and Alicia Noriega is greatly acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00766/abstract

REFERENCES

Adamson, I., Nord, C. E., Lundquist, P., Sjöstedt, S., and Edlund, C. (1999). Comparative effects of omeprazole, amoxycillin plus metronidazole versus omeprazole, clarithromycin plus metronidazole on the oral, gastric and intestinal microflora in *Helicobacter pylori*-infected patients. *J. Antimicrob. Chemother.* 44, 629–640.

Andersson, A., Lindberg, M., Jakobsson, H., Bäckhed, F., Nyrén, P., and Engstrand, L. (2008). Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* 3:e2836. doi: 10.1371/journal.pone.0002836

Bik, E. M., Eckburg, P. B., Gill, S. R., Nelson, K. E., Purdom, E. A., Francois, F., et al. (2006). Molecular analysis of the bacterial microbiota in the human stomach. *Proc. Natl. Acad. Sci. U.S.A.* 103, 732–737. doi: 10.1073/pnas.0506655103

Bruce, M. G., and Maaroos, H. I. (2008). Epidemiology of *Helicobacter pylori* infection. *Helicobacter* 13(Suppl. 1), 1–6. doi: 10.1111/j.1523-5378.2008.00631.x

Charteris, W. P., Kelly, P. M., Morelli, L., and Collins, J. K. (1998). Development and application of an *in vivo* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J. Appl. Microbiol.* 84, 759–768.

Claisse, O., and Lonvaud-Funel, A. (2001). Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. *J. Food Protect.* 64, 833–837.

Cui, Y., Wang, C. L., Liu, X. W., Wang, X. H., Chen, L. L., Zhao, X., et al. (2010). Two stomach-originated *Lactobacillus* strains improve *Helicobacter pylori* infected murine gastritis. *World J. Gastroenterol.* 28, 445–452. doi: 10.3748/wjg.v16.i4.445

Delgado, S., Cabrera-Rubio, R., Mira, A., Suárez, A., and Mayo, B. (2013). Microbiological survey of the human gastric ecosystem using culturing and pyrosequencing methods. *Microbial. Ecol.* 65, 763–772. doi: 10.1007/s00248-013-0192-5

Delgado, S., O'Sullivan, E., Fitzgerald, G., and Mayo, B. (2007). Subtractive screening for probiotic properties of *Lactobacillus* species from the human gastrointestinal tract in the search for new probiotics. *J. Food Sci.* 72, M310–M315. doi: 10.1111/j.1750-3841.2007.00479.x

de Vuyst, L., and Leroy, F. (2007). Bacteriocins from lactic acid bacteria: production, purification, and food applications. *J. Mol. Microbiol. Biotechnol.* 13, 194–199. doi: 10.1159/000104752

EFSA. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.* 10, 2740–2749.

Francavilla, R., Lionetti, E., Castellaneta, S. P., Magistà, A. M., Maurogiovanni, G., Bucci, N., et al. (2008). Inhibition of *Helicobacter pylori* infection in humans by *Lactobacillus reuteri* ATCC 55730 and effect on eradication therapy: a pilot study. *Helicobacter* 13, 127–134. doi: 10.1111/j.1523-5378.2008.00593.x

Gänzle, M. G. (2004). Reutericyclin: biological activity, mode of action, and potential applications. *Appl. Microbiol. Biotechnol.* 64, 326–332. doi: 10.1007/s00253-003-1536-8

Hütt, P., Andreson, H., Kullisaar, T., Vihalemm, T., Unt, E., Kals, J., et al. (2009). Effects of a synbiotic product on blood antioxidative activity in subjects colonized with *Helicobacter pylori*. *Lett. Appl. Microbiol.* 48, 797–800. doi: 10.1111/j.1472-765X.2009.02607.x

Hütt, P., Shchepetova, J., Lõivukene, K., Kullisaar, T., and Mikelsaar, M. (2006). Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *J. Appl. Microbiol.* 100, 1324–1332. doi: 10.1111/j.1365-2672.2006.02857.x

Johnson-Henry, K. C., Mitchell, D. J., Avitzur, Y., Galindo-Mata, E., Jones, N. L., and Sherman, P. M. (2004). Probiotics reduce bacterial colonization and gastric inflammation in *H. pylori*-infected mice. *Dig. Dis. Sci.* 49, 1095–1102. doi: 10.1023/B:DDAS.0000037794.02040.c2

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., et al. (2005). Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, lactococci, pediococci, and bifidobacteria. *Appl. Environ. Microbiol.* 71, 8982–8986. doi: 10.1128/AEM.71.12.8982-8986.2005

Koeuth, T., Versalovic, J., and Lupski, J. R. (1995). Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Res.* 5, 408–418.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., et al. (2002). Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.* 72, 215–224. doi: 10.1016/S0168-1605(01)00674-2

LeBlanc, J. G., Ledue-Clier, F., Bensaada, M., de Giori, G. S., Guerekobaya, T., Sesma, F., et al. (2008). Ability of *Lactobacillus fermentum* to overcome host alpha-galactosidase deficiency, as evidenced by reduction of hydrogen excretion in rats consuming soya alpha-galacto-oligosaccharides. *BMC Microbiol.* 8:22. doi: 10.1186/1471-2180-8-22

Li, X. X., Wong, G. L. H., To, K. F., Wong, V. W. S., Lai, L. H., Chow, D. K. L., et al. (2009). Bacterial microbiota profiling in gastritis without *Helicobacter pylori* infection or non-steroidal anti-inflammatory drug use. *PLoS ONE* 4:e7985. doi: 10.1371/journal.pone.0007985

López-Brea, M., Alarcón, T., Domingo, D., and Díaz-Regañón, J. (2008). Inhibitory effect of Gram-negative and Gram-positive microorganisms against *Helicobacter pylori* clinical isolates. *J. Antimicrob. Chemother.* 61, 139–142. doi: 10.1093/jac/dkm404

Malfertheiner, P., Megraud, F., O'Morain, C., Bazzoli, F., El-Omar, E., Graham, D., et al. (2007). Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III consensus report. *Gut* 56, 772–781. doi: 10.1136/gut.2006.101634

Martín, R., Olivares, M., Marín, M. L., Xaus, J., Fernández, L., and Rodríguez, J. M. (2005). Characterization of a reuterin-producing *Lactobacillus coryniformis* strain isolated from a goat's milk cheese. *Int. J. Food Microbiol.* 104, 267–277. doi: 10.1016/j.ijfoodmicro.2005.03.007

Martín, R., and Suárez, J. E. (2010). Biosynthesis and degradation of H_2O_2 by vaginal lactobacilli. *Appl. Environ. Microbiol.* 76, 400–405. doi: 10.1128/AEM.01631-09

Peek, R. M., and Blaser, M. J. (2002). *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat. Rev. Cancer* 2, 28–37. doi: 10.1038/nrc703

Ross, S., Engstrand, L., and Jonsson, H. (2005). Lactobacillus gastricus sp. nov., Lactobacillus antri sp. nov., Lactobacillus kalixensis sp. nov. and Lactobacillus ultunensis sp. nov., isolated from human stomach mucosa. *Int. J. Syst. Evol. Microbiol.* 55, 77–82. doi: 10.1099/ijs.0.63083-0

Ryan, K. A., Daly, P., Li, Y., Hooton, C., and O'Toole, P. W. (2008b). Strain-specific inhibition of *Helicobacter pylori* by *Lactobacillus salivarius* and other lactobacilli. *J. Antimicrob. Chemother.* 61, 831–834. doi: 10.1093/jac/dkn040

Ryan, K. A., Jarayaman, T., Daly, P., Canchaya, C., Curran, S., Fang, F., et al. (2008a). Isolation of lactobacilli with probiotic properties from the human stomach. *Lett. Appl. Microbiol.* 47, 269–274. doi: 10.1111/j.1472-765X.2008.02416.x

Sgouras, D., Maragkoudakis, P., Petraki, K., Martinez-Gonzalez, B., Eriotou, E., Michopoulos, S., et al. (2004). *In vitro* and *in vivo* inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. *Appl. Environ. Microbiol.* 70, 518–526. doi: 10.1128/AEM.70.1.518-526.2004

Song, Y. L., Kato, N., Matsumiya, Y., Liu, C. X., Kato, H., and Watanabe, K. (1999). Identification of and hydrogen peroxide production by fecal and vaginal lactobacilli isolated from Japanese women and newborn infants. *J. Clin. Microbiol.* 37, 3062–3064.

Sykora, J., Valeckova, K., Amlerova, J., Siala, K., Dedek, P., Watkins, S., et al. (2005). Effects of a specially designed fermented milk product containing probiotic *Lactobacillus casei* DN-114 001 and the eradication of *H. pylori* in children: a prospective randomized double-blind study. *J. Clin. Gastroenterol.* 39, 692–698. doi: 10.1097/01.mcg.0000173855.77191.44

Tuomola, E. M., and Salminen, S. J. (1998). Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int. J. Food Microbiol.* 41, 45–51.

Conflict of Interest Statement: 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.

Received: 31 October 2014; accepted: 16 December 2014; published online: 14 January 2015.

Citation: Delgado S, Leite AMO, Ruas-Madiedo P and Mayo B (2015) Probiotic and technological properties of Lactobacillus spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. Front. Microbiol. ***5****:766. doi: 10.3389/fmicb.2014.00766*

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

frontiers in Microbiology

GENERAL COMMENTARY
published: 19 May 2015
doi: 10.3389/fmicb.2015.00433

Commentary: Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis

*Amit K. Tyagi * and Sahdeo Prasad*

Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Keywords: ***Lactobacillus reuteri***, ***Helicobacter pylori***, **probiotics, inflammation, triple therapy**

OPEN ACCESS

Edited by:
Maria De Angelis, University of Bari Aldo Moro, Italy

Reviewed by:
Ruggiero Francavilla, University of Bari Aldo Moro, Italy

****Correspondence:***
Amit K. Tyagi, akumar6@mdanderson.org; amittyagiiitd@gmail.com

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

Received: *30 December 2014*
Accepted: *22 April 2015*
Published: *19 May 2015*

Citation:
Tyagi AK and Prasad S (2015) Commentary: Probiotic and technological properties of Lactobacillus spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. Front. Microbiol. 6:433. doi: 10.3389/fmicb.2015.00433

A Commentary on

Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis
by Delgado, S., Leite, A. M. O., Ruas-Madiedo, P., and Mayo, B. (2014). Front. Microbiol. 5:766. doi: 10.3389/fmicb.2014.00766

Helicobacter pylori, a curved-shaped, flagellated, microaerophilic, gram-negative bacillus is naturally colonized bacteria in humans. This bacterium can be found in 25–50% of the population in developed countries and in 70–90% in developing countries, probably, due to the poor hygiene (Go, 2002). New epidemiological studies revealed that the prevalence of *H. pylori* is declining. However, *H. pylori*-infected population develops various diseases including peptic ulcer, chronic gastritis, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Ruggiero, 2014). It is the only bacterium that has been linked to the gastric cancer and ulcer disease, and thus associated with significant morbidity and mortality worldwide. Moreover, till now there is no universally effective therapeutic regimen and vaccine are available for the treatment of *H. pylori* associated diseases. Although triple therapy (combining acid suppression with clarithromycin, amoxicillin, or nitroimidazolic compounds) and antibiotics are being used against *H. pylori* infection, prolongation of these therapeutic agents is declining worldwide because of their side effects (such as vomiting, diarrhea, nausea, constipation, headache, etc.) (Yuan et al., 2013) and prevalence of antimicrobial resistance. *H. pylori* eradication treatments are still remain a challenge.

Extensive research on *H. pylori* and associated diseases concluded that probiotics can be used as an alternative or complementary therapy for the management of *H. pylori* infection (Emara et al., 2014), since it does not cause side effects as triple therapy. Probiotics have potential to maintain the disturbed gastroenterological conditions and also beneficial for the patients of inflammatory bowel disease, ulcerative colitis, Crohn's disease, antibiotic-, and rotavirus-associated diarrhea, adenocarcinoma, colorectal cancer, etc. (Goossens et al., 2003). An improvement of *H. pylori* associated gastric inflammation by the use of probiotics could be contributed by various mechanisms (Emara et al., 2014). Accumulated evidences suggested that *H. pylori* could be eradicated from the stomach by selective bacterial–bacterial cell interaction. *Lactobacillus reuteri*

was identified as a highly specific binding antagonist to *H. pylori* among *Lactobacillus* species. *L. reuteri* DSM17648 strain co-aggregates with different *H. pylori* strains and serotypes. However, it does not affect other intestinal and commensal oral bacteria (Holz et al., 2015).

How *L. reuteri* acts against *H. pylori* is not completely uncovered. However, it is reported that *L. reuteri* produces a compound called reuterin, which work as an antimicrobial agent. Other than reuterin, *L. reuteri* also produces some potent antimicrobial compounds, such as reutericin 6 and reutericyclin, but these have no effects on Gram-negative bacteria (Ganzle, 2004). *L. reuteri* also inhibits the binding of *H. pylori* to the putative glycolipid receptors. Mukai et al. (2002) examined the binding competition of *L. reuteri* strains, (JCM1081 and TM105) and *H. pylori* to gangliotetraosylceramide (asialo-GM1) and sulfatide. It was identified as a possible sulfatide-binding protein of the *L. reuteri* strain.

L. reuteri helps to improve the eradication rate of *H. pylori* induced by triple therapy (**Table 1**). In a recent clinical trial, it was observed that triple therapy supplemented with *L. reuteri* increased the eradication rate of *H. pylori* by 8.6%, as well as reduced the side effects and improve the GSRS (Gastrointestinal Symptom Rating Scale) score (Emara et al., 2014). A new probiotic having two strains of *L. reuteri* (DSM 17938 and ATCC PTA 6475) were also studied for the inhibitory effect of *H. pylori* growth, gastritis and prevention of antibiotic-associated side effects, when administered with triple therapy. *L. reuteri* combination increased the eradication rate by 9.1% (Francavilla et al., 2014). Sequential therapy with *L. reuteri* supplementation was also reported in the eradication treatment of *H. pylori* and the intensity of antibiotic-associated side effects (Efrati et al., 2012). *H. pylori* infection was also defined as positive gastric histopathology ^{13}C urea breath test (^{13}C-UBT). Dore et al. (2014) have done an open label single center study, where they found a significant reduction in urease activity with a difference of mean of 38.8 vs. 25.4 assessed before and 4–6 weeks post therapy. In another study, *L. reuteri* DSMZ17648 was also reported to co-aggregates with *H. pylori in vitro* and was shown to reduce ^{13}C-UBT *in vivo* (Mehling and Busjahn, 2013).

Antibiotic-associated gastrointestinal side effects are major drawbacks of all *H. pylori* therapies. One of them is levofloxacin-based second-line therapy. The efficacy of *L. reuteri* supplementation during a second-line levofloxacin triple therapy for *H. pylori* eradication was reported by Ojetti et al. (2012). In this study, The *H. pylori* eradication rate was significantly increased (18%) and incidence of nausea and diarrhea was significantly lowered due to the *L. reuteri* supplementation. The therapeutic role of *L. reuteri* was also compared with a high concentration of probiotics for *H. pylori* eradication, where

TABLE 1 | Selected clinical trials using *Lactobacillus reuteri* for *H. pylori* eradication treatment.

Treatment	Probiotic(s)	Eradication rate	Probiotic efficacy	References
Triple therapy, Omeprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, 14 d	*L. reuteri* ATCC PTA 6475, *L. reuteri* DSM 17938, 14 d during therapy + further 14 d, Control	74.3% (26/35) 65.7% (23/35)	Significantly increase of eradication rate with improved GSRS score and reduction of side effects (taste disorder, diarrhea)	Emara et al., 2014
Three-phase study; pre-eradication (1–28 d), eradication (29–35 d), follow-up (36–96 d), Triple therapy	*L. reuteri* ATCC PTA 6475, *L. reuteri* DSM 17938 Control	75% (37/50) 65.9% (33/50)	Significantly increase of eradication rate but no difference in GSRS score	Francavilla et al., 2014
Pantoprazole 20 mg, 8 weeks	*L. reuteri*, 8 weeks	14.2% (3/21)	Good tolerability with no side effects	Dore et al., 2014
Levofloxacin 500 mg, esomeprazole 20 mg, amoxicillin 1 g, 7 d	*L. reuteri*, during therapy + further 7 d Control	80% (36/45) 62.2% (28/45)	Significantly increase of eradication rates and reduction of side effects (Nausea, diarrhea)	Ojetti et al., 2012
Omeprazole 1 mg/kg, amoxicillin 50 mg/kg, clarithromycin 15 mg/kg, 7 d	*L. plantarum, L. reuteri, L. casei* subsp. *rhamnosus*, *B. infantis*, and *B. longum*, *L. acidophilus, L. salivarius, S. thermophilus, L. sporogenes*, during therapy Control	82.2% (30/34) 76.4% (26/34)	Non-significant increase of eradication rates; significant reduction of side effects (epigastric pain, nausea, vomiting, diarrhea)	Tolone et al., 2012
Pantoprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, Triple therapy, 7 d Sequential regimen, 10 d	*L. reuteri* ATCC55730, during therapy + further 7 or 10 d	63% (52/83) 88% (73/83)	Significantly higher eradication rate and reduction of side effects in Sequential regimen	Efrati et al., 2012
Sequential therapy (Details not describe)	*L. reuteri* ATCC55730, 8 weeks Control	33.8 ± 15% (33) 35.8 ± 15.5% (33)	Significant decrease in Gastrointestinal Symptom	Francavilla et al., 2008
Triple therapy (Details not describe)	*L. reuteri*, 7 d Control	63% 53%	Lowest incidence of side-effects	Scaccianoce et al., 2008
No drug	*L. reuteri* SD2112, 8 weeks	69.7 ± 4% (33)	Significant reduction of ^{13}C-UBT	Imase et al., 2007
Omeprazole 1 mg/kg, amoxicillin 50 mg/kg, clarithromycin 15 mg/kg, Sequential therapy, 10 d	*L. reuteri* ATCC55730 (SD2112) Control	85% (17/20) 80% (16/20)	Significant reduction of GSRS score	Lionetti et al., 2006

GSRS, Gastro intestinal Symptom Rating Scale; 13C-UBT, 13C Urea Breath Test.

the incidence of side effects was lowest in 7-day therapy plus *L. reuteri* (6%) treated group (Scaccianoce et al., 2008). These studies show that *L. reuteri* can be used as a complementary therapy for *H. pylori* infection. *L. reuteri* may compete directly with *H. pylori*, possibly by interference with adherence or by the production of antimicrobial molecules.

In a recent "Frontiers in Microbiology" paper by Delgado et al. (2014), authors isolated 10 strains of different *Lactobacillus* species from the gastric biopsies and stomach juice samples of healthy humans. These all strains were tested for their functional properties, like bile tolerance, acid resistance, adhesion to epithelial gastric cells, production of antimicrobial compounds, antioxidative activity, antibiotic resistance, carbohydrate fermentation, glycosidic activities, and inhibition of *H. pylori*. *In vitro,* two gastric strains (particularly LR32 and LR34) showed good survival under gastrointestinal conditions, along with strong anti-Helicobacter and antioxidative activities. Thus, these strains can be considered as promising probiotic candidates.

The data presented have important implications for different disciplines including gastroenterology, microbial ecology, colorectal cancer, nutrition and health. Thus, based on these findings following intriguing questions can be raised: (1) To what extent *L. reuteri* is helpful for the prevention of *H. pylori*-induced colorectal cancer? (2) If it is available in our stomach or gut, how *L. reuteri* population can be increased inside the gut? (3) What could be the other potential probiotics and nutraceuticals, which can synergize the growth of *L. reuteri* in the gut?

References

Delgado, S., Leite, A. M., Ruas-Madiedo, P., and Mayo, B. (2014). Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. *Front. Microbiol.* 5:766. doi: 10.3389/fmicb.2014.00766

Dore, M. P., Cuccu, M., Pes, G. M., Manca, A., and Graham, D. Y. (2014). *Lactobacillus reuteri* in the treatment of *Helicobacter pylori* infection. *Intern. Emerg. Med.* 9, 649–654. doi: 10.1007/s11739-013-1013-z

Efrati, C., Nicolini, G., Cannaviello, C., O'sed, N. P., and Valabrega, S. (2012). *Helicobacter pylori* eradication: sequential therapy and *Lactobacillus reuteri* supplementation. *World J. Gastroenterol.* 18, 6250–6254. doi: 10.3748/wjg.v18.i43.6250

Emara, M. H., Mohamed, S. Y., and Abdel-Aziz, H. R. (2014). *Lactobacillus reuteri* in management of *Helicobacter pylori* infection in dyspeptic patients: a double-blind placebo-controlled randomized clinical trial. *Therap. Adv. Gastroenterol.* 7, 4–13. doi: 10.1177/1756283X13503514

Francavilla, R., Lionetti, E., and Cavallo, L. (2008). Sequential treatment for Helicobacter pylori eradication in children. *Gut* 57:1178.

Francavilla, R., Polimeno, L., Demichina, A., Maurogiovanni, G., Principi, B., Scaccianoce, G., et al. (2014). *Lactobacillus reuteri* strain combination in *Helicobacter pylori* infection: a randomized, double-blind, placebo-controlled study. *J. Clin. Gastroenterol.* 48, 407–413. doi: 10.1097/MCG.0000000000000007

Ganzle, M. G. (2004). Reutericyclin: biological activity, mode of action, and potential applications. *Appl. Microbiol. Biotechnol.* 64, 326–332. doi: 10.1007/s00253-003-1536-8

Go, M. F. (2002). Review article: natural history and epidemiology of *Helicobacter pylori* infection. *Aliment. Pharmacol. Ther.* 16(Suppl. 1), 3–15. doi: 10.1046/j.1365-2036.2002.0160s1003.x

Goossens, D., Jonkers, D., Stobberingh, E., van den Bogaard, A., Russel, M., and Stockbrugger, R. (2003). Probiotics in gastroenterology: indications and future perspectives. *Scand. J. Gastroenterol. Suppl.* 38, 15–23. 10.1080/00855920310002645

Holz, C., Busjahn, A., Mehling, H., Arya, S., Boettner, M., Habibi, H., et al. (2015). Significant reduction in *Helicobacter pylori* load in humans with non-viable *Lactobacillus reuteri* DSM17648: a pilot study. *Probiotics Antimicrob. Proteins.* 7, 91–100. doi: 10.1007/s12602-014-9181-3

Imase, K., Tanaka, A., Tokunaga, K., Sugano, H., Ishida, H., and Takahashi, S. (2007). Lactobacillus reuteri tablets suppress Helicobacter pylori infection–a double-blind randomised placebo-controlled cross-over clinical study. *Kansenshogaku Zasshi* 81, 387–393. doi: 10.11150/kansenshogakuzasshi1970.81.387

Lionetti, E., Miniello, V. L., Castellaneta, S. P., Magistá, A. M., de Canio, A., Maurogiovanni, G., et al. (2006). Lactobacillus reuteri therapy to reduce side-effects during anti-Helicobacter pylori treatment in children: a randomized placebo controlled trial. *Aliment. Pharmacol. Ther.* 24, 1461–1468. doi: 10.1111/j.1365-2036.2006.03145.x

Mehling, H., and Busjahn, A. (2013). Non-viable *Lactobacillus reuteri* DSMZ 17648 (Pylopass) as a new approach to *Helicobacter pylori* control in humans. *Nutrients* 5, 3062–3073. doi: 10.3390/nu5083062

Mukai, T., Asasaka, T., Sato, E., Mori, K., Matsumoto, M., and Ohori, H. (2002). Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. *FEMS Immunol. Med. Microbiol.* 32, 105–110. doi: 10.1111/j.1574-695X.2002.tb00541.x

Ojetti, V., Bruno, G., Ainora, M. E., Gigante, G., Rizzo, G., Roccarina, D., et al. (2012). Impact of *Lactobacillus reuteri* supplementation on anti-*Helicobacter pylori* levofloxacin-based second-line therapy. *Gastroenterol. Res. Pract.* 2012:740381. doi: 10.1155/2012/740381

Ruggiero, P. (2014). Use of probiotics in the fight against *Helicobacter pylori*. *World J. Gastrointest. Pathophysiol.* 5, 384–391. doi: 10.4291/wjgp.v5.i4.384

Scaccianoce, G., Zullo, A., Hassan, C., Gentili, F., Cristofari, F., Cardinale, V., et al. (2008). Triple therapies plus different probiotics for *Helicobacter pylori* eradication. *Eur. Rev. Med. Pharmacol. Sci.* 12, 251–256.

Tolone, S., Pellino, V., Vitaliti, G., Lanzafame, A., and Tolone, C. (2012). Evaluation of Helicobacter Pylori eradication in pediatric patients by triple therapy plus lactoferrin and probiotics compared to triple therapy alone. *Ital. J. Pediatr.* 38:63. doi: 10.1186/1824-7288-38-63

Yuan, Y., Ford, A. C., Khan, K. J., Gisbert, J. P., Forman, D., Leontiadis, G. I., et al. (2013). Optimum duration of regimens for *Helicobacter pylori* eradication. *Cochrane Database Syst. Rev.* 12:CD008337. doi: 10.1002/14651858.CD008337.pub2

Conflict of Interest Statement: 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

CORRECTION
published: 18 July 2019
doi: 10.3389/fmicb.2019.01628

Corrigendum: Commentary: Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis

Amit K. Tyagi and Sahdeo Prasad*

Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

Keywords: *Lactobacillus reuteri*, *Helicobacter pylori*, probiotics, inflammation, triple therapy

OPEN ACCESS

Edited and reviewed by:
Fabio Minervini,
University of Bari Aldo Moro, Italy

****Correspondence:***
Amit K. Tyagi
akumar6@mdanderson.org;
amittyagiiitd@gmail.com

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

Received: *02 April 2019*
Accepted: *02 July 2019*
Published: *18 July 2019*

Citation:
Tyagi AK and Prasad S (2019) Corrigendum: Commentary: Probiotic and technological properties of Lactobacillus spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. Front. Microbiol. 10:1628. doi: 10.3389/fmicb.2019.01628

A Corrigendum on

Commentary: Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis
by Tyagi, A. K., and Prasad, S. (2015). Front. Microbiol. 6:433. doi: 10.3389/fmicb.2015.00433

In the original article, there was error in **Table 1** as published. The probiotic efficacy for Emara 2014 and Francavilla 2014 reported a significant increase instead of a non-significant increase.

Additionally, the reference for "Francavilla et al., 2008" in **Table 1** was incorrectly written as "Francavilla, R., Lionetti, E., and Cavallo, L. (2008). Sequential treatment for Helicobacter pylori eradication in children. *Gut* 57:1178." It should be "Francavilla, R., Lionetti, E., Castellaneta, S. P., Magistà, A. M., Maurogiovanni, G., Bucci, N., et al. (2008). Inhibition of *Helicobacter pylori* infection in humans by *Lactobacillus* reuteri ATCC 55730 and effect on eradication therapy: a pilot study. *Helicobacter* 13, 127–134. doi: 10.1111/j.1523-5378.2008.00593.x".

The corrected **Table 1** and Reference appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 1 | Selected clinical trials using *Lactobacillus reuteri* for *H. pylori* eradication treatment.

Treatment	Probiotic(s)	Eradication rate	Probiotic efficacy	References
Triple therapy, Omeprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, 14 d	*L. reuteri* ATCC PTA 6475, *L. reuteri* DSM 17938, 14 d during therapy + further 14 d, Control	74.3% (26/35) 65.7% (23/35)	Non-significant increase of eradication rate with improved GSRS score and reduction of side effects (taste disorder, diarrhea)	Emara et al., 2014
Three-phase study; pre-eradication (1–28 d), eradication (29–35 d), follow-up (36–96 d), Triple therapy	*L. reuteri* ATCC PTA 6475, *L. reuteri* DSM 17938, Control	75% (37/50) 65.9% (33/50)	Non-significant increase of eradication rate but no difference in GSRS score	Francavilla et al., 2014
Pantoprazole 20 mg, 8 weeks	*L. reuteri*, 8 weeks	14.2% (3/21)	Good tolerability with no side effects	Dore et al., 2014
Levofloxacin 500 mg, esomeprazole 20 mg, amoxicillin 1 g, 7 d	*L. reuteri*, during therapy + further 7 d Control	80% (36/45) 62.2% (28/45)	Significantly increase of eradication rates and reduction of side effects (Nausea, diarrhea)	Ojetti et al., 2012
Omeprazole 1 mg/kg, amoxicillin 50 mg/kg, clarithromycin15 mg/kg, 7 d	*L. plantarum, L. reuteri*, *L. casei* subsp. *rhamnosus*, *B. infantis, and B. longum*, *L. acidophilus, L. salivarius*, *S. thermophilus, L. sporogenes*, during therapy Control	82.2% (30/34) 76.4% (26/34)	Non-significant increase of eradication rates; significant reduction of side effects (epigastric pain, nausea, vomiting, diarrhea)	Tolone et al., 2012
Pantoprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, Triple therapy, 7 d Sequential regimen, 10 d	*L. reuteri* ATCC55730, during therapy + further 7 or 10 d	63% (52/83) 88% (73/83)	Significantly higher eradication rate and reduction of side effects in sequential regimen	Efrati et al., 2012
Sequential therapy (Details not describe)	*L. reuteri* ATCC55730, 8 weeks Control	33.8 ± 15% (33) 35.8 ± 15.5% (33)	Significant decrease in Gastrointestinal Symptom	Francavilla et al., 2008
Triple therapy (Details not describe)	*L. reuteri*, 7 d Control	63% 53%	Lowest incidence of side-effects	Scaccianoce et al., 2008
No drug	*L. reuteri* SD2112, 8 weeks	69.7 ± 4% (33)	Significant reduction of ^{13}C-UBT	Imase et al., 2007
Omeprazole 1 mg/kg, amoxicillin 50 mg/kg, clarithromycin15 mg/kg, sequential therapy, 10 d	*L. reuteri* ATCC55730 (SD2112) Control	85% (17/20) 80% (16/20)	Significant reduction of GSRS score	Lionetti et al., 2006

GSRS, Gastrointestinal Symptom Rating Scale; ^{13}C-UBT; ^{13}C Urea Breath Test.

REFERENCES

Dore, M. P., Cuccu, M., Pes, G. M., Manca, A., and Graham, D. Y. (2014). *Lactobacillus reuteri* in the treatment of *Helicobacter pylori* infection. *Intern. Emerg. Med.* 9, 649–654. doi: 10.1007/s11739-013-1013-z

Efrati, C., Nicolini, G., Cannaviello, C., O'sed, N. P., and Valabrega, S. (2012). *Helicobacter pylori* eradication: sequential therapy and *Lactobacillus reuteri* supplementation. *World J. Gastroenterol.* 18, 6250–6254. doi: 10.3748/wjg.v18.i43.6250

Emara, M. H., Mohamed, S. Y., and Abdel-Aziz, H. R. (2014). *Lactobacillus reuteri* in management of *Helicobacter pylori* infection in dyspeptic patients: a double-blind placebo-controlled randomized clinical trial. *Therap. Adv. Gastroenterol.* 7, 4–13. doi: 10.1177/1756283X13503514

Francavilla, R., Lionetti, E., Castellaneta, S. P., Magistà, A. M., Maurogiovanni, G., Bucci, N., et al. (2008). Inhibition of *Helicobacter pylori* infection in humans by *Lactobacillus* reuteri ATCC 55730 and effect on eradication therapy: a pilot study. *Helicobacter* 13, 127–134. doi: 10.1111/j.1523-5378.2008.00593.x

Francavilla, R., Polimeno, L., Demichina, A., Maurogiovanni, G., Principi, B., Scaccianoce, G., et al. (2014). *Lactobacillus reuteri* strain combination in *Helicobacter pylori* infection: a randomized, double-blind, placebo-controlled study. *J. Clin. Gastroenterol.* 48, 407–413. doi: 10.1097/MCG.0000000000000007

Imase, K., Tanaka, A., Tokunaga, K., Sugano, H., Ishida, H., and Takahashi, S. (2007). Lactobacillus reuteri tablets suppress Helicobacter pylori infection–a double-blind randomised placebo-controlled cross-over clinical study. *Kansenshogaku Zasshi* 81, 387–393. doi: 10.11150/kansenshogakuzasshi1970.81.387

Lionetti, E., Miniello, V. L., Castellaneta, S. P., Magistá, A. M., de Canio, A., Maurogiovanni, G., et al. (2006). Lactobacillus reuteri therapy to reduce side-effects during anti-Helicobacter pylori treatment in children: a randomized placebo controlled trial. *Aliment. Pharmacol. Ther.* 24, 1461–1468. doi: 10.1111/j.1365-2036.2006.03145.x

Ojetti, V., Bruno, G., Ainora, M. E., Gigante, G., Rizzo, G., Roccarina, D., et al. (2012). Impact of *Lactobacillus reuteri* supplementation on anti-*Helicobacter pylori* levofloxacin-based second-line therapy. *Gastroenterol. Res. Pract.* 2012:740381. doi: 10.1155/2012/740381

Scaccianoce, G., Zullo, A., Hassan, C., Gentili, F., Cristofari, F., Cardinale, V., et al. (2008). Triple therapies plus different probiotics for

Helicobacter pylori eradication. *Eur. Rev. Med. Pharmacol. Sci.* 12, 251–256.

Tolone, S., Pellino, V., Vitaliti, G., Lanzafame, A., and Tolone, C. (2012). Evaluation of Helicobacter Pylori eradication in pediatric patients by triple therapy plus lactoferrin and probiotics compared to triple therapy alone. *Ital. J. Pediatr*. 38:63. doi: 10.1186/1824-7288-38-63

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 August 2015
doi: 10.3389/fmicb.2015.00768

Edited by:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Baltasar Mayo, Consejo Superior de Investigaciones Científicas, Spain
Rasu Jayabalan, National Institute of Technology, Rourkela, India
Fatemeh Nejati, Islamic Azad University, Iran

***Correspondence:**
Seung Hwan Yang, Graduate School of Interdisciplinary Program of Biomodulation, College of Natural Science, Center for Nutraceutical and Pharmaceutical Materials, Myongji University (Science Campus), Cheoin-gu, Yongin, Gyeonggi-do 449-728, South Korea
ymichigan@mju.ac.kr;
Joo-Won Suh, Division of Bioscience and Bioinformatics, College of Natural Science, Center for Nutraceutical and Pharmaceutical Materials, Myongji University (Science Campus), Cheoin-gu, Yongin, Gyeonggi-do 449-728, South Korea
jwsuh@mju.ac.kr

†These authors have contributed equally to this work.

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

Received: *09 April 2015*
Accepted: *14 July 2015*
Published: *04 August 2015*

Citation:
Damodharan K, Lee YS, Palaniyandi SA, Yang SH and Suh J-W (2015) Preliminary probiotic and technological characterization of Pediococcus pentosaceus strain KID7 and in vivo assessment of its cholesterol-lowering activity. Front. Microbiol. 6:768. doi: 10.3389/fmicb.2015.00768

Preliminary probiotic and technological characterization of *Pediococcus pentosaceus* strain KID7 and *in vivo* assessment of its cholesterol-lowering activity

***Karthiyaini Damodharan*[1,2†], *Young Sil Lee*[2†], *Sasikumar A. Palaniyandi*[2,3†], *Seung Hwan Yang*[2,3*] *and Joo-Won Suh*[1,2*]**

[1] Division of Biosciences and Bioinformatics, Myongji University, Yongin, South Korea, [2] Center for Nutraceutical and Pharmaceutical Materials, Myongji University, Yongin, South Korea, [3] Graduate School of Interdisciplinary Program of Biomodulation, College of Natural Science, Myongji University, Yongin, South Korea

The study was aimed to characterize the probiotic properties of a *Pediococcus pentosaceus* strain, KID7, by *in vitro* and *in vivo* studies. The strain possessed tolerance to oro-gastrointestinal transit, adherence to the Caco-2 cell line, and antimicrobial activity. KID7 exhibited bile salt hydrolase activity and cholesterol-lowering activity, *in vitro*. *In vivo* cholesterol-lowering activity of KID7 was studied using atherogenic diet-fed hypercholesterolemic mice. The experimental animals (C57BL/6J mice) were divided into 4 groups viz., normal diet-fed group (NCD), atherogenic diet-fed group (HCD), atherogenic diet- and KID7-fed group (HCD-KID7), and atherogenic diet- and *Lactobacillus acidophilus* ATCC 43121-fed group (HCD-*L.ac*) as positive control. Serum total cholesterol (T-CHO) level was significantly decreased by 19.8% in the HCD-KID7 group ($P < 0.05$), but not in the HCD-*L.ac* group compared with the HCD group. LDL cholesterol levels in both HCD-KID7 and HCD-*L.ac* groups were decreased by 35.5 and 38.7%, respectively, compared with HCD group (both, $P < 0.05$). Glutamyl pyruvic transaminase (GPT) level was significantly lower in the HCD-KID7 and HCD-*L.ac* groups compared to HCD group and was equivalent to that of the NCD group. Liver T-CHO levels in the HCD-KID7 group were reduced significantly compared with the HCD group ($P < 0.05$) but not in the HCD-*L.ac* group. Analysis of expression of genes associated with lipid metabolism in liver showed that low-density lipoprotein receptor (*LDLR*), cholesterol-7α-hydroxylase (*CYP7A1*) and apolipoprotein E (*APOE*) mRNA expression was significantly increase in the HCD-KID7 group compared to the HCD group. Furthermore, KID7 exhibited desired viability under freeze-drying and subsequent storage conditions with a combination of skim milk and galactomannan. *P. pentosaceus* KID7 could be a potential probiotic strain, which can be used to develop cholesterol-lowering functional food after appropriate human clinical trials.

Keywords: *Pediococcus*, probiotic, oro-gastrointestinal transit, cholesterol-lowering, bile salt hydrolase, LDL-receptor, cholesterol 7α-hydroxylase, storage viability

Introduction

High blood cholesterol (hypercholesterolemia) is a risk factor for cardiovascular diseases, which remains one of the largest causes of death worldwide (Ishimwe et al., 2015). It has been shown that a 1% reduction in serum cholesterol is associated with an estimated reduction of 2–3% in the risk of coronary heart disease (CHD) (Ishimwe et al., 2015). Statin drugs are predominantly prescribed for the reduction of serum cholesterol level and in turn the risk of CHD. There is an increasing interest in non-drug therapies for lowering serum cholesterol and the risk of CHD due to several adverse side effects of statin drugs reported in the literature and by patients (Sultan and Hynes, 2013). An alternative is probiotics with cholesterol-lowering activity. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Recently, several studies have shown that probiotics could be used as alternative supplements to exert cholesterol-lowering effects in humans (Trautvetter et al., 2012; Fuentes et al., 2013; Guardamagna et al., 2014). Probiotics have been suggested to reduce cholesterol via various mechanisms (Ishimwe et al., 2015). Available literature suggests that probiotics with bile salt hydrolase (BSH) activity show cholesterol-lowering activity *in vivo* (Kumar et al., 2011; Jones et al., 2012, 2014; Pavlovic et al., 2012; Degirolamo et al., 2014). Other mechanisms such as cholesterol adsorption to cell surface, cholesterol assimilation into bacterial cell membrane (Liong and Shah, 2005a) and co-precipitation with deconjugated bile acids (Liong and Shah, 2005b) are also proposed/demonstrated *in vitro* without evidence of their occurrence *in vivo*.

Several species and strains belonging to the orders *Lactobacillales* and *Bifidobacteriales* are widely recognized, approved and used as probiotics with some yeast and *Bacillus* sp. as well. The beneficial effects of probiotics include but are not limited to gastro-intestinal microbial balance, suppression of pathogens, immunomodulatory activity, hypocholesterolemic activity, and alleviation of certain conditions such as diarrhea, allergy, lactose intolerance, irritable bowel syndrome, inflammatory bowel disease (IBD), and colon cancer (reviewed in Nagpal et al., 2012). However, a single probiotic microbe cannot provide all of these beneficial effects and efficacy and the activity of probiotic strains vary considerably. For example, certain probiotic strains show efficacy against antibiotic-associated diarrhea but there is less evidence for their efficacy against IBD.

Since, the probiotic property of microbes differs from one strain to another, new strains must be assessed for their putative probiotic properties according to FAO/WHO guidelines (FAO-WHO, 2002). The FAO/WHO guideline recommends certain testing methods to establish the health benefits of a microbe to be called probiotic. The testing methods include *in vitro* and *in vivo* study of oro-gastrointestinal transit tolerance, production of antimicrobial substances, beneficial probiotic characters such as cholesterol-lowering activity, anti-hypertensive activity, anti-diabetic activity etc., and adherence to human intestinal cells, before testing the microbe in human clinical trials (FAO-WHO, 2002). The guidelines also insist on the characterization of a putative probiotic microbe for its safety that the strain should not possess any transferrable antibiotic resistance (FAO-WHO, 2002). Additionally, any probiotic microbe should maintain its viability and probiotic activity during industrial manufacturing practices such as drying, and storage in various products.

The objective of this study was to establish the various probiotic properties and cholesterol-lowering activity of a *Pediococcus pentosaceus* strain KID7, through *in vitro* and *in vivo* studies and technological characterization of strain KID7 for its ability to maintain desired viability during manufacturing and storage condition.

Materials and Methods

Microorganisms and Culture Conditions

Strain KID7 was isolated from fermented finger millet (*Eleusine coracana*) gruel obtained from a household in Yongin, Korea. The gruel was serial-10-fold diluted and 10^{-4} to 10^{-6} dilutions were plated on de Man Rogosa and Sharpe (MRS) agar medium and incubated at 37°C for 24 h to pick as single colony of strain KID7. The probiotic strains *Lactobacillus rhamnosus* GG and*Lactobacillus acidophilus* ATCC 43121 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and the type strain *Pediococcus pentosaceus* KACC 12311 was obtained from the Korean Agricultural Culture Collection (KACC), Republic of Korea. The strains were cultured in MRS agar medium and incubated at 37°C for 24 h before being used for experiments. The strains were stored as glycerol stocks (20% glycerol in MRS broth) at −80°C. The probiotic strains and type strain *P. pentosaceus* KACC 12311 were used as reference strains for comparison of probiotic and biochemical characteristics, respectively.

Pathogenic microbes used in this study were obtained from the Korean Collection for Type Cultures (KCTC), KACC and Korean Culture Center of Microorganisms (KCCM), Republic of Korea. The pathogenic strains were routinely cultured in Luria-Bertani (LB) agar medium and stored as glycerol stocks (20% glycerol in LB broth, v/v) at −80°C.

Strain Identification and Biochemical Test

KID7 was identified by gram staining, microscopic examination and the API 50 CHL kit (Biomerieux S.A., La Balme les Grottes, France). In addition, the fermentation pattern of KID7 was identified using homo- and hetero-fermentation (HHD) medium (Mcdonald et al., 1987). Enzyme activities such as β-galactosidase, β-glucosidase and protease activity were studied following previous reports (Vidhyasagar and Jeevaratnam, 2013; Lee et al., 2014). KID7 was studied for its growth in the presence of NaCl (1–10%, w/v) in MRS broth. Genomic DNA of strain KID7 was isolated using a genomic DNA isolation kit (GeneALL, Seoul, South Korea), following the manufacturer's protocol. PCR amplification of the 16S rRNA gene from KID7 was performed with the primers 27F and 1492R (Borges et al., 2013) and sequencing of the PCR product was done with 27F and 785F (5′-GGATTAGATACCCTGGTA-3′) primers to get a partial sequence of the 16S rRNA gene. Sequencing service was provided by Solgent Co. Ltd. (Seoul, South Korea). The

sequence was searched for similarities in the EzTaxon database using the BLAST program. A phylogenetic tree was constructed using the closely related sequences by multiple alignment using Clustal X followed by neighbor-joining phylogenetic tree construction using MEGA 6 software (Tamura et al., 2013).

In vitro Study of Probiotic Properties

Safety assessment

KID7 was subjected to safety assessment such as biogenic amine production, hemolytic activity, and degradation of type III mucin from porcine stomach (Sigma, St. Louis, MO, USA). The biogenic amine production was tested according to the procedure described by Bover-Cid and Holzapfel (1999) using decarboxylase agar medium with or without amino acids such as L-phenylalanine, L-lysine, L-tryptophan, L-tyrosine, L-arginine, L-ornithine, or L-histidine. Hemolytic activity and mucin degradation were tested by petri dish-based methods described by Borges and Teixeira (2014) and Zhou et al. (2001), respectively. The minimum inhibitory concentration (MIC) of various antibiotics for strain KID7 was tested by 2-fold broth microdilution method (Wiegand et al., 2008). Susceptibility of the strain to a particular antibiotic was determined according to the cut-off MIC values given by European Food Safety Authority (EFSA, 2012).

Oro-gastrointestinal transit assay

Oro-gastrointestinal transit (OGT) tolerance assay was performed according to the method described by Bove et al. (2012) with modification. The assay mimic the physiological conditions of oral, gastric and intestinal stress. The strain KID7 was subjected to oral stress for 10 min in electrolyte solution (sodium chloride, 6.2 g; potassium chloride, 2.2 g; calcium chloride, 0.22 g; sodium bicarbonate, 1.2 g) (Marteau et al., 1997) with 150 mg/L lysozyme (Sigma). This was followed by gastric stress for a 30-min incubation in electrolyte solution of pH 3 + 0.3% pepsin (Sigma) and another 30-min incubation in electrolyte solution of pH 2 + 0.3% pepsin. Finally, the cells were subjected to a 120-min incubation in intestinal electrolyte solution (sodium chloride, 5 g; potassium chloride, 0.6 g; calcium chloride, 0.25 g; pH 7) (Marteau et al., 1997) with 0.3% bile oxgall (BD) and 0.1% pancreatin (Sigma-Aldrich). The strains in PBS without stress were used as control. The cells were separated from the stress solution after each stress by centrifugation at 5000 rpm for 5 min and either subjected to next stress or used for viability analysis. The viability was calculated from colony-forming units (cfu) of appropriate dilutions from the control and stress-treated bacterial cells plated on MRS agar medium incubated for 48 h at 37°C. The bacterial viability at the end of each stress was also monitored using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Oregon, USA) following the manufacturer's protocol. The live and dead bacteria were visualized as green and red fluorescent cells, respectively under a fluorescence microscope (Olympus BX50, Tokyo, Japan) and photomicrographs were captured using a digital camera (Olympus DP70, Tokyo, Japan).

Caco-2 cell culture and adhesion assay

Caco-2 cell line was obtained from KCTC (Korea) and routinely cultured in minimum essential medium (MEM) high-glucose medium (Gibco) supplemented with 20% (v/v) inactivated fetal bovine serum and 100 U of antibiotics penicillin-streptomycin. The culture was incubated at 37°C with 5% CO_2.For theadhesion assay, the Caco-2 cell line was used between the 39th and 41st passage, which phenotypically resembled the enterocytes and expressed tight junction. The Caco-2 cells were seeded at the concentration of 2×10^5 cfu/ml in 12-well cell culture plates and incubated for 21 days to get polarized monolayer and 100% confluence. The medium was changed every day.

The adhesion of lactic acid bacteria (LAB) strains to Caco-2 cells was determined by the method described by Lee et al. (2014) with minor modification. Briefly, the Caco-2 cells were replaced with non-antibiotic-supplemented MEM medium 2 h prior to the adhesion assay. 0.5 ml of 18-h bacterial suspension in PBS was mixed with an equal volume of non-antibiotic-supplemented cell culture medium. The final concentration of bacteria was 1×10^8 cfu/ml, added to each well of the tissue culture plate containing the Caco-2 monolayer, and incubated at 37°C in 5% CO_2 for 2 h. After incubation, the number of adhered bacterial cells was determined according to Lee et al. (2014).

Antimicrobial activity

The antimicrobial activity of strain KID7 was determined against human, animal and food-borne pathogens listed in **Table 2**. Strain KID7 was cultured in MRS broth for 48 h at 37°C and the culture filtrate was concentrated 10-fold by lyophilization. One hundred microliter of concentrated crude culture filtrate was loaded on to an 8-mm paper disc (ADVANTEC, Japan) and dried. The disc was placed on the pathogen- spread LB agar plate and incubated for 18 h and the zone of inhibition was measured. The entire assay was performed in triplicates to check the reproducibility. Hydrogen peroxide production by the LAB strains was determined according to the method of Saito et al. (2007).

Bile salt hydrolase activity

Bile salt hydrolase activity of KID7 was detected by thin-layer chromatography (TLC). The reaction mixtures were prepared by a method described by Guo et al. (2011) with slight modification. MRS reaction mixture was prepared with 5 mM taurodeoxycholic acid (TDCA), or 5 mM glycocholic acid (GCA), or 5 mM taurocholic acid (TCA) in sodium phosphate buffer (pH 7.4). One milliliter of 18-h culture was mixed with an equal volume of phosphate-buffered MRS + conjugated bile acid and incubated for 36 h at 37°C. After incubation, the mixture was lyophilized, dissolved in 1 ml of methanol and centrifuged to remove precipitates. Three microliter of sample was spotted on TLC (silica gel 60, 20 × 20 cm) and developed using a mobile phase of hexane:methylethlyketone:glacial acetic acid (56:36:8, v/v/v) (Chavez and Krone, 1976). After development, the plates were dried with hot air, sprayed with 10% sulfuric acid in distilled water, and heated at 110°C for 10 min in a hot air oven to detect the free cholic acid, deoxycholic acid, and conjugated bile acids. The spots were visualized under UV illumination.

Determination of cholesterol-lowering activity

The LAB strains were studied for cholesterol-lowering activity in MRS liquid broth supplied with cholesterol. MRS broth was initially prepared with 0.05% cysteine-HCl to create microaerophilic condition and autoclaved, followed by addition of cholesterol (CHO) dissolved in ethanol:Tween 80 (3:1, v/v) to a final concentration of 500 μg/ml, w/v, in the MRS medium. The ethanol concentration should not exceed 5%. The MRS-CHO broth was inoculated with log phase bacterial culture (2% v/v of A_{600} = 0.5 OD culture) and incubated at 37°C37°C for 24 h. After the incubation, bacterial cells were pelleted out by centrifugation at 1500 × g at 4°C4°C for 10 min. The cholesterol concentration in spent medium was estimated by enzymatic method using the BCS total cholesterol kit (Bioclinical System, Seoul, South Korea) following the manufacturer's protocol and compared with uninoculated MRS-CHO broth as control and the percentage cholesterol removal was calculated. The cholesterol-lowering activity of LAB was also studied in the presence of 0.3 and 0.5% bile. Additionally, growth of the LAB strains was also measured in MRS-CHO, and MRS-CHO-bile (0.3 and 0.5%) broth.

In vivo Study of Cholesterol-Lowering Activity of KID7

Experimental animals

Male C57BL/6J mice (7–8 weeks-old) were purchased from Orient Bio (Sung-Nam, South Korea). The mice were housed under temperature (23 ± 3°C) and humidity-controlled conditions (40 ± 6%) with a 12-h light/dark cycle and were given free access to water and food throughout the experiment. After acclimatization, the mice were fed an atherogenic diet (HCD, 40 kcal % fat, 1.25% cholesterol and 0.5% cholic acid, Cat #101556; Research Diets, NJ, USA) for 4 weeks to induce hypercholesterolemia before the oral administration of LAB. One group was continued with a normal diet (NCD). After 4 weeks, HCD-fed mice were assigned to one of the following three groups for 4–5 weeks: HCD-control (Con); HCD administered with 3×10^8 cfu/ml of KID7 (HCD-KID7); or HCD administered with 3×10^8 cfu/ml of *L. acidophilus* ATCC 43121 (HCD-*L.ac*). KID7 and *L. acidophilus* ATCC 43121 were mixed with distilled water and administered by oral gavage once daily for 32 days and the control NCD and HCD groups were administered distilled water alone. The body weight was measured once per week for 6 weeks. Food intake (g/mouse/day) was determined by subtracting the remaining food weight from the initial food weight of the previous feeding day, and dividing by the number of mice housed in the cage. The experimental design was approved by the Ethical Committee of the Myongji Bioeffeciency Research Center in Myongji University (Yongin-Si, Gyeonggi-do, Republic of Korea), and the mice were maintained in accordance with standard guidelines.

Collection of tissues

At the end of the 32-days treatment period, the mice were sacrificed by cervical dislocation, and the liver and white adipose tissue (WAT) were excised immediately, rinsed, weighed, frozen in liquid nitrogen, and stored at −80°C until analysis.

Serum biochemical analysis

For biochemical analyses of serum, blood samples were collected from tail vein at 16-h-fasted state. Serum was obtained from the blood by centrifugation (3000 rpm, 10 min, 4°C) and was stored at −80°C until analysis. Serum total cholesterol (T-CHO), triglyceride (TG), HDL-cholesterol, glucose, glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) levels were determined by using commercial assay kits (Asan Pharm, Seoul, Korea). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula (Friedewald et al., 1972).

Liver and fecal lipid contents

Lipids from the isolated liver and feces was extracted using the Folch method (Folch et al., 1957). The extracted lipid fraction was used to measure the T-CHO and TG levels using commercial kit mentioned above. Fecal cholic acid content was measured following the method of Kumar et al. (2011).

Total RNA isolation and gene expression analysis

Total RNA was extracted from liver using the RNeasy mini kit (Qiagen Korea, Seoul, South Korea) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed to cDNA with the PrimeScript RT reagent kit (Takara, Shiga, Japan). The mRNA expression levels of cholesterol metabolisms-related genes were evaluated by real-time PCR using iQTM SYBR Green Supermix and the real-time PCR iQTM5 system (Bio-Rad Laboratories, Hercules, CA, USA). Relative gene expression levels were calculated by the difference in Ct value after normalization with β-actin (*ACTB*) and were expressed as the fold-change relative to the HCD control group. The primers used in the experiments are listed in **Table 1**.

Technological Characterization

Viability of strain KID7 under freeze-drying and storage conditions

KID7 was grown in MRS broth at 37°C for 30 h (late log-phase culture) and the cells were harvested by centrifugation at 3000 × g for 15 min at 4°C. The cell pellet was washed and resuspended in PBS (control) and additives to give a final cell concentration of 1.8×10^9 cfu/ml. The additives used were 1% (w/v) skim milk, 1% (w/v) galactomannan (Sigma-Aldrich), 1% (w/v) wheat bran hydrolysate, 0.5% (w/v) skim milk + 0.5% (w/v) galactomannan, and 0.5% (w/v) galactomannan + 0.5% (w/v) wheat bran hydrolysate, prepared separately in sterile distilled water. Wheat bran hydrolysate was prepared following the method of Wang et al. (2009). The cell suspension in PBS and additives were frozen at −80°C. The frozen suspensions were subjected to freeze-drying using a lab-scale freeze dryer (IlShin lab Co., ltd, Korea), pre-cooled to −80°C. The samples were dried under reduced pressure (5 mbar) for 24 h. The freeze-dried samples were rehydrated using sterile distilled water and serially diluted and plated on MRS agar to determine the viable count. Alternatively, the freeze-dried samples were stored at three different temperature viz., −20, 4, and 25°C for up to 6 months. The viable cell count present in each sample was determined as described above. The number of

TABLE 1 | The primer sequences used for quantitative real-time PCR.

Gene name	Forward (5′ → 3′)	Reverse (5′ → 3′)	Reference
SREBF2	GGCCTCTCCTTTAACCCCTT	CACCATTTACCAGCCACAGG	Ahn et al., 2013
LDLR	GCGTATCTGTGGCTGACACC	TGTCCACACCATTCAAACCC	
HMGCR	GGGACCAACCTTCTACCTC	GCCATCACAGTGCCACATA	Wang et al., 2014
CYP7A1	TCTCAACGATACACTCTCCACC	CTTCAGAGGCTGCTTTCATTGC	
APOE	GAACCGCTTCTGGGATTACCT	TCAGTGCCGTCAGTTCTTGTG	Ding et al., 2012
ACTB	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA	

ACTB, β-actin; SREBF2, sterol regulatory element binding protein 2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, Low-density lipoprotein receptor; CYP7A1, cytochrome P450 family 7 subfamily A polypeptide 1 (cholesterol 7 α-hydroxylase); APOE, apolipoprotein E.

colonies on MRS agar was calculated and the total cfu was calculated for each sample stored at various temperatures and time points.

Statistical analysis

Data are expressed as means ± SEM. The differences among treatment groups were analyzed by One-Way ANOVA, followed by Duncan test, using Origin 7 Software (MicroCal Software, Northampton, MA, USA). Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Identification of Strain KID7 Based on 16S rDNA Sequence and Biochemical Test

BLAST search of the EzTaxon database showed that the 16S rDNA sequence of KID7 (Genbank accession number KJ810576) (1508 bp) showed 99.3% (difference of 10/1499 bp) similarity with *P. pentosaceus* type strain DSM 20336. All other *Pediococcus* species showed less than 98.5%, which is the cut-off value for species-level comparison studies (Stackebrandt, 2011). Phylogenetic analysis of the 16S rRNA gene sequence of KID7 with that of various *Pediococcus* species confirmed its close relatedness to *P. pentosaceus* DSM 20336 (Figure S1). Hence, *P. pentosaceus* KACC 12311 (=DSM 20336) was used for comparative biochemical test with KID7. The biochemical tests of strain KID7 and the closely related strain *P. pentosaceus* KACC 12311 are shown in Table S1. The strain KID7 possess various enzymatic activities such as protease, β-galactosidase and β-glucosidase similar to that of *P. pentosaceus* KACC 12311. It showed difference in the carbohydrate utilization pattern, which is considered as strain-level difference between KID7 and *P. pentosaceus* KACC 12311. Strain KID7 grew well in the presence of NaCl, however, a reduction in cell growth was observed with increasing concentration of NaCl (Figure S2).

In vitro Probiotic Properties of KID7

In vitro safety assessment of KID7

Strain KID7 was negative for hemolytic activity on blood agar (Figure S3B), mucin degradation on mucin-supplied medium (Figure S3C), and bioamine production on various amino acid-supplied decarboxylase agar media (Figure S3A). The minimum inhibitory concentration (MIC) of strain KID7 for selected antibiotics was shown in **Table 2**. The strain KID7 was sensitive to all antibiotics except kanamycin B sulfate and streptomycin sulfate.

Survival of KID7 under simulated oro-gastrointestinal transit condition

The strain KID7 showed good tolerance to oral, gastric and intestinal stress based on the results of fluorescence microscopic analysis for live and dead/damaged cells (**Figure 1A**) and plate count obtained after each stress treatment (**Figure 1B**). We observed a high amount of red fluorescent cells (indicating dead/damaged cells) in the gastric stress (pH 2) and intestinal stress in the fluorescence microscopic analysis (**Figure 1A**). However, we observed that these were only damaged cells, as they recovered during culture on MRS agar, which was evidenced by the number of colony-forming units in the gastric stress and intestinal stress (**Figure 1B**). Overall, a reduction of 1 log unit cfu ($P < 0.05$) was observed at the end of the OGT assay compared to the initial count (**Figure 1B**).

Adherence of KID7 to Caco-2 cell monolayer

The adherence of KID7, *L. rhamnosus* GG, and *L. acidophilus* ATCC 43121 to Caco-2 cell monolayer was calculated as the percentage of cells that remained attached to the Caco-2 monolayer after two washes compared to the initial amount of LAB added (**Figure 2**). Plate count of the attached bacterial cells revealed high adherence of KID7 cells (34.7%) (**Figure 2**) compared to *L. rhamnosus* GG (14.2%) and *L. acidophilus* ATCC 43121 (5%) (**Figure 2**).

Antimicrobial activity of strain KID7

The concentrated culture filtrate from KID7 showed a broad-spectrum antimicrobial activity against human, animal and food-borne pathogens used in this study (**Table 3**). The antimicrobial activity was observed against both gram-positive and gram-negative pathogenic bacteria, whereas no antimicrobial activity was observed against LAB strains tested. We observed that KID7 (Figure S4A) and the reference strain *P. pentosaceus* KACC 12311 (Figure S4B) did not produce H_2O_2, whereas, *L. rhamnosus* GG produces H_2O_2, which is observed as a zone of blue color around the colony on Prussian blue agar (Figure S4C).

Bile salt hydrolase and cholesterol-lowering activity of KID7

The strain KID7 possessed bile salt hydrolase activity that deconjugated TDCA, GCA and TCA (**Figure 3A**). We

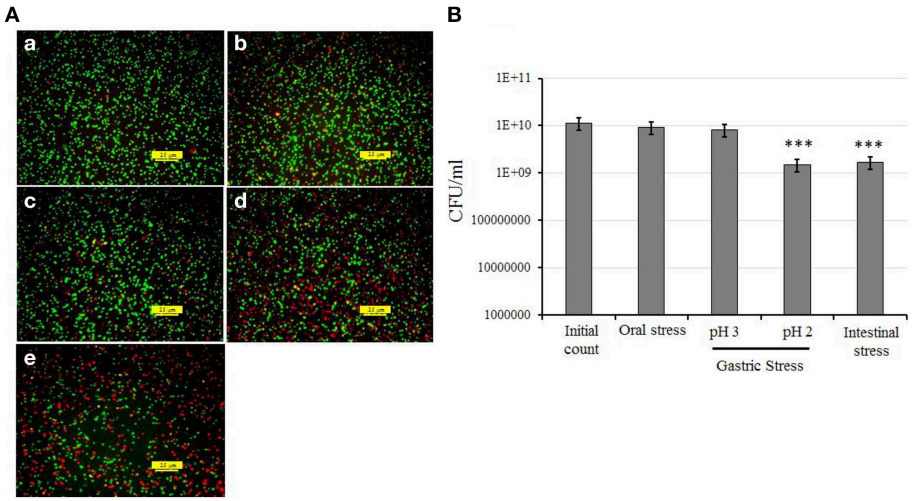


FIGURE 1 | *In vitro* assessment of oro-gastrointestinal transit tolerance of strain KID7 under simulated conditions. (A) Fluorescence photomicrograph of KID7 cells after various stages of simulated oro-gastrointestinal transit: (a) initial, (b) oral stress, (c) gastric stress pH 3, (d) gastric stress pH 2, and (e) intestinal stress. The live, and dead/damaged cells were identified by using the LIVE/DEAD BacLight bacterial viability kit after oral, gastric and intestinal stress treatment. Green color indicates viable bacteria; red color indicates damaged or dead bacteria. **(B)** Viable count of KID7 after each stages of simulated oro-gastrointestinal transit. The viable count of KID7 was determined by plating the cells from each stage on MRS agar and incubation at 37°C for 48 h. Error bar represents standard error of mean of three independent experiments. *** Significantly different from initial count at $P < 0.005$.

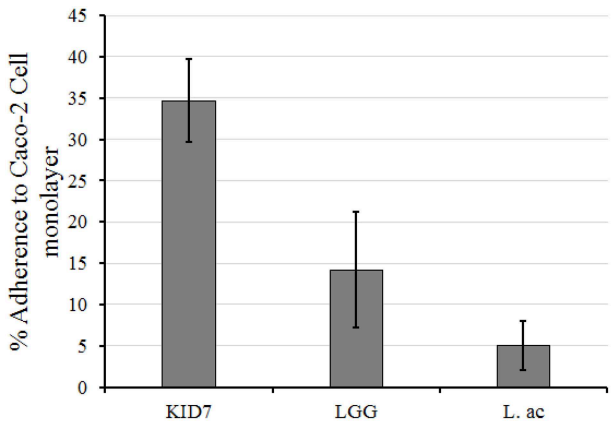


FIGURE 2 | Adherence of *P. pentosaceus* strain KID7, *L. rhamnosus* GG, and *L. acidophilus* ATCC 43121 to Caco-2 cell monolayer. The error bars indicate standard deviation of three independent experiments.

TABLE 2 | Antibiotic susceptibility of *P. pentosaceus* KID7.

Antibiotics	Minimum inhibitory concentration (MIC) (mg/L)	EFSA cut-off (MIC) (mg/L)
Chloramphenicol	1 (S)	4
Tetracyclin hydrochloride	4 (S)	8
Erythromycin	0.25 (S)	1
Streptomycin sulfate	256 (R)	64
Gentamycin C	8 (S)	16
Clindamycin	0.5 (S)	1
Ampicillin	1 (S)	4
Kanamycin B sulfate	1024 (R)	64

Bacterial susceptibility to antibiotic was determined according to the cut-off values given by EFSA (2012). S, Susceptible; R, Resistant.

observed a 40% decrease in *in vitro* cholesterol concentration in MRS-CHO broth after the growth of KID7 for 24 h. However, this reduction level was decreased in the presence of bile oxgal—0.3% (33% cholesterol reduction) and—0.5% (17% cholesterol reduction) (**Figure 3B**). The percentage of cholesterol reduction by KID7 was comparatively higher than that of *P. pentosaceus* KACC 12311 (**Figure 3B**). The reduction in cholesterol-lowering activity of KID7 and *P. pentosaceus* KACC 12311 in the presence of 0.3 and 0.5% bile oxgal may be due to reduced cell growth in the presence of bile compared to bile- non-supplied MRS-CHO broth (**Figure 3C**). These observations suggest that cell concentration is the key factor that affects cholesterol reduction in liquid broth.

In vivo Cholesterol-Lowering Activity of KID7

Effects of KID7 and L. acidophilus on body weight gain, food intake, and organ weight

The body weight gain, food intake, and organ weight are shown in **Table 4**. Body weight gain was not significantly different among all groups. Food intake of the NCD group was higher than that of the HCD control group ($P < 0.005$), but there was no significant difference in food intake among the HCD control and HCD-LAB groups. WAT weight did not differ among all groups. Liver weights in the NCD group were reduced vs. that of the

TABLE 3 | Antimicrobial activity of culture filtrate of strain KID7.

Test strains	Zone of inhibition (mm)*
GRAM POSITIVE PATHOGENS	
Staphylococcus aureus KCCM 11335	15
Staphylococcus epidermidis KCTC 1917	12
Listeria monocytogenes KACC 10764	20
Staphylococcus aureus KCCM 40510 (Methicillin- resistant)	11
Bacillus cereus KACC 11240	10
GRAM NEGATIVE PATHOGENS	
Salmonella typhi KCTC 2514	12
Salmonella choleraesuis KCTC 2932	15
Pseudomonas aeruginosa KCCM 11802	18
Yersinia enterocolitica ssp. *enterocolitica* KACC 15320	20
Salmonella gallinarum KCTC 2931	26
Shigella boydii KACC 10792	14
Escherichia coli O138 KCTC 2615	11
E. coli O1 KCTC 2441	12
LACTIC ACID BACTERIA (GRAM POSITIVE)	
Enterococcus durans KACC 10787	–
Lactobacillus fermentum KACC 11441	–
Lactobacillus plantarum ssp. *plantarum* KACC 11451	–
Lactobacillus brevis KACC 11433	–
Lactobacillus paracasei KACC 12361	–

**Concentrated crude culture filtrate of strain KID7 was used for the antimicrobial assay. –, Indicates no zone of inhibition.*

HCD control group, but did not differ in the HCD control and HCD-LAB groups.

Effects of KID7 and L. acidophilus on the serum biochemical levels

As shown in **Figure 4**, T-CHO and LDL-CHO levels in the NCD group were significantly lower compared to the HCD control group (all, $P < 0.005$). T-CHO levels were significantly decreased by 19.8% in the HCD-KID7 group ($P < 0.05$), but did not in the HCD-*L.ac* group (**Figure 4A**). LDL-CHO levels in both the HCD-KID7 and HCD-*L.ac* group were decreased by an average of 35.5 and 38.7%, respectively, compared with the HCD control group (both, $P < 0.05$) (**Figure 4B**). HDL-CHO and TG level gain was not significantly different among all groups (**Figures 4C,D**). GPT levels in the NCD, HCD-KID7, and HCD-*L.ac* groups were reduced by 30, 30, and 28%, respectively, vs. the HCD control group (all, $P < 0.05$) (**Figure 4E**). GOT levels in the HCD-KID7 and HCD-*L.ac* groups showed the tendency to reduce, but not statistically significant (**Figure 4F**).

Effects of KID7 and L. acidophilus on lipid content in liver and feces

Liver T-CHO levels and TG in the NCD group were lower than that of the HCD control group ($P < 0.001$ and $P < 0.05$, respectively) **Figure 5**. Liver T-CHO levels in the HCD-KID7 group were reduced significantly compared with the HCD control group ($P < 0.05$) (**Figure 5A**). Liver TG levels tended to decrease in the HCD-KID7 group compared with the HCD control group, but not statistically significant (**Figure 5B**). Fecal T-CHO levels tended to increase in the HCD-KID7 group compared with the HCD control group, but not statistically significant ($P = 0.056$) (**Figure 5C**). A significant increase ($P < 0.05$) in fecal cholic acid content was observed in the HCD-KID7 group compared to HCD group. In the HCD-*L.ac* group no significant change in liver total cholesterol, liver triglyceride content, fecal cholesterol and fecal cholic acid contents was noted compared to the HCD control group (**Figures 5A–D**).

Effects of KID7 and L. acidophilus on expression levels of genes associated with cholesterol metabolism in liver

We examined the expression levels of cholesterol metabolism-related genes to understand the mechanism of action of KID7 compared to *L. acidophilus* ATCC 43121 in improving hypercholesterolemia (**Figure 6**). The NCD feeding was found to significantly increase the *SREBF2*, *HMGCR*, *LDLR*, and *APOE* gene expression levels compared to those of the HCD group. *SREBF2* gene expression level did not change in the HCD-KID7 group, but significantly increased in the HCD-*L.ac* group compared to the HCD group (**Figure 6**). *LDLR* expression level was significantly increased in the HCD-KID7 and in the HCD-*L.ac* group. Meanwhile, no significant difference was observed in *HMGCR* expression in the HCD-KID7 compared to HCD group (**Figure 6**). A significant increase in *CYP7A1* mRNA expression level was observed in the HCD-KID7 but not in HCD-*L.ac* group compared to HCD. *APOE* mRNA expression level was significantly increased in the HCD-KID7 and HCD-*L.ac* groups vs. that of the HCD group (**Figure 6**).

Technological Characteristic of KID7

Viability of KID7 under freeze-drying and storage conditions

The viability of strain KID7 after freeze-drying condition in the presence of various food grade additives was given in **Figure 7A**. Among the tested additives, 0.5% skim milk + 0.5% galactomannan (SMG), protected the bacterial cells during freeze-drying condition and the viability was retained at 99.79%, which was better than in the presence of 1% skim milk alone (viability = 99.6%) (**Figure 7A**). Further, we tested KID7 cells in SMG combination for storage at different temperature conditions in freeze-dried form for up to 6 months. The viability of KID7 cells in SMG combination was high at 4°C followed by −20°C and 25°C (**Figure 7B**). After 6 months of storage, KID7 cells in SMG matrix retained >90% viability at 4°C and =89% viability at −20°C, which resulted in 1-log-order decrease in viable cells (**Figure 7B**) whereas at 25°C the viability was reduced to 68% with 2-log-order decrease in the viable cells (**Figure 7B**). These results suggest that KID7 combined with SMG matrix could be stored long term at 4°C in freeze-dried form and demonstrate the technological properties of the strain.

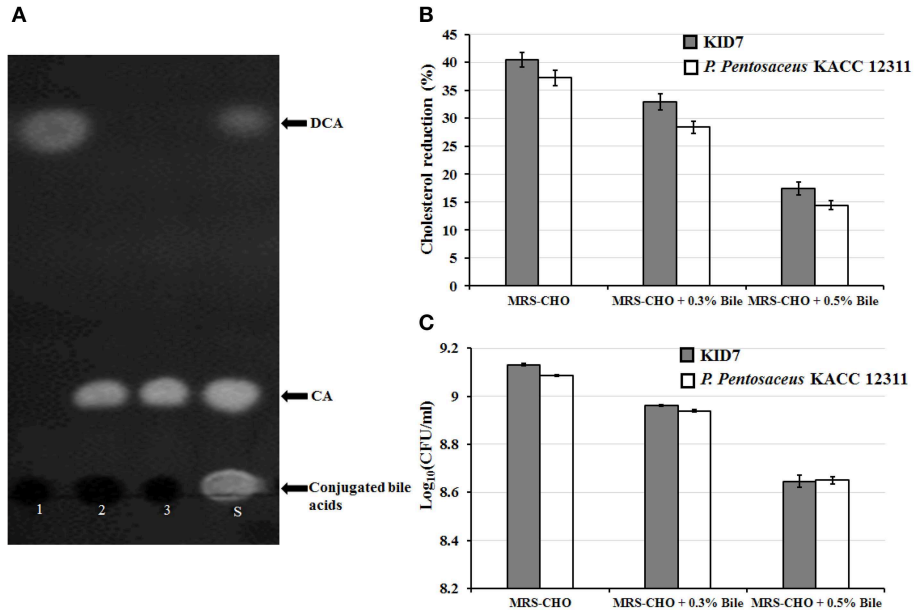


FIGURE 3 | *In vitro* cholesterol-lowering activity of *P. pentosaceus* KID7. (A) Thin-layer chromatography analysis of free cholic acid present in the culture filtrate of KID7 grown in MRS containing various conjugated bile salts. 1, MRS+5 mM TDCA; 2, MRS+5 mM TCA; 3, MRS+5 mM GCA; S, Standard mixture of TDCA, TCA, GCA, cholic acid (CA), and deoxycholic acid (DCA). **(B)** Cholesterol reduction activity of strain KID7 and *P. pentosaceus* KACC 12311 with or without bile-oxgall. **(C)** Growth of KID7 and *P. pentosaceus* KACC 12311 in MRS-CHO medium with or without bile-oxgall represented in log cfu/ml. The data are representation of mean values of three independent assays and error bars indicates ± standard deviation.

TABLE 4 | Effects of KID7 and *L. acidophilus* on body weight, food intake, and tissues weight in mice.

Parameters	Dietary groups			
	NCD	HCD	HCD + KID7	HCD + *L. acidophilus*
BODY WEIGHT (g)				
Initial	21.77 ± 0.48	22.24 ± 0.49	20.89 ± 0.52	21.03 ± 0.37
Final	23.24 ± 0.49	22.01 ± 0.36	21.97 ± 0.50	22.64 ± 0.45
Gain	1.88 ± 0.35	1.56 ± 0.39	1.68 ± 0.40	1.8 ± 0.70
Food intake rate (g/mouse/day)	3.24 ± 0.18	2.50 ± 0.23	2.5 ± 0.21	2.42 ± 0.17
TISSUES WEIGHT (g)				
Liver	0.9 ± 0.03***	1.29 ± 0.05	1.27 ± 0.04	1.21 ± 0.52
Epididymal fat	0.16 ± 0.03	0.16 ± 0.019	0.17 ± 0.015	0.20 ± 0.033

NCD, Normal diet; HCD, high-cholesterol diet; KID7, P. pentosaceus KID7; L.ac, L. acidophilus. Data are expressed as means ± SEM (n = 8/group). *** *Significantly different at P < 0.005 vs. the HCD group.*

Discussion

P. pentosaceus has been approved as an animal feed additive in the United States, European Union, China, Thailand, Australia, and New Zealand (Lim and Tan, 2009). In recent years it has received increased attention and has been characterized for its possible utilization as probiotic in humans (Osmanagaoglu et al., 2012; Zhao et al., 2012; Borges et al., 2013; Vidhyasagar and Jeevaratnam, 2013; Borges and Teixeira, 2014; Garcia-Ruiz et al., 2014; Lee et al., 2014).

Microbes used as probiotics should satisfy certain safety criteria such as being negative for mucin degradation activity (Abe et al., 2010), since microbes degrading mucin can translocate from the intestinal lumen to other body parts and cause bacteremia (Abe et al., 2010). Generally, members of LAB does not possess hemolytic activity. However, *Enterococcus* and *Bacillus* strains possess hemolytic potential and such strains should be adequately tested and confirmed for non-hemolytic activity (FAO-WHO, 2002). Testing for bioamine production is an important safety test, since most of the LAB have the ability to produce bioamines (Bover-Cid and Holzapfel, 1999). These are toxic substances generated from the decarboxylation of amino acids present in foods, the most important being diamines, which are precursors of carcinogenic nitrosamines (Bover-Cid and Holzapfel, 1999). Bover-Cid and Holzapfel (1999) observed that pediococci do not show any ability to produce bioamines,

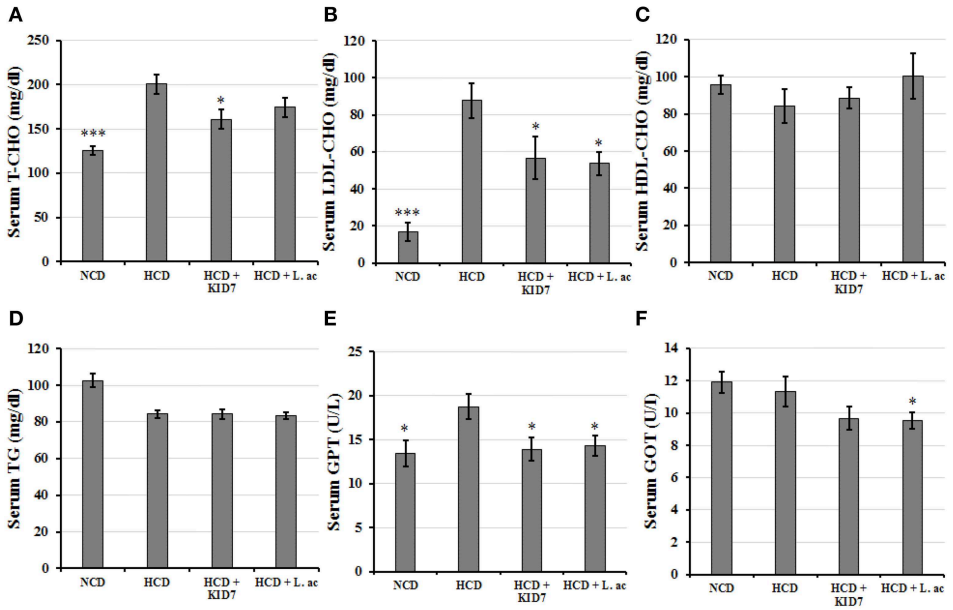


FIGURE 4 | Effects of KID7 and *L. acidophilus* on the serum biochemical parameters. Mice were fed either normal diet, atherogenic diet, or atherogenic diet supplemented with KID7 or *L. acidophilus* for 32 days. **(A)** T- CHO; **(B)** LDL-CHO; **(C)** HDL-CHO; **(D)** TG; **(E)** GPT; **(F)** GOT. NCD: normal diet; HCD: high-cholesterol diet; HCD-KID7: HCD + *P. pentosaceus* KID7; HCD-*L.ac*: HCD + *L. acidophilus*. Data are expressed as means ± SEM ($n = 8$/group). *, *** indicate significantly lower at $P < 0.05$ and $P < 0.005$, respectively, vs. the HCD group.

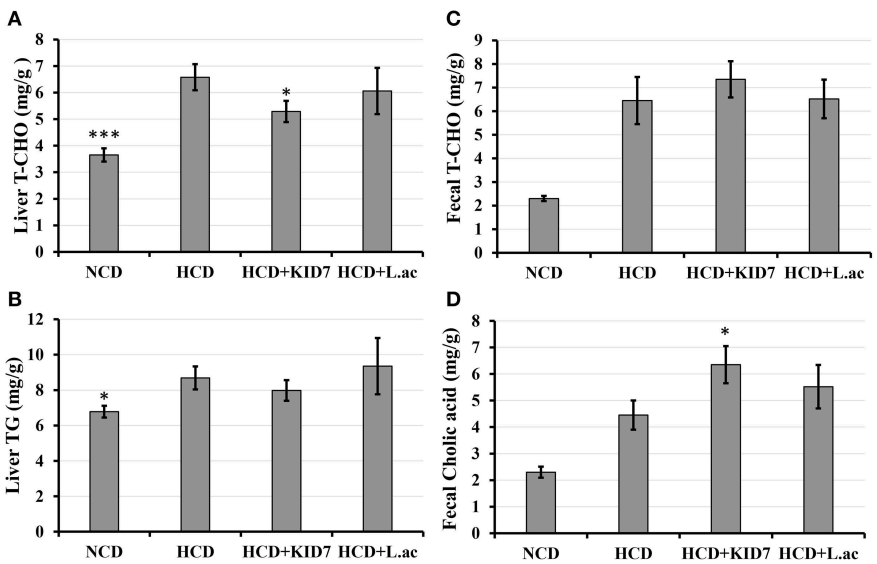


FIGURE 5 | Effects of *P. pentosaceus* KID7 and *L. acidophilus* on hepatic and fecal lipid levels. Mice were fed either normal diet, atherogenic diet or atherogenic diet supplemented with KID7 or *L. acidophilus* for 32 days. **(A)** Liver T-CHO; **(B)** Liver TG; **(C)** Fecal T-CHO, and **(D)** Fecal cholic acid contents. NCD: normal diet; HCD: high-cholesterol diet; HCD-KID7: HCD + *P. pentosaceus* KID7; HCD-*L.ac*: HCD + *L. acidophilus*. Data are expressed as means ± SEM ($n = 8$/group). *, *** indicate significantly different (lower for **A** and **B**, higher for **C** and **D**) at $P < 0.05$ and $P < 0.005$, respectively, vs. the HCD group.

which is in accordance with our observation that KID7 did not produce bioamine on any of the amino acids tested in this study (Figure S2). Antibiotic susceptibility is another key requirement of a probiotic strain used for human or animal consumption (FAO-WHO, 2002; EFSA, 2012). Ingestion of probiotic bacteria with transmissible (plasmid or transposon-borne) antibiotic-resistant genes may lead to generation of new antibiotic-resistant pathogens in the host gut by horizontal gene

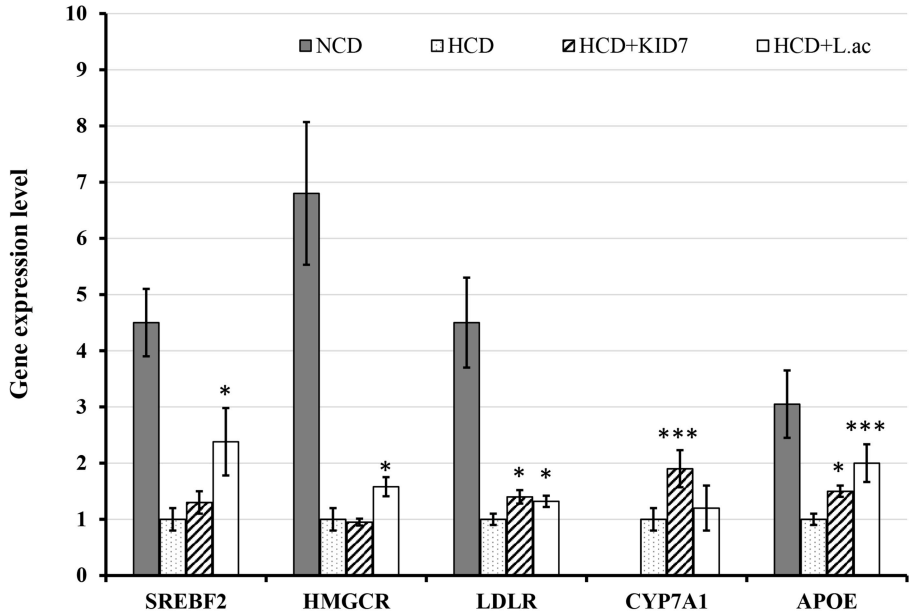


FIGURE 6 | Effects of KID7 and *L. acidophilus* on expression levels of lipid metabolism-related genes in mice liver. The mRNA expression levels were evaluated by quantitative real-time PCR. Data were normalized to β-actin RNA expression levels and then compared to the HCD group, which was assigned a value of 1.0. NCD: normal diet; HCD: high-cholesterol diet; HCD-KID7: HCD + *P. pentosaceus* KID7; HCD-*L.ac*: HCD + *L. acidophilus*. Data are expressed as means ± SEM ($n = 8$/group). *, *** indicate significantly higher at $P < 0.05$ and $P < 0.005$, respectively, vs. the HCD group.

transfer (EFSA, 2012). However, ingestion of probiotic bacteria containing non-transmissible drug resistance genes pose a low potential for horizontal transfer and generally may be used as feed-additive (EFSA, 2012). The strain KID7 was resistant to kanamycin and streptomycin. *Pediococcus pentosaceus* strains were shown to have MICs of 256 to >256 and up to 192 μg/ml for kanamycin and streptomycin, respectively (Danielsen et al., 2007), which are higher than the EFSA recommended cut-off values. Strains showing higher MIC than the EFSA cut-off values are considered resistant and must be studied for the genetic basis of resistance and must be of non-transmissible nature; otherwise, those strains should not be used as feed additives (EFSA, 2012). The aminoglycoside resistance gene *aac(6′)Ie-aph(2″)Ia* has been reported in some *Pediococcus* species (Ammor et al., 2007), which codes for the bifunctional aminoglycoside-modifying enzyme 69-N-aminoglycoside acetyltransferase-20-O-aminoglycoside phosphotransferase (Tenorio et al., 2001). The *aac(6′)Ie-aph(2″)Ia* gene confers high level of resistance to gentamicin in addition to other aminoglycoside antibiotics (Tenorio et al., 2001), however, KID7 was susceptible to gentamicin. The genetic basis of streptomycin and kanamycin resistance in strain KID7 is not known and needs further study to assess the nature of resistance to these aminoglycoside antibiotics.

Several previous reports on *P. pentosaceus* studied the acid and bile tolerance separately (Jonganurakkun et al., 2008; Osmanagaoglu et al., 2010; Vidhyasagar and Jeevaratnam, 2013; Garcia-Ruiz et al., 2014; Lee et al., 2014); however, in real situations, the consumed probiotic should pass through the physiological events of ingestion and digestion of the human gastrointestinal tract (GIT), which include passage through the oral cavity, stomach and the small intestine (Bove et al., 2012). Tolerance to the GIT is one of the selection criterion recommended by FAO-WHO (2002); hence, we studied the tolerance of KID7 to progressive oral, gastric, and intestinal stress (OGT assay) in order to mimic the physiological condition. Tolerance of *L. plantarum* strains to oral and gastric stress up to pH 3 was reported previously (Bove et al., 2012). However, when the pH reduced from 3 to 2 a drastic drop of viability was observed for all *L. plantarum* strains and *L. acidophilus* LA5 (Bove et al., 2012) and at the end of the OGT assay a tendency to recover viability was noticed for bacterial samples (Bove et al., 2012). In our study, we observed KID7 strain was able to recover from cell damage caused by the OGT transit (**Figure 1**), which is in accordance with Bove et al. (2012). Survival and tolerance of the strains are essential to express the probiotic functions in the intestine (Kumar et al., 2011).

Adherence and colonization of LAB strains in the intestine can be studied using human intestinal epithelial cell models such as Caco-2 and HT-29 cell lines, *in vitro* (Tuo et al., 2013). KID7 showed higher adherence to Caco-2 cells as compared to *L. rhamnosus* GG and *L. acidophilus* ATCC 43121. A good adherence capacity can promote gut residence time of a probiotic bacteria, aids in pathogen exclusion and interaction with host cells for the protection of epithelial cells and immune modulation (Lebeer et al., 2008). Several previous reports demonstrated that

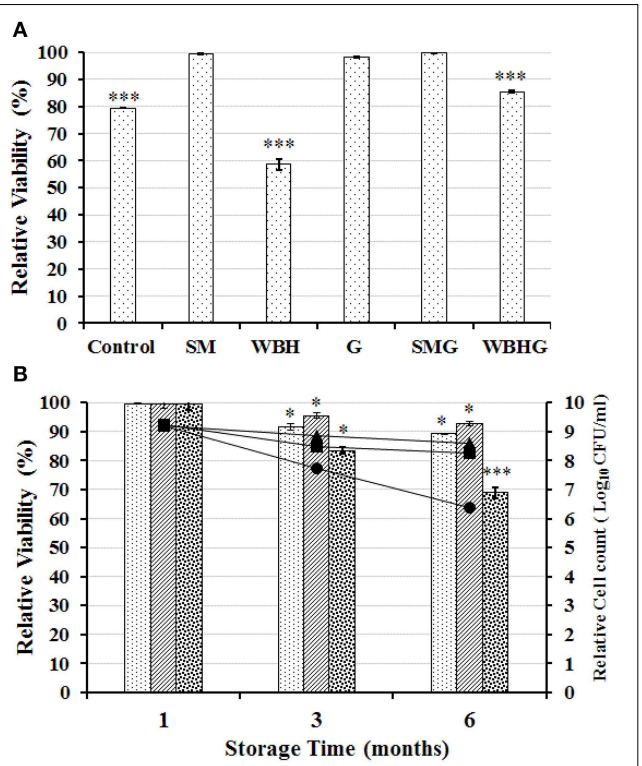


FIGURE 7 | Viability of strains KID7 under freeze-drying and storage conditions. (A) Viability of strain KID7 after freeze-drying with various combinations of food grade additives such as SM, skim milk (1%); G, galactomannan (1%); WBH, wheat bran hydrolysate (1%); SMG, skim milk (0.5%) + galactomannan (0.5%); WBHG, wheat bran hydrolysate (0.5%) + galactomannan (0.5%); and Control, KID7 cells in PBS without food grade additives. **(B)** Viability of strain KID7 combined with SMG, skim milk (0.5 %) + galactomannan (0.5%) under different storage conditions such as −20°C (▦, ■), 4°C (▨, ▲), and 25°C (▩, ●), up to 6 months. Histogram represents percentage viability of KID7 under different storage condition. Line graph represents relative logarithmic value of colony-forming units of KID7 under different storage condition. Error bars represent standard error of mean of three independent experiments. *$P < 0.05$ and ***$P < 0.005$ significantly different at the *P*-values compared to initial count.

P. pentosaceus strains adhered to intestinal epithelial cells better than *Lactobacillus* strains (Turpin et al., 2012; Thirabunyanon and Hongwittayakorn, 2013; Garcia-Ruiz et al., 2014).

Antimicrobial activity of LAB against pathogens can be due to the production of organic acids, hydrogen peroxide (H_2O_2), and bacteriocins (Vesterlund, 2009). KID7 does not produce H_2O_2 (Figure S5). The antimicrobial activity of culture filtrate from KID7 against gram-positive and -negative pathogenic bacteria and not against LAB strains suggest that the antimicrobial activity could be due to the production of organic acids.

BSH enzymes deconjugate the conjugated bile acids into the amino acids (glycine or taurine) and free bile acids (cholic acid or deoxycholic acid) (Joyce et al., 2014). It has been reported that high-level expression of BSH activity by gut microbiota resulted in host weight reduction and cholesterol reduction (Joyce et al., 2014). Hence, BSH activity is an important selection criterion for probiotic microbes to be used for cholesterol reduction. Furthermore, BSH activity is also among the selection criteria recommended by FAO-WHO (2002) for screening potential probiotic strains. Bile acids are synthesized in the liver using cholesterol as precursor. BSH activity of probiotics in the gut could modify the conjugated bile acids and excretion of the free bile acids, which results in *de novo* synthesis of conjugated bile acids from cholesterol to maintain hepatic bile acid pool (Herrema et al., 2010; Degirolamo et al., 2014). Thus, bacterial BSH activity in the gut acts as a sink for cholesterol (Joyce et al., 2014).

Several LAB strains have been reported to possess *in vitro* cholesterol assimilation/uptake activity. LAB exhibit mechanisms, such as cholesterol adsorption to cell wall, and cholesterol assimilation into cell membrane (Liong and Shah, 2005a) and co-precipitation with deconjugated bile acids (Liong and Shah, 2005b) in *in vitro* assays. Cholesterol removal from culture medium by KID7 could involve any of these mechanisms. Previous reports on cholesterol reduction by *Pediococcus* strains showed varying levels (58–73%) of cholesterol reduction in liquid medium supplied with 100–200 μg/ml of cholesterol in the presence of 0.3% bile (Vidhyasagar and Jeevaratnam, 2013). In the present study, we observed a reduction in cholesterol-lowering activity in the presence of 0.3 and 0.5% bile oxgall, dose-dependently (**Figure 3B**). This reduction in the cholesterol assimilation activity is due to reduction in cell growth of KID7 in 0.3 and 0.5% bile oxgall (**Figure 3C**). The reduction in cell growth in the presence of oxgall was also reported for a *L. fermentum* strain (Pereira et al., 2003), however, the cholesterol assimilation activity increased despite low growth rate. In our case, lower growth led to lower cholesterol assimilation by KID7 cells. Although, KID7 tolerated bile during brief exposure of 2 h and showed good viability in the OGT assay, growth of the strain was significantly affected during longer exposure to bile as is the case with cholesterol-lowering activity in the presence of bile. The type strain *P. pentosaceus* KACC 12311 also followed the same pattern as KID7; however, it showed relatively lower growth and cholesterol assimilation activity in the presence and absence of bile oxgall. The lower growth of the KID7 and *P. pentosaceus* KACC 12311 in bile-supplied medium could be due to bile salt-deconjugating activity of the strains, which leads to accumulation of free bile acids that are more toxic than conjugated bile (Pereira et al., 2003). However, these *in vitro* assays do not reflect the true nature of probiotic strains in cholesterol-lowering activity, since the conditions *in vivo* are completely different. Hence, the probiotic activity of any strain must be validated through *in vivo* studies.

We tested the strain KID7 *in vivo* to confirm the cholesterol-lowering activity in atherogenic diet-induced hypercholesterolemic mice in comparison with the previously reported cholesterol-lowering probiotic strain *L. acidophilus* ATCC 43121 as positive control. Supplementation of hypercholesterolemic mice with strain KID7 significantly lowered the serum total cholesterol level, LDL level and hepatic total cholesterol level. Reduction in the serum LDL level and cholesterol level correlated with our observation of increased expression of genes related to cholesterol metabolism in liver such as LDLR and ApoE (**Figure 6**). Increased expression of

LDL-receptor will lead to increased absorption of LDL by hepatocytes. The absorbed LDL is degraded and the cholesterol is added to the cholesterol pool (Cohen, 2008). An increase in cholesterol pool will down regulate HMG CoA-reductase (*HMGCR*) and LDL receptor (*LDLR*) expression. In our study, we did not observe any significant down regulation in *HMGCR* expression in the HCD-KID7 group compared to HCD group, whereas *LDLR* expression and *APOE* expression increased in the HCD-KID7 group. This could be due to sinking cholesterol level in cholesterol pool due to expression of *CYP7A1* (**Figure 6**), which codes for the enzyme cholesterol 7-α-hydroxylase, which catalyzes a rate-limiting step in the *de novo* synthesis of bile acids from cholesterol (Cohen, 2008). Bile salts are secreted into bile duct at a rate of ∼24 g/day, but their synthesis is only a fraction of that rate, because most of the bile salts are recycled to the liver from the ileum (Cohen, 2008). Reduction in bile salt reabsorption due to bile salt hydrolase activity in the gut will cause bile synthesis from cholesterol to maintain the bile salts pool size. The increased expression of *CYP7A1* and a decrease in hepatic cholesterol pool also correlated with an increase in total cholic acid excretion in feces of the HCD-KID7 group (**Figure 5D**). These results suggests that cholesterol-lowering activity of KID7 *in vivo* could be due to its bile salt hydrolase activity. We also observed a significant increase in the level of hepatic LDL mRNA expression in the HCD-*L.ac* group, which is consistent with Park et al. (2007). In addition to the *LDLR* gene, we observed the expression of *SREBF2*, and *APOE* genes in the HCD-*L.ac* group, which was not previously reported in studies using *L. acidophilus* ATCC 43121.

A previous study (Moon et al., 2014) on lipid-lowering effect of *Pediococcus acidilactici* strain M76 and *P. acidilactici* DSM 20284 reported a reduction in serum cholesterol level of 12 and 2.4%, respectively, and a reduction in total hepatic cholesterol level of 30.5 and 14.9%, respectively for the two *P. acidilactici* strains used in their study. Compared to those results, KID7 showed a 19.8% reduction in serum total cholesterol level, a 35.5% reduction in LDL cholesterol level, and a reduction in liver total cholesterol level of 19.7%. Our results indicated that KID7 could be used as a potential cholesterol-lowering probiotic that needs to be confirmed by clinical trials in humans.

Probiotic supplements are usually in the form of dry powders to reduce water activity and protect viability. The preferred drying method is freeze-drying as it preserves viability of the probiotic bacteria. However, not all strains tolerate drying and may show significant loss of viability (Ross et al., 2005). Hence, protective agents and stabilizing agents are added while freeze-drying (Ross et al., 2005). The combination of skim milk and galactomannan increased the viability of KID7 after freeze-drying and subsequent storage conditions. It was reported that skim milk acts as a cryoprotectant and prevents the cells from the damage during freeze-drying (Ross et al., 2005). Additionally, galactomannan from locust bean is a natural polysaccharide used as emulsifying, thickening, and stabilizing agent in the food industry (Cheow et al., 2014). It has been reported elsewhere that spray-drying of probiotic *Lactobacillus paracasei* NFBC in a milk medium supplemented with gum acacia provided better protection to the cells than milk powder alone (Desmond et al., 2002).

In conclusion, the *P. pentosaceus* KID7 reported in this study exhibited cholesterol-lowering activity *in vivo* and possessed the essential characteristics to become a potential probiotic. Furthermore, KID7 maintained good viability during freeze-drying and storage when formulated with a combination of skim milk and galactomannan, which can be utilized in the large-scale production of *P. pentosaceus* KID7-based probiotic products after validation of the probiotic ability in human clinical trials.

Acknowledgments

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01133402)" Rural Development Administration, Republic of Korea.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00768

References

Abe, F., Muto, M., Yaeshima, T., Iwatsuki, K., Aihara, H., Ohashi, Y., et al. (2010). Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability. *Anaerobe* 16, 131–136. doi: 10.1016/j.anaerobe.2009.07.006

Ahn, T. G., Lee, J. Y., Cheon, S. Y., An, H. J., and Kook, Y. B. (2013). Protective effect of Sam-Hwang-Sa-Sim-Tang against hepatic steatosis in mice fed a high-cholesterol diet. *BMC Complement. Altern. Med.* 13:366. doi: 10.1186/1472-6882-13-366

Ammor, M. S., Flórez, A. B., and Mayo, B. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiol.* 24, 559–570. doi: 10.1016/j.fm.2006.11.001

Borges, S., Barbosa, J., Silva, J., and Teixeira, P. (2013). Evaluation of characteristics of *Pediococcus* spp. to be used as a vaginal probiotic. *J. Appl. Microbiol.* 115, 527–538. doi: 10.1111/jam.12232

Borges, S., and Teixeira, P. (2014). *Pediococcus pentosaceus* SB83 as a potential probiotic incorporated in a liquid system for vaginal delivery. *Benef. Microbes* 5, 421–426. doi: 10.3920/BM2013.0084

Bove, P., Gallone, A., Russo, P., Capozzi, V., Albenzio, M., Spano, G., et al. (2012). Probiotic features of *Lactobacillus plantarum* mutant strains. *Appl. Microbiol. Biotechnol.* 96, 431–441. doi: 10.1007/s00253-012-4031-2

Bover-Cid, S., and Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.* 53, 33–41. doi: 10.1016/S0168-1605(99)00152-X

Chavez, M. N., and Krone, C. L. (1976). Silicic acid thin-layer chromatography of conjugated and free bile acids. *J. Lipid Res.* 17, 545–547.

Cheow, W. S., Kiew, T. Y., and Hadinoto, K. (2014). Controlled release of *Lactobacillus rhamnosus* biofilm probiotics from alginate-locust bean gum microcapsules. *Carbohydr. Polym.* 103, 587–595. doi: 10.1016/j.carbpol.2014.01.036

Cohen, D. E. (2008). Balancing cholesterol synthesis and absorption in the gastrointestinal tract. *J. Clin. Lipidol.* 2, S1–S3. doi: 10.1016/j.jacl.2008.01.004

Danielsen, M., Simpson, P. J., O'Connor, E. B., Ross, R. P., and Stanton, C. (2007). Susceptibility of *Pediococcus* spp. to antimicrobial agents. *J. Appl. Microbiol.* 102, 384–389. doi: 10.1111/j.1365-2672.2006.03097.x

Degirolamo, C., Rainaldi, S., Bovenga, F., Murzilli, S., and Moschetta, A. (2014). Microbiota modification with probiotics induces hepatic bile acid synthesis via downregulation of the Fxr-Fgf15 axis in mice. *Cell Rep.* 7, 12–18. doi: 10.1016/j.celrep.2014.02.032

Desmond, C., Ross, R. P., O'callaghan, E., Fitzgerald, G., and Stanton, C. (2002). Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *J. Appl. Microbiol.* 93, 1003–1011. doi: 10.1046/j.1365-2672.2002.01782.x

Ding, X., Fan, S., Lu, Y., Zhang, Y., Gu, M., and Zhang, L. (2012). *Citrus ichangensis* peel extract exhibits anti-metabolic disorder effects by the inhibition of PPARγ and LXR signaling in high-fat diet-induced C57BL/6 mouse. *Evid. Based Complement. Alternat. Med.* 2012:678592. doi: 10.1155/2012/678592

EFSA. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.* 10:2740. doi: 10.2903/j.efsa.2012.2740

FAO-WHO. (2002). *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food.* Food and Agricultural Organization of the United Nations. Available online at: ftp://ftp.fao.org/es/esn/food/wgreport2.pdf

Folch, J., Lees, M., and Stanley, G. H. S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509.

Friedewald, W. T., Levy, R. I., and Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* 18, 499–502.

Fuentes, M. C., Lajo, T., Carrión, J. M., and Cuñé, J. (2013). Cholesterol-lowering efficacy of *Lactobacillus plantarum* CECT 7527, 7528 and 7529 in hypercholesterolaemic adults. *Br. J. Nutr.* 109, 1866–1872. doi: 10.1017/S000711451200373X

García-Ruiz, A., González De Llano, D., Esteban-Fernández, A., Requena, T., Bartolomé, B., and Moreno-Arribas, M. V. (2014). Assessment of probiotic properties in lactic acid bacteria isolated from wine. *Food Microbiol.* 44, 220–225. doi: 10.1016/j.fm.2014.06.015

Guardamagna, O., Amaretti, A., Puddu, P. E., Raimondi, S., Abello, F., Cagliero, P., et al. (2014). Bifidobacteria supplementation: effects on plasma lipid profiles in dyslipidemic children. *Nutrition* 30, 831–836. doi: 10.1016/j.nut.2014.01.014

Guo, C. F., Zhang, L. W., Han, X., Li, J. Y., Du, M., Yi, H. X., et al. (2011). A sensitive method for qualitative screening of bile salt hydrolase-active lactobacilli based on thin-layer chromatography. *J. Dairy Sci.* 94, 1732–1737. doi: 10.3168/jds.2010-3801

Herrema, H., Meissner, M., Van Dijk, T. H., Brufau, G., Boverhof, R., Oosterveer, M. H., et al. (2010). Bile salt sequestration induces hepatic *de novo* lipogenesis through farnesoid X receptor and liver X receptor alpha-controlled metabolic pathways in mice. *Hepatology* 51, 806–816. doi: 10.1002/hep.23408

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

Ishimwe, N., Daliri, E. B., Lee, B. H., Fang, F., and Du, G. (2015). The perspective on cholesterol-lowering mechanisms of probiotics. *Mol. Nutr. Food. Res.* 59, 94–105. doi: 10.1002/mnfr.201400548

Jones, M. L., Martoni, C. J., Parent, M., and Prakash, S. (2012). Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active *Lactobacillus reuteri* NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *Br. J. Nutr.* 107, 1505–1513. doi: 10.1017/S0007114511004703

Jones, M. L., Tomaro-Duchesneau, C., and Prakash, S. (2014). The gut microbiome, probiotics, bile acids axis, and human health. *Trends Microbiol.* 22, 306–308. doi: 10.1016/j.tim.2014.04.010

Jonganurakkun, B., Wang, Q., Xu, S. H., Tada, Y., Minamida, K., Yasokawa, D., et al. (2008). *Pediococcus pentosaceus* NB-17 for probiotic use. *J. Biosci. Bioeng.* 106, 69–73. doi: 10.1263/jbb.106.69

Joyce, S. A., Macsharry, J., Casey, P. G., Kinsella, M., Murphy, E. F., Shanahan, F., et al. (2014). Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. *Proc. Natl. Acad. Sci. U.S.A.* 111, 7421–7426. doi: 10.1073/pnas.1323599111

Kumar, R., Grover, S., and Batish, V. K. (2011). Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillus plantarum* strains in Sprague-Dawley rats. *Br. J. Nutr.* 105, 561–573. doi: 10.1017/S0007114510003740

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. Rev.* 72, 728–764. doi: 10.1128/MMBR.00017-08

Lee, K. W., Park, J. Y., Sa, H. D., Jeong, J. H., Jin, D. E., Heo, H. J., et al. (2014). Probiotic properties of *Pediococcus* strains isolated from jeotgals, salted and fermented Korean sea-food. *Anaerobe* 28, 199–206. doi: 10.1016/j.anaerobe.2014.06.013

Lim, A., and Tan, H.-M. (2009). "Probiotics: legal status and regulatory issues section 1.8.2. animal probiotics" in *Handbook of Probiotics and Prebiotics*, eds Y. K. Lee and S. Salminen (Hoboken, NJ: John Wiley & Sons, Inc.). 123–139.

Liong, M. T., and Shah, N. P. (2005a). Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* strains. *J. Dairy Sci.* 88, 55–66. doi: 10.3168/jds.S0022-0302(05)72662-X

Liong, M. T., and Shah, N. P. (2005b). Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. *Int. Dairy J.* 15, 391–398. doi: 10.1016/j.idairyj.2004.08.007

Marteau, P., Minekus, M., Havenaar, R., and Huis in't Veild, J. H. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J. Diary Sci.* 80, 1031–1037. doi: 10.3168/jds.S0022-0302(97)76027-2

Mcdonald, L. C., Mcfeeters, R. F., Daeschel, M. A., and Fleming, H. P. (1987). A differential medium for the enumeration of homofermentative and heterofermentative lactic acid bacteria. *Appl. Environ. Microbiol.* 53, 1382–1384.

Moon, Y. J., Baik, S. H., and Cha, Y. S. (2014). Lipid-lowering effects of *Pediococcus acidilactici* M76 isolated from Korean traditional makgeolli in high fat diet-induced obese mice. *Nutrients* 6, 1016–1028. doi: 10.3390/nu6031016

Nagpal, R., Kumar, A., Kumar, M., Behare, P. V., Jain, S., and Yadav, H. (2012). Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS Microbiol. Lett.* 334, 1–15. doi: 10.1111/j.1574-6968.2012.02593.x

Osmanagaoglu, O., Kiran, F., and Ataoglu, H. (2010). Evaluation of *in vitro* probiotic potential of *Pediococcus pentosaceus* OZF isolated from human breast milk. *Probiotics Antimicrob. Proteins* 2, 162–174. doi: 10.1007/s12602-010-9050-7

Osmanagaoglu, O., Kiran, F., Yagci, F. C., and Gursel, I. (2012). Immunomodulatory function and *in vivo* properties of *Pediococcus pentosaceus* OZF, a promising probiotic strain. *Ann. Microbiol.* 63, 1311–1318. doi: 10.1007/s13213-012-0590-9

Park, Y. H., Kim, J. G., Shin, Y. W., Kim, S. H., and Whang, K. Y. (2007). Effect of dietary inclusion of *Lactobacillus acidophilus* ATCC 43121 on cholesterol metabolism in rats. *J. Microbiol. Biotechnol.* 17, 655–662.

Pavlovic, N., Stankov, K., and Mikov, M. (2012). Probiotics interactions with bile acids and impact on cholesterol metabolism. *Appl. Biochem. Biotechnol.* 168, 1880–1895. doi: 10.1007/s12010-012-9904-4

Pereira, D. I., Mccartney, A. L., and Gibson, G. R. (2003). An *in vitro* study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Appl. Environ. Microbiol.* 69, 4743–4752. doi: 10.1128/AEM.69.8.4743-4752.2003

Ross, R. P., Desmond, C., Fitzgerald, G. F., and Stanton, C. (2005). Overcoming the technological hurdles in the development of probiotic foods. *J. Appl. Microbiol.* 98, 1410–1417. doi: 10.1111/j.1365-2672.2005.02654.x

Saito, M., Seki, M., Iida, K.-I., Nakayama, H., and Yoshida, S.-I. (2007). A novel agar medium to detect hydrogen peroxide-producing bacteria based on the prussian blue-forming reaction. *Microbiol. Immunol.* 51, 889–892. doi: 10.1111/j.1348-0421.2007.tb03971.x

Stackebrandt, E. (2011). Molecular taxonomic parameters. *Microbiol. Aust.* 2, 59–61.

Sultan, S., and Hynes, N. (2013). The ugly side of statins. Systemic appraisal of the contemporary un-known unknowns. *Open J. Endocr. Metab. Dis.* 03, 179–185. doi: 10.4236/ojemd.2013.33025

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tenorio, C., Zarazaga, M., Martinez, C., and Torres, C. (2001). Bifunctional enzyme 6′-*N*-aminoglycoside acetyltransferase-2"-O- aminoglycoside phosphotransferase in *Lactobacillus* and *Pediococcus* isolates of animal origin. *J. Clin. Microbiol.* 39, 824–825. doi: 10.1128/JCM.39.2.824–825.2001

Thirabunyanon, M., and Hongwittayakorn, P. (2013). Potential probiotic lactic acid bacteria of human origin induce antiproliferation of colon cancer cells via synergic actions in adhesion to cancer cells and short-chain fatty acid bioproduction. *Appl. Biochem. Biotechnol.* 169, 511–525. doi: 10.1007/s12010-012-9995-y

Trautvetter, U., Ditscheid, B., Kiehntopf, M., and Jahreis, G. (2012). A combination of calcium phosphate and probiotics beneficially influences intestinal lactobacilli and cholesterol metabolism in humans. *Clin. Nutr.* 31, 230–237. doi: 10.1016/j.clnu. 2011.09.013

Tuo, Y., Yu, H., Ai, L., Wu, Z., Guo, B., and Chen, W. (2013). Aggregation and adhesion properties of 22 *Lactobacillus* strains. *J. Dairy Sci.* 96, 4252–4257. doi: 10.3168/jds.2013-6547

Turpin, W., Humblot, C., Noordine, M. L., Thomas, M., and Guyot, J. P. (2012). *Lactobacillaceae* and cell adhesion: genomic and functional screening. *PLoS ONE* 7:e38034. doi: 10.1371/journal.pone.0038034

Vesterlund, S. (2009). "Mechanisms of probiotics: production of antimicrobial substances," in *Handbook of Probiotics and Prebiotics*, eds Y. K. Lee, and S. Salminen (Hoboken, NJ: JohnWiley & Sons, Inc.).

Vidhyasagar, V., and Jeevaratnam, K. (2013). Evaluation of *Pediococcus pentosaceus* strains isolated from Idly batter for probiotic properties *in vitro*. *J. Func. Foods* 5, 235–243. doi: 10.1016/j.jff.2012.10.012

Wang, H., Chen, G., Ren, D., and Yang, S. T. (2014). Hypolipidemic activity of Okra is mediated through inhibition of lipogenesis and upregulation of cholesterol degradation. *Phytother. Res.* 28, 268–273. doi: 10.1002/ptr.4998

Wang, J., Yuan, X., Sun, B., Tian, Y., and Cao, Y. (2009). Scavenging activity of enzymatic hydrolysates from wheat bran. *Food Technol. Biotechnol.* 47, 39–46.

Wiegand, I., Hilpert, K., and Hancock, R. E. (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

Zhao, X., Higashikawa, F., Noda, M., Kawamura, Y., Matoba, Y., Kumagai, T., et al. (2012). The obesity and fatty liver are reduced by plant-derived *Pediococcus pentosaceus* LP28 in high fat diet-induced obese mice. *PLoS ONE* 7:e30696. doi: 10.1371/journal.pone.0030696

Zhou, J. S., Gopal, P. K., and Gill, H. S. (2001). Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin *in vitro*. *Int. J. Food Microbiol.* 63, 81–90. doi: 10.1016/S0168-1605(00)00398-6

Conflict of Interest Statement: 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: 30 October 2015
doi: 10.3389/fmicb.2015.01177

Milk kefir: composition, microbial cultures, biological activities, and related products

*Maria R. Prado[1], Lina Marcela Blandón[1], Luciana P. S. Vandenberghe[1], Cristine Rodrigues[1], Guillermo R. Castro[2], Vanete Thomaz-Soccol[1] and Carlos R. Soccol[1]**

[1] Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná, Curitiba, Brazil, [2] Nanobiomaterials Laboratory, Institute of Applied Biotechnology – School of Sciences, Universidad Nacional de la Plata, La Plata, Argentina

In recent years, there has been a strong focus on beneficial foods with probiotic microorganisms and functional organic substances. In this context, there is an increasing interest in the commercial use of kefir, since it can be marketed as a natural beverage that has health promoting bacteria. There are numerous commercially available kefir based-products. Kefir may act as a matrix in the effective delivery of probiotic microorganisms in different types of products. Also, the presence of kefir's exopolysaccharides, known as kefiran, which has biological activity, certainly adds value to products. Kefiran can also be used separately in other food products and as a coating film for various food and pharmaceutical products. This article aims to update the information about kefir and its microbiological composition, biological activity of the kefir's microflora and the importance of kefiran as a beneficial health substance.

Keywords: kefir, biological activity, polysaccharides, kefiran, microbial composition

OPEN ACCESS

Edited by:
Maria De Angelis, University of Bari Aldo Moro, Italy

Reviewed by:
Theo Varzakas, Technological Educational Institute of Peloponnese, Greece
Cristiana Garofalo, Università Politecnica delle Marche, Italy

****Correspondence:***
Carlos R. Soccol
soccol@ufpr.br

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

Received: *22 April 2015*
Accepted: *12 October 2015*
Published: *30 October 2015*

Citation:
Prado MR, Blandón LM, Vandenberghe LPS, Rodrigues C, Castro GR, Thomaz-Soccol V and Soccol CR (2015) Milk kefir: composition, microbial cultures, biological activities, and related products. Front. Microbiol. 6:1177. doi: 10.3389/fmicb.2015.01177

INTRODUCTION

Kefir is an acidic-alcoholic fermented milk product with little acidic taste and creamy consistency that was originated in the Balkans, in Eastern Europe, and in the Caucasus (Fontán et al., 2006; Serafini et al., 2014). Kefir can be produced by fermenting milk with commercial freeze-dried kefir starter cultures, traditional kefir grains, and the product that remains after the removal of kefir grains (Bensmira et al., 2010). Kefir grains are a kind of yogurt starter, which are white to yellow – white, gelatinous, and variable in size (varying from 0.3–3.5 cm in diameter) and are composed by a microbial symbiotic mixture of lactic acid bacteria (10^8 CFU/g), yeast (10^6–10^7 CFU/g), and acetic acid bacteria (10^5 CFU/g) that stick to a polysaccharide matrix (Garrote et al., 2010; Chen et al., 2015). After successive fermentations, kefir grains can break up to new generation grains, which have the same characteristics as the old ones (Gao et al., 2012).

Commercial kefir is produced by two methods: The "Russian method" and the pure cultures. In the "Russian method" kefir is produced on a larger scale, using a series fermentation process, beginning with the fermentation of the grains and using the percolate. The other method employs pure cultures isolated from kefir grains or commercial cultures (Leite et al., 2013). Also, the industrial or commercial process uses direct-to-vat inoculation (DVI) or direct-to-vat set (DVS) kefir starter cultures. In addition, *Bifidobacterium* sp., *Lactobacillus* sp. and probiotic yeast (*Saccharomyces boulardii*) may be used as adjunct cultures when blended with kefir grains or kefir

DVI cultures (Wszolek et al., 2006). On the other hand, whey may be a practical base for kefir culture production, and fermented whey has shown to be a suitable cryoprotective medium during freeze-drying. The freeze-dried culture retains a high survival rate and shows good metabolic activity and fermentation efficiency, indicating a good potential for its use as a value-added starter culture in dairy technology. All of these studies have shown promising perspectives for the application of kefir grains in whey valorization strategies (Bensmira et al., 2010; Cheirsilp and Radchabut, 2011).

Traditionally, kefir is manufactured using cow, ewe, goat, or buffalo milk. However, in some countries, animal milk is scarce, expensive, or minimally consumed due to dietary constraints, preferences, or religious customs. Therefore, there have been many attempts to produce kefir from a variety of food sources such as soy milk (Botelho et al., 2014). Historically, kefir has been linked with health, for example, in Soviet countries, kefir has been recommended for consumption by healthy people to restrain the risk of some diseases (Saloff-Coste, 1996; St-Onge et al., 2002; Farnworth and Mainville, 2003). The consumption of this fermented milk has been related to a variety of health benefits (Vujičič et al., 1992; McCue and Shetty, 2005; Rodrigues et al., 2005a) not only linked to its microflora, but also due to the presence of some metabolic products as organic acids (Garrote et al., 2001; Ismaiel et al., 2011). In addition, kefir cultures have the ability to assimilate cholesterol in milk (Yanping et al., 2009). On the other hand, there is a growing commercial interest in using kefir as a suitable food matrix for supplementation with health-promoting bacteria. Kefir may not only be a natural probiotic beverage, but also acts as an effective matrix for the delivery of probiotic microorganisms (Vinderola et al., 2006; Medrano et al., 2008; Oliveira et al., 2013).

In kefir grains the main polysaccharide is kefiran, which is a heteropolysaccharide composed by equal proportions of glucose and galactose and is mainly produced by *Lactobacillus kefiranofaciens* (Zajšek et al., 2011). It has been demonstrated that kefiran improves the viscosity and viscoelastic properties of acid milk gels (Rimada and Abraham, 2006), and is able to form gels that have interesting viscoelastic properties at low temperatures, because of that, kefiran can also be used as an additive in fermented products. Besides, kefiran can enhance the rheological properties of chemically acidified skim milk gels increasing their apparent viscosity (Zajšek et al., 2013).

Compared with other polysaccharides, kefiran has outstanding advantages such as antitumor, antifungal, antibacterial properties (Cevikbas et al., 1994; Wang et al., 2008) immunomodulation or epithelium protection (Serafini et al., 2014), anti-inflammatory (Rodrigues et al., 2005b), healing (Rodrigues et al., 2005a), and antioxidant activity (Chen et al., 2015).

This review presents the most recent advances about kefir and kefiran, their production and microbial cultures involved, biological activities and potential applications in health and food industries.

MICROBIAL COMPOSITION OF KEFIR GRAINS AND KEFIR

Kefir grains have a complex composition of microbial species such as the predominance of lactic acid bacteria, acetic bacteria, yeasts, and fungi (Jianzhong et al., 2009; Pogačić et al., 2013). This microbial species are classified into four groups: homofermentative and heterofermentative lactic acid bacteria and lactose and non-lactose assimilating yeast (Cheirsilp and Radchabut, 2011). In that way, *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus plantarum*, and *L. kefiranofaciens* are predominant species. However, these species represent only 20% of the *Lactobacillus* in the final fermented beverage, with the remainder consisting of *Lactobacillus kefiri* (80%; Yüksekdag et al., 2004; Zanirati et al., 2015). *Acetobacter aceti* and *A. rasens* have also been isolated, such as the fungus *Geotrichum candidum*. More than 23 different yeast species have been isolated from kefir grains and from fermented beverages of different origins. However, the predominant species are *Saccharomyces cerevisiae, S. unisporus, Candida kefyr*, and *Kluyveromyces marxianus* ssp. *marxianus* (Witthuhn et al., 2004; Diosma et al., 2014; Zanirati et al., 2015; **Table 1**).

The microbial composition may vary according to kefir origin, the substrate used in the fermentation process and the culture maintenance methods. Tibetan kefir, which is used in China, is composed of *Lactobacillus, Lactococcus,* and yeast. Additionally, acetic acid bacteria have been identified in Tibetan kefir, depending on the region in China from where it was obtained (Gao et al., 2012), additionally, Tibetan kefir composition differs from that of Russian kefir, Irish kefir, Taiwan kefir, Turkey fermented beverage with kefir; however, it is known that this microbial diversity is responsible for the physicochemical features and biological activities of each kefir (Jianzhong et al., 2009; Kabak and Dobson, 2011; Gao et al., 2012; Altay et al., 2013).

Wang et al. (2012) examined a section of a whole kefir grain and found in the outer layer of the grain, lactococci, and yeasts, and, in the inner layer of the grain, the quantity of lactobacilli were much higher and more yeasts cells were found. There are little information about the mechanism of grain formation, so the same authors, proposed a hypothesis to explain that. "Initially, *Lactobacillus kefiranofaciens* and *Saccharomyces turicensis* start to auto-aggregate and co-aggregated to small granules." The aggregation is enhanced when the pH drops. The biofilm producers, *Lactobacillus kefiri*, *Kluyveromyces marxianus* HY1, and *Pichia fermentans* HY3 then adhere to the surface of these small granules due to their cell surface properties and their strong aggregation ability, which gives rise to thin biofilms. After biofilm formation, the kefir yeasts and *Lactobacillus* continue to co-aggregated with the granule strains and associate with the granule biofilm to become a three dimensional microcolony. As the cell density due to the growth of kefir yeasts and *Lactobacillus* increases, cells and milk components that are present in the liquid phase accumulate on the granule surface

TABLE 1 | Microbial compositions found in kefir and kefir grains of different origins.

Microorganism	Source – Country	Reference
Lactobacillus kefir, Lactobacillus kefiranofaciens, Lactobacillus paracasei, Lactobacillus plantarum, Lactococcus lactis ssp. *lactis, Kluyveromyces marxianus, Lactobacillus parakefir, Saccharomyces cerevisiae, Saccharomyces unisporus, Leuconostoc mesenteroides, Acetobacter* sp., *Saccharomyces* sp., *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*, *Lactococcus lactis, Lactobacillus kefiri, Lactobacillus parakefiri*	Kefir grains and beverage – Argentina	Garrote et al., 2001; Londero et al., 2012; Hamet et al., 2013; Diosma et al., 2014.
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactococcus lactis ssp. *cremoris, Gluconobacter frateurii, Acetobacter orientalis, Acetobacter lovaniensis, Kluyveromyces marxianus, Naumovozyma* sp.*, Kazachastania khefir*	Kefir grains and beverage – Belgium	Korsak et al., 2015
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus paracasei, Lactobacillus helveticus, Gluconobacter japonicus, Lactobacillus uvarum, Acetobacter syzygii, Lactobacillus satsumensis, Saccharomyces cerevisiae., Leuconostoc sp., *Streptococcus* sp., *Acetobacter* sp., *Bifidobacterium* sp., *Halococcus* sp., *Lactobacillus amylovorus, Lactobacillus buchneri, Lactobacillus crispatus, Lactobacillus kefiranofaciens* ssp. *kefiranofaciens, Lactobacillus kefiranofaciens* ssp. *kefirgranum, Lactobacillus parakefiri*	Kefir grains – Brazil	Miguel et al., 2010; Leite et al., 2012; Zanirati et al., 2015
Lactobacillus brevis, Lactobacillus delbrueckii ssp. *bulgaricus, Lactobacillus helveticus, Streptococcus thermophilus, Lactobacillus casei* ssp. *pseudoplantarum, Kluyveromyces marxianus* var. *lactis, Saccharomyces cerevisiae, Candida inconspicua, Candida maris, Lactobacillus lactis* ssp. *lactis*	Kefir grains and beverage – Bulgaria	Simova et al., 2002
Lactobacillus paracasei, Lactobacillus parabuchneri, Lactobacillus casei, Lactobacillus kefiri, Lactococcus lactis, Acetobacter lovaniensis, Kluyveromyces lactis, Kazachstania aerobia, Saccharomyces cerevisiae, Lachancea meyersii	Kefir beverage – Brazil	Magalhães et al., 2011
Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus helveticus, Kluyveromyces marxianus, Saccharomyces cerevisiae, Pseudomonas sp.*, Kazachstania unispora, Kazachstania exigua, Lactobacillus kefiri, Lactobacillus casei, Bacillus subtilis, Pichia kudriavzevii, Leuconostoc lactis, Lactobacillus plantarum, Acetobacter fabarum, Pichia guilliermondii, Lactococcus* sp., *Lactobacillus* sp., *Acetobacter* sp., *Shewanella* sp., *Leuconostoc* sp., *Streptococcus* sp, *Acinetobacter* sp., *Pelomonas* sp., *Dysgonomonas* sp., *Weissella* sp., *Shewanella* sp.	Kefir grains (Tibet)– China	Jianzhong et al., 2009; Gao et al., 2012, 2013a
Acetobacter acetic, Enterococcus faecalis, Enterococcus durans, Lactococcus lactis ssp. *cremoris, Leuconostoc pseudomesenteroides, Leuconostoc paramesenteroides, Lactobacillus brevis, Lactobacillus acidophilus, Saccharomyces* sp., *Brettanomyces* sp., *Candida* sp., *Saccharomycodes* sp., *Acetobacter rancens*	Kefir beverage – China	Yang et al., 2007
Lactobacillaceae and *Streptococcaceae*	Kefir grains and beverage – Ireland	Dobson et al., 2011
Lactobacillus kefiranofaciens, Dekkera anomala, Streptococcus thermophilus, Lactococcus lactis, Acetobacter sp.*, Lactobacillus lactis, Enterococcus* sp.*, Bacillus* sp.*, Acetobacter fabarum, Acetobacter lovaniensis, Acetobacter orientalis*	Kefir grains – Italy	Garofalo et al., 2015
Leuconostoc sp.*, Lactococcus* sp.*, Lactobacillus* sp., *Lactobacillus plantarum, Zygosaccharomyces* sp.*, Candida* sp.*, Candida lambica, Candida krusei, Saccharomyces* sp., *Cryptococcus* sp.	Kefir grains and beverage – South Africa	Witthuhn et al., 2005
Lactobacillus sp., *Leuconostoc* sp., *Lactococcus* sp., *Zygosaccharomyces* sp., *Candida* sp., *Saccharomyces* sp.	Kefir grains – South Africa	Witthuhn et al., 2004
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Escherichia coli, Pseudomonas sp., *Saccharomyces turicensis,*	Kefir grains – Taiwan	Wyder et al., 1999; Chen et al., 2008; Wang et al., 2012;
Lactobacillus kefiri, Leuconostoc mesenteroides, Lactococcus lactis, Streptococcus thermophilus, Lactobacillus kefiranofaciens, Lactobacillus acidophilus	Kefir grains and beverage – Turkey	Guzel-Seydim et al., 2005; Kesmen and Kacmaz, 2011
Lactobacillus helveticus, Lactobacillus buchneri, Lactobacillus kefiranofaciens, Lactobacillus acidophilus, Lactobacillus helveticus, Streptococcus thermophilus, Bifidobacterium bifidum, Kluyveromyces marxianus	Kefir grains – Turkey	Kok-Tas et al., 2012; Nalbantoglu et al., 2014
Lactococcus cremoris, Lactococcus lactis, Streptococcus thermophilus, Streptococcus durans	Kefir beverage – Turkey	Yüksekdag et al., 2004

TABLE 2 | Kefir microorganisms and their biological activities.

Organism of interest	Origin	Biological activity	Reference
Lactobacillus plantarum MA2	Tibetan kefir	Hypocholesterolemic effect	Yanping et al., 2009
Lactobacillus plantarum Lp27	Tibetan kefir	Inhibited cholesterol absorption	Ying et al., 2013
Lactobacillus plantarum CIDCA 83114	Kefir grains – Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *C. difficile* toxins on eukaryotic cells	Bolla et al., 2013
Lactobacillus kefir CIDCA 8348	Kefir grains – Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *C. difficile* toxins on eukaryotic cells	Bolla et al., 2013
Lactobacillus plantarum ST8KF	Kefir grains – South Africa	Bactericida effect against: *Lactobacillus casei, Lactobacillus salivarius, Lactobacillus curvatus, Listeria innocua*	Powell et al., 2007
Lactobacillus kefiranofaciens K1	Kefir grains – Taiwanese milk	Antiallergenic effect	Chen et al., 2008; Wei-Sheng et al., 2010
Lactobacillus kefiranofaciens M1	Kefir grains – Taiwanese milk	Immunoregulatory effects – anticolitis effect	Hong et al., 2009; Chen et al., 2012
Lactobacillus lactis CIDCA 8221	Kefir grains – Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *Clostridium difficile* toxins on eukaryotic cells	Bolla et al., 2013
Kluyveromyces marxianus CIDCA 8154	Kefir grains – Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *Clostridium difficile* toxins on eukaryotic cells	Bolla et al., 2013
Saccharomyces cerevisiae CIDCA 8112	Kefir grains – Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *Clostridium difficile* toxins on eukaryotic cells	Bolla et al., 2013
Lactobacillus lactis ssp. *cremoris*	Kefir grains – India	Activity against food spoilage bacteria	Raja et al., 2009

Source: Soccol et al., 2014.

TABLE 3 | Biological activity of kefiran.

Exopolysaccharide	Biological activity	Reference
Kefiran	Reduction of blood pressure induced by hypertension	Maeda et al., 2004
	Favors the activity of peritoneal macrophages	
	Increase in peritoneal IgA	Duarte et al., 2006
	Antitumoral activity	Liu et al., 2002
	Antimicrobial activity	Rodrigues et al., 2005a
	Modulation of the intestinal immune system and protection of epithelial cells against *Bacillus cereus* exocellular factors	Medrano et al., 2008; Piermaria et al., 2010

and the kefir grains are formed. There is a symbiotic relation between the microorganisms present in kefir grains, wherein the bacteria and yeast survive and share their bioproducts as power sources and microbial growth factors. This microorganism association is responsible for lactic and alcoholic fermentation (Witthuhn et al., 2005; Wang et al., 2012; Hamet et al., 2013).

After receiving its actual/present denomination, some of the microorganisms isolated and identified in kefir cultures were classified using the product name, as in *Lactobacillus kefiri, L. kefiranofaciens, L. kefirgranum, Lactobacillus parakefir*, and *Candida kefyr* (Wyder et al., 1999; Kwon et al., 2003; Yang et al., 2007; Kok-Tas et al., 2012). **Table 1** demonstrates the microbial composition, which has been isolated from kefir and kefir grains of different origins.

BIOLOGICAL ACTIVITY OF KEFIR

Due to its composition, kefir is mainly considered a probiotic resource (Nalbantoglu et al., 2014). "Probiotics are microbial cell preparations or components of microbial cells with a beneficial effect on the health of the host" (Lopitz et al., 2006). Some studies suggest that probiotic bacteria in kefir consumers' gut are abundant and are correlated with health improvement (Ahmed et al., 2013; Zheng et al., 2013); in that way, it had been demonstrated that the cell-free fraction of kefir enhances the ability to digest lactose relieving symptoms (Farnworth, 2005; Rizk et al., 2009).

Another reason for the increased interest in probiotic strains from kefir is its capacity to lower cholesterol levels. There are different ways in which bacteria can alter serum cholesterol: (i) through the binding to and absorption into the cell before it can

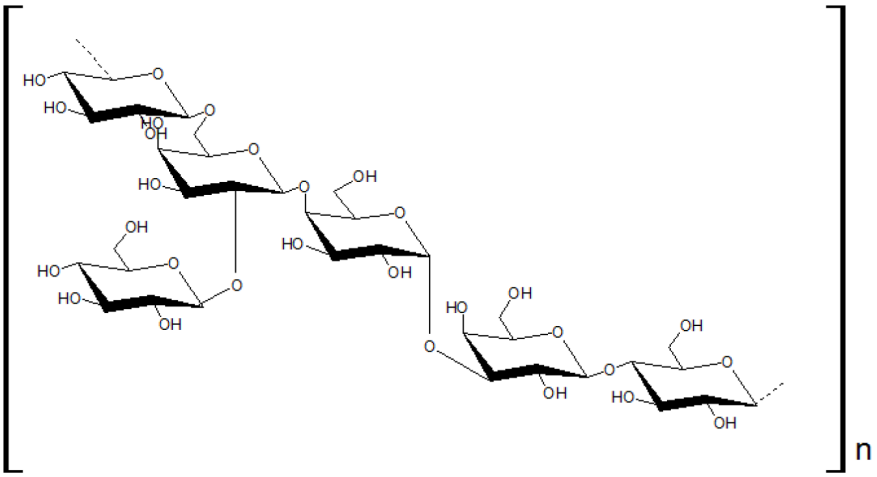


FIGURE 1 | Kefiran structure.

be absorbed into the body; (ii) producing free and deconjugating bile acids; (iii) inhibiting the enzyme HMG-CoA reductase (Yanping et al., 2009).

The microorganisms in the kefir grains produce lactic acid, antibiotics and bactericides, which inhibit the development of degrading and pathogenic microorganisms in kefir milk (Liu et al., 2002). Kefir acts against the pathogenic bacteria *Salmonella, Helicobacter, Shigella, Staphylococcus, Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Bacillus subtilis, Micrococcus luteus, Listeria monocytogenes, Streptococcus pyrogenes*, (Lopitz et al., 2006), *Streptococcus faecalis* KR6, *Fusarium graminearum* CZ1 (Ismaiel et al., 2011), and the fungus *Candida albicans*. On the other hand, it has been demonstrated that a mixture of kefir isolated bacteria and yeast is able to prevent diarrhea and enterocolitis triggered by *Clostridium difficile* (Bolla et al., 2013). Besides, kefir showed good efficacy in inhibiting spore formation and aflatoxin B1 produced by the fungus *Aspergillus flavus*, which is a toxic compound formed either in the field or during food storage. Therefore, kefir appears as a promising safe alternative natural food preservative offering protection against intoxication with aflatoxin B1 (Ismaiel et al., 2011).

It had been proved that many species of lactobacilli present in kefir have S-layer proteins. Surface layers (S-layers) can be aligned in unit cells on the outermost surface of many prokaryotic microorganisms (Mobili et al., 2009). It has been demonstrated that these S-layer proteins can apply a protective action inhibiting the grown of *Salmonella enterica* serovar *Enteritidis* in Caco-2 cells, and also have the ability to antagonize the effects of toxins from *Clostridium difficile* on eukaryotic/eukaryotic cells *in vitro* (Carasi et al., 2012).

However, there are other important bioactivities that have been tested with kefir grains, the cell-free fraction of kefir or acid lactic bacteria isolated from kefir, such as antitumoral (Gao et al., 2013b), anti-inflammatory (Diniz et al., 2003), antimicrobial (Anselmo et al., 2010) immunoregulatory (Hong et al., 2009), antiallergenic (Wei-Sheng et al., 2010), wound healing (Huseini et al., 2012), antidiabetic (Young-In et al., 2006) antimutagenic (Guzel-Seydim et al., 2006), and antigenotoxic (Grishina et al., 2011). In that way, it had been demonstrated that kefir cell-free fraction has antiproliferative effects on human gastric cancer SGC7901 cells (Gao et al., 2013b), colon adenocarcinoma cells (Khoury et al., 2014), HuT–102 malignant T lymphocytes, sarcoma 180 in mice, Lewis lung carcinoma and human mammary cancer (Rizk et al., 2009), and reduce oxidative stress (Punaro et al., 2014). Another study has shown that suspensions after 24 h fermentation and mechanically disintegrated kefir grains cause a significant inhibition of granuloma tissue formation and a 43% inhibition of the inflammatory process (Diniz et al., 2003).

Nevertheless, there are other important studies performed with some microorganisms isolated from different types of kefir. Some microorganisms with their biological activities and origin are shown in **Table 2**.

KEFIRAN, A POTENTIAL EXOPOLYSACCHARIDE

The increased search for natural polysaccharides has been very significant due to their use in the food, pharmaceutical, and cosmetic industries as additives, bio-absorbents, metal removal agents, bioflocculants, and medicine delivery agents, among other functions (De Vuyst et al., 2001; Welman and Maddox, 2003; Badel et al., 2011). Many microorganisms, such as bacteria, fungi, and weeds, have the capacity/ability to synthesize and excrete extracellular polysaccharides, and these polysaccharides can be either soluble or insoluble (Wang et al., 2010; Badel et al., 2011).

The polysaccharides that are commonly used as food additives are xanthan, dextran, gellan, and alginates, while the exopolysaccharides (EPSs) produced by lactic acid bacteria

TABLE 4 | Marketed kefir-based products and their information.

Companies	Product	General information
Lifeway • United States • Canada • Great Britain	Low Fat Kefir	All-natural 99% lactose-free Gluten-free 12 probiotic cultures
	Non-Fat Kefir	High in protein and calcium
	Veggie Kefir	All-natural 99% lactose-free Gluten-free 12 probiotic cultures Oat fiber enriched High in protein and calcium
	Kefir Oats	All-natural 99% lactose-free Gluten-free 12 probiotic cultures No added sugar High in protein and calcium
	Perfect 12 Kefir	USDA Certified Organic Oregon Tilth Certified Organic 99% lactose-free Gluten-free 12 probiotic cultures High in protein and calcium
	Traditional Kefir	USDA Certified Organic Oregon Tilth Certified Organic 99% lactose-free Gluten-free 12 probiotic cultures No added sugar
	Greek Style Kefir	USDA Certified Organic Oregon Tilth Certified Organic 99% lactose-free Gluten-free Seven probiotic cultures Contains Inulin
	Low Fat Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% lactose-free Gluten-free 12 probiotic cultures Phytoboost = 1 serving of vegetables
	Whole Milk Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% lactose-free Gluten-free 12 probiotic cultures No-spill pouch
	Helios Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% lactose-free Gluten-free 12 probiotic cultures High in protein and calcium
	Green Kefir (Organic)	All-natural 99% lactose-free Gluten-free 10 probiotic cultures High in protein and calcium
	ProBugs (organic)	All-natural 99% lactose-free Gluten-free 12 probiotic cultures
	ProBugs Blast (Organic)	All-natural 99% lactose-free Gluten-free 10 probiotic cultures 60 calories per serving 0.5 g of fat
	Frozen ProBugs (Organic)	All-natural 99% lactose-free Gluten-free 12 probiotic cultures 20 Billion units of probiotics 99% lactose-free Gluten-free High in protein and calcium
	Frozen Kefir	99% lactose-free Gluten-free High in protein and calcium
Evolve Kefir • United States	Evolve Kefir	11 probiotic cultures. Natural fruit flavors. Fiber. Protein and calcium
Wallaby Organic • Australia	Lowfat Kefir	12 different strains of Live and Active Kefir cultures.
CocoKefir • United States	CocoKefir App le Cinnamon CocoKefir Citrus CocoKefir CocoYo Body Ecology Coconut Kefir	Dairy, gluten, soy, and fat free Low calorie Contains valuable nutrients such as potassium, manganese, and magnesium. Beneficial probiotic strains

show good physicochemical characteristics for their use as food additives. In addition to these characteristics, EPSs are obtained from microorganisms classified as GRAS (generally recognized as safe), such as lactic acid bacteria (Wang et al., 2008; Saija et al., 2010; Badel et al., 2011).

Many reports have demonstrated that the quantity and properties of EPSs depend on the microorganisms used in the fermentation process and on the fermentation conditions and the composition of the culture media (Kim et al., 2008). EPSs have physicochemical and rheological properties that make them suitable as additives, which can be used as stabilizers, emulsifiers, gelling agents, and viscosity improvers. Additionally, EPSs possess biological properties suggesting their use as antioxidants, antitumor agents, antimicrobial agents, and immunomodulators, among other roles (Suresh Kumar et al., 2008; Bensmira et al., 2010; Piermaria et al., 2010).

The EPS kefiran is produced by *Lactobacillus kefiranofaciens* (Kooiman, 1968; Wang et al., 2010) from kefir grains, which are composed of proteins, polysaccharides, and a complex symbiotic microbial mixture (Witthuhn et al., 2005; Jianzhong et al., 2009). These microorganisms grow in kefiran, which is a polysaccharide matrix consisting of glucose and galactose. Despite good kefiran production by *L. kefiranofaciens* alone, it has been observed that

the addition of *Saccharomyces* sp. to the culture improves the net quantity of kefiran, illustrating the importance of the symbiosis between the bacteria and yeast that are present in kefir (Cheirsilp et al., 2003).

Lactic acid bacteria can synthesize homopolysaccharides or heteropolysaccharides. The synthesized homopolysaccharides are glucans or fructans, which are composed of only one type of monosaccharide (glucose or fructose, respectively; Van Hijum et al., 2006; Badel et al., 2011), whereas the heteropolysaccharides contain different types of monosaccharides in different proportions (mainly glucose, galactose, and rhamnose), (De Vuyst and Degeest, 1999; Ruas-Madiedo et al., 2002).

Similarly to lactic acid bacteria, *Lactobacillus* sp. also produces glucan and fructan. The homopolysaccharides show a much higher performance compared with heteropolysaccharide production (Welman and Maddox, 2003; Badel et al., 2011).

The heteropolysaccharides excreted by *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, and *Lactobacillus helveticus* contain galactose, glucose, and rhamnose as the main monosaccharides, with other monosaccharides being present in smaller concentrations. They are also highly branched with different types of linkages, and their denominations are complex and generally dependent on the main monosaccharide (De Vuyst and Degeest, 1999; Badel et al., 2011).

Lactobacillus plantarum isolated from Tibetan kefir excretes EPS classified as heteropolysaccharides composed of galactose, glucose, and mannose. This EPS has the capacity/ability to reduce blood cholesterol and form a biofilm shape (Zhang et al., 2009; Wang et al., 2010).

Kefiran is an EPS classified as a heteropolysaccharide comprising glucose and galactose in high concentrations, and it is classified as a water-soluble glucogalactan, which makes it suitable to be used as an additive (Wang et al., 2008, 2010). Kefiran has excellent rheological properties and can significantly improve the viscosity of lacteous products by favoring and maintaining gel properties and avoiding the loss of water during storage (Rimada and Abraham, 2006). With respect to the biological activity of kefiran, several studies have demonstrated that this EPS can be used as a nutraceutical, as described in **Table 3**.

The first study about kefiran structure was published by Kooiman (1968), who proposed a structure composed of two units: kefiran (polysaccharide) and kefirose (pentasaccharide). Then, some authors analyzed the polysaccharide structure with current techniques such chromatography and infrared spectroscopy (Wang et al., 2008; Chen et al., 2015) and nuclear magnetic resonance (NMR; Ghasemlou et al., 2012). The kefiran structure, according to them, is shown in **Figure 1**.

KEFIR-BASED PRODUCTS

Nowadays, the interest in developing functional foods is increasing because people want to improve their health and prevent diseases. Keeping in mind that kefir is a beverage with high probiotic activity, among other bioactivities, new companies are emerging around the world. One of the biggest kefir companies known is Lifeway, which started in 1986; their products can be obtained in the United States, Canada, and Great Britain, all of them based in kefir beverages, frozen, and cheese.

Other companies are Evolve Kefir with its principal product, a smoothie; Wallaby Yogurt Company with Low Fat Kefir; and CocoKefir LLC, which provides drinks/beverages based mainly on coconut water cultured with a comprehensive blend of probiotics. **Table 4** summarizes the products provided these companies with some general information about each one.

CONCLUSION

Kefir, the traditional beverage, is now recognized as a potential source of probiotics and molecules with highly interesting healthy properties. The careful and detailed characterization of kefir composition has helped the scientific community to find new possibilities for its application. Kefiran, the EPS of kefir, has very important physicochemical and rheological properties. Besides, its biological properties suggest its use as antioxidant, antitumor agent, antimicrobial agent, and immunomodulator, among other roles. Research is constantly being conducted to consolidate kefir and kefiran properties for the development of new important products to preserve consumer's health.

ACKNOWLEDGMENT

Authors want to thank CNPq and CAPES for the financial support.

REFERENCES

Ahmed, Z., Wang, Y., Ahmad, A., Khan, S. T., Nisa, M., Ahmad, H., et al. (2013). Kefir and health: a contemporary perspective. *Crit. Rev. Food Sci. Nutr.* 53, 422–434. doi: 10.1080/10408398.2010.540360

Altay, F., Karbancıoglu-Güler, F., Daskaya-Dikmen, C., and Heperkan, D. (2013). A review on traditional Turkish fermented non-alcoholic beverages: microbiota, fermentation process and quality characteristics. *Int. J. Food Microbiol.* 167, 44–56. doi: 10.1016/j.ijfoodmicro.2013.06.016

Anselmo, R. J., Viora, S. S., Ojeda, P. A., and Lausada, L. I. (2010). Efecto antagónico del kefir sobre endosporas y células vegetativas de *Bacillus cereus* y *Clostridium perfringens*. (Spanish) Antagonistic effect of the kefir on endospores and vegetative cells of *Bacillus cereus* and *Clostridium perfringens*. *Inf. Tecnol.* 21, 131–138.

Badel, S., Bernardi, T., and Michaud, P. (2011). New perspective for Lactobacilli exopolysaccharides. *Biotecnol. Adv.* 29, 54–66. doi: 10.1016/j.biotechadv.2010.08.011

Bensmira, M., Nsabimana, C., and Jiang, B. (2010). Effects of fermentation conditions and homogenization pressure on the rheological properties of Kefir. *Food Sci. Technol.* 43, 1180–1184.

Bolla, P. A., Carasi, P., Bolla Mde, L., De Antoni, G. L., and Serradell Mde, L. (2013). Protective effect of a mixture of kefir-isolated

lactic acid bacteria and yeasts in a hamster model of *Clostridium difficile* infection. *Anaerobe* 21, 28–33. doi: 10.1016/j.anaerobe.2013.03.010

Botelho, P. S., Maciel, M. I., Bueno, L. A., Marques Mde, F., Marques, D. N., and Sarmento Silva, T. M. (2014). Characterization of a new exopolysaccharide obtained from of fermented kefir grains in soymilk. *Carbohydr. Polym.* 107, 1–6. doi: 10.1016/j.carbpol.2014.02.036

Carasi, P., Trejo, F. M., Pérez, P. F., De Antoni, G. L., and Serradell Mde, L. (2012). Surface proteins from *Lactobacillus* kefir antagonize in vitro cytotoxic effect of *Clostridium difficile* toxins. *Anaerobe* 18, 135–142. doi: 10.1016/j.anaerobe.2011.11.002

Cevikbas, A., Yemni, E., Ezzedenn, F. W., Yardimici, T., Cevikbas, U., and Stohs, S. J. (1994). Antitumoural, antibacterial and antifungal activities of kefir and kefir grain. *Phytother. Res.* 8, 78–82. doi: 10.1002/ptr.2650080205

Cheirsilp, B., and Radchabut, S. (2011). Use of whey lactose from dairy industry for economical kefiran production by *Lactobacillus kefiranofaciens* in mixed cultures with yeasts. *N. Biotechnol.* 28, 574–580. doi: 10.1016/j.nbt.2011.01.009

Cheirsilp, B., Shimizu, H., and Shioya, S. (2003). Enhanced kefiran production by mixed culture of *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*. *J. Biotechnol.* 100, 43–53. doi: 10.1016/S0168-1656(02)00228-6

Chen, H. C., Wang, S. Y., and Chen, M. J. (2008). Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiol.* 25, 492–501. doi: 10.1016/j.fm.2008.01.003

Chen, Y. P., Hsiao, P. J., Hong, W. S., Dai, T. Y., and Chen, M. J. (2012). *Lactobacillus kefiranofaciens* M1 isolated from milk kefir grains ameliorates experimental colitis in vitro and in vivo. *J. Dairy Sci.* 95, 63–74. doi: 10.3168/jds.2011-4696

Chen, Z., Shi, J., Yang, X., Nan, B., Liu, Y., and Wang, Z. (2015). Chemical and physical characteristics and antioxidant activities of the exopolysaccharide produced by Tibetan kefir grains during milk fermentation. *Int. Dairy J.* 43, 15–21. doi: 10.1016/j.idairyj.2014.10.004

De Vuyst, L., and Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol. Rev.* 23, 153–177. doi: 10.1111/j.1574-6976.1999.tb00395.x

De Vuyst, L., De Vin, F., and Kamerling, J. P. (2001). Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* 11, 687–707. doi: 10.1016/S0958-6946(01)00114-5

Diniz, R., Garla, L., Schneedorf, J., and Carvalho, J. C. (2003). Study of anti-inflammatory activity of Tibetan mushroom, a symbiotic culture of bacteria and fungi encapsulated into a polysaccharide matrix. *Pharmacol. Res.* 47, 49–52. doi: 10.1016/S1043-6618(02)00240-2

Diosma, G., Romanin, D. E., Rey-Burusco, M. F., Londero, A., and Garrote, G. L. (2014). Yeasts from kefir grains: isolation, identification, and probiotic characterization. *World J. Microbiol. Biotechnol.* 30, 43–53. doi: 10.1007/s11274-013-1419-9

Dobson, A., O'Sullivan, O., Cotter, P. D., Ross, P., and Hill, C. (2011). High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. *FEMS Microbiol. Lett.* 320, 56–62. doi: 10.1111/j.1574-6968.2011.02290.x

Duarte, J., Vinderola, G., Ritz, B., Perdigon, G., and Matar, C. (2006). Immunomodulating capacity of commercial fish protein hydrolysate for diet supplementation. *Immunobiology* 211, 341–350. doi: 10.1016/j.imbio.2005.12.002

Farnworth, E. R. (2005). Kefir – a complex probiotic. *Food Sci. Technol. Bull. Funct. Foods* 2, 1–17. doi: 10.1616/1476-2137.13938

Farnworth, E. R., and Mainville, I. (2003). "Kefir: a fermented milk product," in *Handbook of Fermented Functional Foods*, ed. E. R. Farnworth (Boca Raton, FL: CRC Press), 77–112.

Fontán, M. C. G., Martínez, S., Franco, I., and Carballo, J. (2006). Microbiological and chemical changes during the manufacture of Kefir made from cows' milk, using a commercial starter culture. *Int. Dairy J.* 16, 762–767. doi: 10.1016/j.idairyj.2005.07.004

Gao, J., Gu, F., Abdella, N. H., Ruan, H., and He, G. (2012). Investigation on culturable microflora in Tibetan kefir grains from different areas of China. *J. Food Sci.* 77, 425–433. doi: 10.1111/j.1750-3841.2012.02805.x

Gao, J., Gu, F., He, J., Xiao, J., Chen, Q., Ruan, H., et al. (2013a). Metagenome analysis of bacterial diversity in Tibetan kefir grains. *Eur. Food Res. Technol.* 236, 549–556. doi: 10.1007/s00217-013-1912-2

Gao, J., Gu, F., Ruan, H., Chen, Q., He, J., and He, G. (2013b). Induction of apoptosis of gastric cancer cells SGC7901 in vitro by a cell-free fraction of Tibetan kefir. *Int. Dairy J.* 30, 14–18. doi: 10.1016/j.idairyj.2012.11.011

Garofalo, C., Osimani, A., Milanovič, V., Aquilanti, L., De Filippis, F., Stellato, G., et al. (2015). Bacteria and yeast microbiota in milk kefir grains from different Italian regions. *Food Microbiol.* 49, 123–133. doi: 10.1016/j.fm.2015.01.017

Garrote, G. L., Abraham, A. G., and De Antoni, G. L. (2001). Chemical and microbiological characterisation of kefir grains. *J. Dairy Res.* 68, 639–652. doi: 10.1017/S0022029901005210

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.

Ghasemlou, M., Khodaiyan, F., Jahanbin, K., Mohammad, S., Gharibzahedi, T., and Taheri, S. (2012). Structural investigation and response surface optimisation for improvement of kefiran production yield from a low-cost culture medium. *Food Chem.* 133, 383–389. doi: 10.1016/j.foodchem.2012.01.046

Grishina, A., Kulikova, I., Alieva, L., Dodson, A., Rowland, I., and Jing, J. (2011). Antigenotoxic effect of kefir and ayran supernatants on fecal water-induced DNA damage in human colon cells. *Nutr. Cancer* 63, 73–79. doi: 10.1080/01635581.2010.516873

Guzel-Seydim, Z. B., Seydim, A. C., Greene, A. K., and Ta, T. (2006). Determination of antimutagenic properties of acetone extracted fermented milks and changes in their total fatty acid profiles including conjugated linoleic acids. *Int. J. Dairy Technol.* 59, 209–215. doi: 10.1111/j.1471-0307.2006.00265.x

Guzel-Seydim, Z., Wyffels, J. T., Seydim, A. C., and Greene, A. K. (2005). Turkish kefir and kefir grains: microbial enumeration and electron microscopic observation. *Int. J. Dairy Technol.* 58, 25–29. doi: 10.1111/j.1471-0307.2005.00177.x

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

Hong, W. S., Chen, H. C., Chen, Y. P., and Chen, M. J. (2009). Effects of kefir supernatant and lactic acid bacteria isolated from kefir grain on cytokine production by macrophage. *Int. Dairy J.* 19, 244–251. doi: 10.1016/j.idairyj.2008.10.010

Huseini, H. F., Rahimzadeh, G., Fazeli, M. R., Mehrazma, M., and Salehi, M. (2012). Evaluation of wound healing activities of kefir products. *Burns* 38, 719–723. doi: 10.1016/j.burns.2011.12.005

Ismaiel, A. A., Ghaly, M. F., and El-Naggar, A. K. (2011). Milk kefir: ultrastructure, antimicrobial activity and efficacy on aflatoxin b1 production by *Aspergillus flavus*. *Curr. Microbiol.* 62, 1602–1609. doi: 10.1007/s00284-011-9901-9

Jianzhong, Z., Xiaoli, L., Hanhu, J., and Mingsheng, D. (2009). Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. *Food Microbiol.* 26, 770–775. doi: 10.1016/j.fm.2009.04.009

Kabak, B., and Dobson, A. D. (2011). An introduction to the traditional fermented foods and beverages of Turkey. *Crit. Rev. Food Sci. Nutr.* 51, 248–260. doi: 10.1080/10408390903569640

Kesmen, Z., and Kacmaz, N. (2011). Determination of lactic microflora of kefir grains and kefir beverage by using culture-dependent and culture-independent methods. *J. Food Sci.* 76, 276–283. doi: 10.1111/j.1750-3841.2011.02191.x

Khoury, N., El-Hayek, S., Tarras, O., El-Sabban, M., El-Sibai, M., and Rizk, S. (2014). Kefir exhibits anti-proliferative and pro-apoptotic effects on colon adenocarcinoma cells with no significant effects on cell migration and invasion. *Int. J. Oncol.* 45, 2117–2127. doi: 10.3892/ijo.2014.2635

Kim, Y., Kim, J. U., Oh, S., Kim, Y. J., Kim, M., and Kim, S. H. (2008). Technical optimization of culture conditions for the production of exopolysaccharide (EPS) by *Lactobacillus rhamnosus* ATCC 9595. *Food Sci. Biotechnol.* 17, 587–593.

Kok-Tas, T., Ekinci, F. Y., and Guzel eSeydim, Z. B. (2012). Identification of microbial flora in kefir grains produced in Turkey using PCR. *Int. J. Dairy Technol.* 65, 126–131. doi: 10.1111/j.1471-0307.2011.00733.x

Kooiman, P. (1968). The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. *Carbohydr. Res.* 7, 220–221. doi: 10.1016/S0008-6215(00)81138-6

Korsak, N., Taminiau, B., Leclercq, M., Nezer, C., Crevecoeur, S., Ferauche, C., et al. (2015). Short communication: evaluation of the microbiota of kefir samples using metagenetic analysis targeting the 16S and 26S ribosomal DNA fragments. *J. Dairy Sci.* 98, 3684–3689. doi: 10.3168/jds.2014-9065

Kwon, C. S., Park, M. Y., Cho, J. S., Choi, S. T., and Chang, D. S. (2003). Identification of effective microorganisms from kefir fermented milk. *Food Sci. Biotechnol.* 12, 476–479.

Leite, A. M. O., Mayoa, B., Rachid, C. T., Peixoto, R. S., Silva, J. T., Paschoalin, V. M., et al. (2012). Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food Microbiol.* 31, 215–221. doi: 10.1016/j.fm.2012.03.011

Leite, A. M. O., Miguel, M. A., Peixoto, R. S., Rosado, A. S., Silva, J. T., and Paschoalin, V. M. (2013). Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. *Braz. J. Microbiol.* 44, 341–349. doi: 10.1590/S1517-83822013000200001

Liu, J. R., Wang, S. Y., Lin, Y. Y., and Lin, C. W. (2002). Antitumor activity of milk, kefir and soya milk kefir in tumor bearing mice. *Nutr. Cancer* 44, 183–187. doi: 10.1207/S15327914NC4402_10

Londero, A., Hamet, M. F., De Antoni, G. L., Garrote, G. L., and Abraham, A. G. (2012). Kefir grains as a starter for whey fermentation at different temperatures: chemical and microbiological characterisation. *J. Dairy Res.* 79, 262–271. doi: 10.1017/S0022029912000179

Lopitz, F. O., Rementeria, A., Elguezabal, N., and Garaizar, J. (2006). Kefir: una comunidad simbiótica de bacterias y levaduras con propiedades saludables. *Rev. Iberoam. Micol.* 23, 67–74. doi: 10.1016/S1130-1406(06)70016-X

Maeda, H., Zhu, X., Suzuki, S., Suzuki, K., and Kitamura, S. (2004). Structural characterization and biological activities of an exopolysaccharide kefiran produced by *Lactobacillus kefiranofaciens* WT-2B(T). *J. Agric. Food Chem.* 52, 5533–5538. doi: 10.1021/jf049617g

Magalhães, K. T., Pereira, G. V. M., Campos, C. R., Dragone, G., and Schwan, R. F. (2011). Brazilian kefir: structure, microbial communities and chemical composition. *Braz. J. Microbiol.* 42, 693–702. doi: 10.1590/S1517-838220110002000034

McCue, P. P., and Shetty, K. (2005). Phenolic antioxidant mobilization during yogurt production from soymilk using Kefir cultures. *Process Biochem.* 40, 1791–1797. doi: 10.1016/j.procbio.2004.06.067

Medrano, M., Pérez, P. F., and Abraham, A. G. (2008). Kefiran antagonizes cytopathic effects of *Bacillus cereus* extracellular factors. *Int. J. Food Microbiol.* 122, 1–7. doi: 10.1016/j.ijfoodmicro.2007.11.046

Miguel, M. G. C. P., Cardoso, P. G., Lago, L. A., and Schwan, R. F. (2010). Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent methods. *Food Res. Int.* 43, 1523–1528. doi: 10.1016/j.foodres.2010.04.031

Mobili, P., Serradell Mde, L. A., Trejo, S. A., Avilés Puigvert, F. X., Abraham, A. G., et al. (2009). Heterogeneity of S-layer proteins from aggregating and non-aggregating *Lactobacillus* kefir strains. *Antonie Van Leeuwenhoek* 95, 363–372. doi: 10.1007/s10482-009-9322-y

Nalbantoglu, U., Cakar, A., Dogan, H., Abaci, N., Ustek, D., Sayood, K., et al. (2014). Metagenomic analysis of the microbial community in kefir grains. *Food Microbiol.* 41, 42–51. doi: 10.1016/j.fm.2014.01.014

Oliveira, A. M. L., Miguel, M. A. L., Peixoto, R. S., Rosado, A. S., Silva, J. T., and Paschoalin, V. M. F. (2013). Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. *Braz. J. Microbiol.* 44, 341–349. doi: 10.1590/S1517-83822013000200001

Piermaria, J., Bosch, A., Pinotti, A., Yantorno, O., Garcia, M. A., and Abraham, A. G. (2010). Kefiran films plasticized with sugars and polyols: water vapor barrier and mechanical properties in relation to their microstructure analyzed by ATR/ FT-IR spectroscopy. *Food Hydrocoll.* 25, 1261–1269. doi: 10.1016/j.foodhyd.2010.11.024

Pogačić, T., Sinko, S., Zamberlin, S., and Samarzija, D. (2013). Microbiota of kefir grains. *Mljekarstvo* 63, 3–14.

Powell, J. E., Witthuhn, R. C., Todorov, S. D., and Dicks, L. M. T. (2007). Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. *Int. Dairy J.* 17, 190–198. doi: 10.1016/j.idairyj.2006.02.012

Punaro, G. R., Maciel, F. R., Rodrigues, A. M., Rogero, M. M., Bogsan, C. S. B., Oliveira, M. N., et al. (2014). Kefir administration reduced progression of renal injury in STZ-diabetic rats by lowering oxidative stress. *Nitric Oxide* 37, 53–60. doi: 10.1016/j.niox.2013.12.012

Raja, A., Gajalakshmi, P., Raja, M. M. M., and Imran, M. M. (2009). Effect of *Lactobacillus* laceis cremoris isolated from Kefir against food spoilage bacteria. *Am. J. Food Technol.* 4, 201–209. doi: 10.3923/ajft.2009.201.209

Rimada, P. S., and Abraham, A. G. (2006). Effects of different fermentation parameters on quality characteristics of kefir. *Int. Dairy J.* 16, 33–39. doi: 10.3168/jds.2012-5753

Rizk, S., Maalouf, K., and Baydoun, E. (2009). The Antiproliferative effect of kefir cell-free fraction on HuT-102 malignant T lymphocytes. *Clin. Lymphoma Myeloma* 9, 198–203. doi: 10.3816/CLM.2009.s.012

Rodrigues, K. L., Caputo, L. R. G., Carvalho, J. C. T., Evangelista, J., and Schneedorf, J. M. (2005a). Antimicrobial and healing activity of kefir and kefiran extract. *Int. J. Antimicrob. Agents* 25, 404–408. doi: 10.1016/j.ijantimicag.2004.09.020

Rodrigues, K. L., Carvalho, J. C. T., and Schneedorf, J. M. (2005b). Anti-inflammatory properties of kefir and its polysaccharide extract. *Inflammopharmacology* 13, 485–492. doi: 10.1163/156856005774649395

Ruas-Madiedo, P., Hugenholtz, J., and Zoon, P. (2002). An overview of the functionality of exopolysaccharides produced by lacti acid bacteria. *Int. Dairy J.* 12, 163–171. doi: 10.1016/S0958-6946(01)00160-1

Saija, N., Welman, A., and Bennett, R. (2010). Development of a dairy-based exopolysaccharide bioingredient. *Int. Dairy J.* 20, 603–608. doi: 10.1016/j.idairyj.2010.03.011

Saloff-Coste, C. J. (1996). Kefir. Nutritional and health benefits of yoghurt and fermented milks. *Dannone World Newsl.* 11, 1–7.

Serafini, F., Turroni, P., Ruas-Madiedo, G. A., Lugli, C., Milani, S., Duranti, N., et al. (2014). Kefir fermented milk and kefiran promote growth of *Bifidobacterium bifidum* PRL2010 and modulate its gene expression. *Int. J. Food Microbiol.* 178, 50–59. doi: 10.1016/j.ijfoodmicro.2014.02.024

Simova, E., Beshkova, D., Angelov, A., Hristozova, T., Frengova, G., and Spasov, Z. (2002). Lactic acid bacteria and yeasts in kefir grains and kefir made from them. *J. Ind. Microbiol. Biotechnol.* 28, 1–6. doi: 10.1038/sj.jim.7000186

Soccol, C. R., Prado, M. R. M., Garcia, L. M. B., Rodrigues, C., and Medeiros, A. B. P. (2014). Current developments in probiotics. *J. Microb. Biochem. Technol.* 7, 011–020. doi: 10.4172/1948-5948.1000175

St-Onge, M. P., Farnworth, E. R., Savard, T., Chabot, D., Mafu, A., and Jones, P. J. (2002). Kefir consumption does not alter plasma lipid levels or cholesterol fractional synthesis rates relative to milk in hyperlipidemic men: a randomized controlled trial. *BMC Complement. Altern. Med.* 2:1. doi: 10.1186/1472-6882-2-1

Suresh Kumar, A., Mody, K., and Jha, B. (2008). Bacterial exopolysaccharides – a perception. *J. Basic Microbiol.* 47, 103–117. doi: 10.1002/jobm.200610203

Van Hijum, S. A., Kralj, S., Ozimek, L. K., Dijkhuizen, L., and Van Geel-Achutten, I. G. (2006). Structural-function relationships of glucansucrases and fructan enzymes from lactic acid bacteria. *Microbiol. J. Biol. Macromol.* 70, 157–176.

Vinderola, G., Perdigon, G., Duarte, J., Farnworth, E., and Matar, C. (2006). Effects of the oral administration of the products derived from milk fermentation by kefir microflora on immune stimulation. *J. Dairy Res.* 73, 472–479. doi: 10.1017/S002202990600197X

Vujičič, I. F., Vulić, M., and Könyves, T. (1992). Assimilation of cholesterol in milk by kefir cultures. *Biotechnol. Lett.* 14, 847–850. doi: 10.1007/BF01029151

Wang, S. Y., Chen, K. N., Lo, Y. M., Chiang, M. L., Chen, H. C., Liu, J. R., et al. (2012). Investigation of microorganisms involved in biosynthesis of the kefir grain. *Food Microbiol.* 32, 274–285. doi: 10.1016/j.fm.2012.07.001

Wang, Y., Ahmed, Z., Feng, W., Li, C., and Song, S. (2008). Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir. *Int. J. Biol. Macromol.* 43, 283–288. doi: 10.1016/j.ijbiomac.2008.06.011

Wang, Y. P., Li, C., Liu, P., Zaheer, A., Xiao, P., and Bai, X. (2010). Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet Kefir. *Carbohydr. Polym.* 82, 895–903. doi: 10.1016/j.carbpol.2010.06.013

Wei-Sheng, H., Yen-Po, C., and Ming-Ju, C. (2010). The Antiallergic effect of kefir Lactobacilli. *J. Food Sci.* 75, 244–253. doi: 10.1111/j.1750-3841.2010.01787.x

Welman, A. D., and Maddox, I. S. (2003). Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends Biotechnol.* 21, 269–274. doi: 10.1016/S0167-7799(03)00107-0

Witthuhn, R. C., Schoeman, T., and Britz, T. J. (2004). Isolation and characterisation of the microbial population of different South African kefir grains. *Int. J. Dairy Technol.* 57, 33–37. doi: 10.1111/j.1471-0307.2004.00126.x

Witthuhn, R. C., Schoeman, T., and Britz, T. J. (2005). Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation. *Int. Dairy J.* 15, 383–389. doi: 10.1016/j.idairyj.2004.07.016

Wszolek, M., Kupiec-Teahan, B., Skov Gulard, H., and Tamime, A. Y. (2006). "Production of kefir, koumiss and other related products," in *Fermented Milks*, ed. A. Y. Tamime (Oxford: Blackwell Publishing), 174–216.

Wyder, M. T., Meile, L., and Teuber, M. (1999). Description of *Saccharomyces turicensis* sp. nov., a new species from kefyr. *Syst. Appl. Microbiol.* 3, 420–425. doi: 10.1016/S0723-2020(99)80051-4

Yang, X. J., Fan, M. T., Shi, J. L., and Dang, B. (2007). Isolation and identification of preponderant flora in Tibetan kefir. *China Brewing* 171, 52–55.

Yanping, W., Nv, X., Aodeng, X., Zaheer, A., Bin, Z., and Xiaojia, B. (2009). Effects of *Lactobacillus plantarum* MA2 isolated from Tibet kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. *Appl. Microbiol. Biotechnol.* 84, 341–347. doi: 10.1007/s00253-009-2012-x

Ying, H., Fei, W., Xiaojun, W., Yujie, S., Longfei, Y., and Jinfeng, W. (2013). Characterization of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains: a potential probiotic bacterium with cholesterol-lowering effects. *J. Dairy Sci.* 96, 2816–2825. doi: 10.3168/jds.2012-6371

Young-In, K., Apostolidis, E., and Shetty, K. (2006). Anti-diabetes functionality of kefir culture-mediated fermented soymilk supplemented with Rhodiola extracts. *Food Biotechnol.* 20, 13–29. doi: 10.1080/08905430500522055

Yüksekdag, Z. N., Beyatli, Y., and Aslim, B. (2004). Determination of some characteristics coccoid forms of lactic acid bacteria isolated from Turkish kefirs with natural probiotic. *Food Sci. Technol.* 37, 663–667.

Zajšek, K., Goršek, A., and Kolar, M. (2013). Cultivating conditions effects on kefiran production by the mixed culture of lactic acid bacteria imbedded within kefir grains. *Food Chem.* 139, 970–977. doi: 10.1016/j.foodchem.2012.11.142

Zajšek, K., Kolar, M., and Goršek, A. (2011). Characterisation of the exopolysaccharide kefiran produced by lactic acid bacteria entrapped within natural kefir grains. *Int. J. Dairy Technol.* 64, 544–548. doi: 10.1111/j.1471-0307.2011.00704.x

Zanirati, D. F., Abatemarco, M., Cicco Sandesb, S. H., Nicolia, J. R., Nunes, A. C., and Neumanna, E. (2015). Selection of lactic acid bacteria from Brazilian kefir grains for potential use as starter or probiotic cultures. *Anarobe* 32, 70–76. doi: 10.1016/j.anaerobe.2014.12.007

Zhang, B., Xu, N., Xi, A. D., and Wang, Y. P. (2009). Screening and identification of cholesterol-reducing KF5 strain. *J. Tianjin Univ. Sci. Technol.* 24, 17–20.

Zheng, Y., Lu, Y., Wang, J., Yang, L., Pan, C., and Huang, Y. (2013). Probiotic properties of *Lactobacillus* strains isolated from Tibetan Kefir grains. *PLoS ONE* 8:e69868. doi: 10.1371/journal.pone.0069868

Conflict of Interest Statement: 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: 14 July 2015
doi: 10.3389/fmicb.2015.00685

Probiotic-based strategies for therapeutic and prophylactic use against multiple gastrointestinal diseases

*Natallia V. Varankovich, Michael T. Nickerson and Darren R. Korber**

Department of Food and Bioproduct Sciences, University of Saskatchewan, Saskatoon, SK, Canada

Probiotic bacteria offer a number of potential health benefits when administered in sufficient amounts that in part include reducing the number of harmful organisms in the intestine, producing antimicrobial substances and stimulating the body's immune response. However, precisely elucidating the probiotic effect of a specific bacterium has been challenging due to the complexity of the gut's microbial ecosystem and a lack of definitive means for its characterization. This review provides an overview of widely used and recently described probiotics, their impact on the human's gut microflora as a preventative treatment of disease, human/animal models being used to help show efficacy, and discusses the potential use of probiotics in gastrointestinal diseases associated with antibiotic administration.

Keywords: probiotic, human gut, gastrointestinal disorders, antibiotic resistance, gut microbiota

OPEN ACCESS

Edited by:
Maria De Angelis,
University of Bari Aldo Moro, Italy

Reviewed by:
Maria Guadalupe Vizoso Pinto,
National University of Tucumán, Argentina
Aldo Corsetti,
University of Teramo, Italy

****Correspondence:***
Darren R. Korber,
Department of Food and Bioproduct Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK S7N 5A8, Canada
darren.korber@usask.ca

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

Received: *09 January 2015*
Accepted: *22 June 2015*
Published: *14 July 2015*

Citation:
Varankovich NV, Nickerson MT and Korber DR (2015) Probiotic-based strategies for therapeutic and prophylactic use against multiple gastrointestinal diseases. Front. Microbiol. 6:685. doi: 10.3389/fmicb.2015.00685

Microbial Ecology of the Human Gastrointestinal Tract

The human intestinal microbiota is a complex ecosystem with considerable impact on human health and well-being, contributing to maturation of the immune system and providing a direct barrier against pathogen colonization (Doré and Corthier, 2010). It consists of bacteria, archaea, some protozoa, anaerobic fungi, and different bacteriophages and viruses, and it has been estimated that more than 1000 species of microbes inhabit the human intestine (Tuohy et al., 2012). The presence of a great number of microbes (up to 5×10^{11} bacterial cells per gram of intestinal contents) suggests strong regulatory effects on the human host, and recent findings suggest that gut microbiota can have a considerable impact on both our weight and mood (Duca et al., 2014; Naseribafrouei et al., 2014). The composition and function of human microbial populations associated with various body sites have been studied with the help of metagenomic tools as part of two recent initiatives – the NIH Human Microbiome Project (HMP) and the European Metagenomics of the Human Intestine (metaHIT) project (NIH HMP Working Group et al., 2009; Dusko Ehrlich and MetaHIT Consortium, 2011). These massive molecular approaches have already revealed the presence of three different clusters, or enterotypes, which correspond to one of three most abundant genera of human intestine – *Bacteroides*, *Prevotella,* and *Ruminococcus* (Arumugam et al., 2013).

Bacteria that initially colonize the large gut of an infant are facultative anaerobes, such as *Escherichia coli* and *Streptococcus* sp. These species metabolize oxygen in the gut, thereby creating anaerobic conditions. Subsequent colonization largely depends on food profile and environmental factors (i.e., sanitary conditions). After the full formation of the gastrointestinal microflora, its

composition has been shown to include such genera as *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Lactobacillus, Fusobacterium,* and various Gram-positive cocci (Fooks et al., 1999; Wallace et al., 2011).

Within the gastrointestinal tract (GIT), the microbiota provides various functions, such as digestion of essential nutrients and maturation of intestinal epithelial cells. Studies on mice have shown a number of significant effects of microbiota on the host: in ex-germ-free reconventionalized mice, their intestinal epithelium was thicker, kinetics of enterocytes – faster, short-chain fatty acids were produced at significantly higher concentrations, and there was a normal level of immunological activity present, compared to germ-free animals (Aureli et al., 2011). Microbes also have the ability to affect physiologic parameters, providing systemic effects on blood lipids and generally influencing the immune system, as well as inhibiting harmful bacteria (Mikelsaar, 2011). Pathogen inhibition by human intestinal microbiota may provide significant human health benefits through protection against infection as a natural barrier against pathogen exposure in the GIT (Wallace et al., 2011). Factors such as food contamination by pathogens, as well as the high load of antibiotics in soil and animal feed, can influence the microbial ecology of human GIT (Sapkota et al., 2007). Using molecular genetic tools, it has been shown that antibiotics could induce significant alterations in the dominant colonic microbiota that are not detectable using bacteriological (culture-based) techniques, with effects lasting for up to 2 months (Mangin et al., 1999). Several more specific disorders involve disruption of the human microflora ecology: acute gastroenteritis, *Clostridium difficile* infection (CDI), necrotising enterocolitis in neonates, irritable bowel syndrome and *Helicobacter pylori* infection (Kotzampassi and Giamarellos-Bourboulis, 2012). Probiotics are currently being examined for their potential treatments of these aforementioned disorders.

Probiotic Bacteria

According to the popularized definition by the Food and Agriculture Organization/World Health Organization, and as grammatically modified by Hill et al. (2014), probiotics are defined as "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). The most common probiotics include representatives of lactobacilli, enterococci, bifidobacteria, and yeasts (**Table 1**). In addition, bacterial mixtures may be used to achieve the complex beneficial effect of probiotics (Caballero-Franco et al., 2007).

Presumed health benefits of probiotics include reducing harmful organisms in the intestine, producing antimicrobial factors, and stimulating the body's immune response (Collado et al., 2007; Foligné et al., 2010; Konieczna et al., 2013). Some of the beneficial effects of probiotics (e.g., lowering of cholesterol level) are yet to be substantiated by well-controlled clinical trials. However, there are a growing number of studies providing data on effects of probiotic bacteria on the human immune system and on microflora of the GIT (Holzapfel and Schillinger, 2002; Foligne et al., 2007; Verdú et al., 2009; Wen et al., 2012). Increasingly, reports of the human/animal microbiome playing a central role in other key aspects of health functionality are emerging, including beneficial impacts on the treatment of metabolic disorders, such as obesity and type 2 diabetes, improvement of bowel function in patients with colorectal cancer, potential cognitive, and mood-enhancing benefits, antidepressant, and anxiolytic (antianxiety) activity (Desbonnet et al., 2008; Bravo et al., 2011; DiBaise et al., 2012; Lee et al., 2014a; Owen et al., 2014). The latter anxiolytic effect has even led to the emergence of the new term, psychobiotic, coined by Dinan et al. (2013) as a "live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness."

Products containing probiotic bacteria generally include supplements and foods. Live probiotics are commonly available in fermented dairy products and probiotic-fortified foods. These bacteria are added into numerous foods and beverages, ranging from yogurts to breakfast cereals. There are also tablets, capsules, powders, and sachets containing probiotics in freeze-dried form. Functional foods, defined as food preparations with various health-related properties, often include bacterial strains with declared probiotic properties (Turroni et al., 2011). The scientific interest in probiotics is growing exponentially: the search for published papers featuring the keyword "probiotic" in NIH PubMed database revealed 7265 articles for the period from 2000 to 2010, with 953 of them being clinical trials. Within the following 5 years (up to May 20th 2015), the frequency of publications doubled with 7979 papers being published, including 778 clinical trials.

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming cocci, coccobacilli, or rods, which generally have non-respiratory (fermentative) metabolism and lack true catalase. Unlike bifidobacteria, which are active in lower parts of the colon, lactobacilli are prevalent in the upper GIT (Turroni et al., 2011). This group is also a normal member of the human microflora, found in the oral cavity, the small intestine, and the vaginal epithelium, where it is thought to play beneficial roles (Gomes and Malcata, 1999). Among the beneficial effects, lactobacilli can improve digestion, absorption, and availability of nutrients (Wallace et al., 2011). Furthermore, LAB are capable of hydrolyzing compounds that limit the bioavailability of minerals, like tannin and phytate, due to tannin acylhydrolase and phytase activities (Turpin et al., 2010). In addition, it was shown that some lactobacilli strains could enhance mineral absorption in Caco-2 cells and improve the nutritional status of the host by producing B-group vitamins. More recently, the role of lactobacilli in energy homeostasis, particularly in obese patients, has been the object of an increased interest (Guo et al., 2010; Mikelsaar, 2011). A further potential positive impact of LAB is their ability to inhibit or kill *H. pylori*, which is now regarded as the major cause of gastritis and peptic ulcers and is a risk factor for gastric malignancy (Hamilton-Miller, 2003). In addition, both *Lactobacillus* sp. and

TABLE 1 | Microorganisms with reported probiotic potential.

Probiotic group	Species	Details of a representative study	Reference
Bifidobacteria	*Bifidobacterium animalis*	Fermented oat milk with *B. animalis* sp. *lactis* BB-12®; double-blind randomized placebo-controlled clinical trial; $n = 209$; 35% improvement in bowel movement.	Pitkala et al. (2007), Jungersen et al. (2014)
	B. breve	*B. breve* M16-V powder with or without starch; F344/Du rat pups; $n = 46$; downregulation of the expression of inflammatory molecules.	Shimakawa et al. (2003)
	B. adolescentis	*B. adolescentis* ATCC 101; female germ-free rats; $n = 30$; significant modulation of both systemic and the intestinal immune response to *Bacteroides thetaiotaomicron* DSMZ 2079.	Scharek et al. (2000)
	B. longum	*B. longum,* isolated from human GIT; double-blind randomized placebo-controlled clinical trial; $n = 29$; significant inhibitory effect on viral gastroenteritis symptoms.	Lee et al. (2014b)
	B. infantis	*B. infantis* 35624 was administered by gavage; C57BL/6 mice; $n = 64$; decrease in the severity of dextran sulfate sodium-induced colitis, immunomodulation.	Konieczna et al. (2013)
Lactobacilli	*L. acidophilus*	*L. acidophilus* – SDC, administered in capsules; double-blind randomized placebo-controlled clinical trial; $n = 40$; 23.6% reduction (compared to placebo) in the severity (pain, discomfort) of Irritable Bowel Syndrome.	Sinn et al. (2008)
	L. fermentum, L. amylovorus	Microencapsulated bacteria; double-blind randomized placebo controlled clinical trial; $n = 28$, obese adults; *L. fermentum* – 3% loss in total fat mass, *L. amylovorus* – 4% loss in total fat mass, a significant reduction in the abundance of Clostridial cluster IV.	Omar et al. (2013)
	L. rhamnosus	*L. rhamnosus GG* powder; double-blind randomized clinical trial; n = 559; decrease in frequency and duration of acute watery diarrhea.	Basu et al. (2009)
	L. paracasei	*L. paracasei* ST11, lyophilized form; double-blind randomized placebo-controlled clinical trial; $n = 230$, male infants and young children; significant benefit in the management of children with non-rotavirus-induced diarrhea.	Sarker et al. (2005)
	L. johnsonii	*L. johnsonii* La1 in dietary product; double-blind randomized placebo-controlled clinical trial; $n = 326$, children, found positive for *Helicobacter pylori*; significant decrease in $\sigma^{13}CO_2$ above baseline values (outcome of a test for *H. pylori*).	Cruchet et al. (2003)
	L. reuteri	*L. reuteri* SD 2112; randomized controlled clinical tral; $n = 40$, infants and children; significant decrease in diarrhea symptoms.	Shornikova et al. (1997)
	Pediococcus pentosaceus	*P. pentosaceus* NB-17; mouse spleen cells were co-cultivated with heat-killed bacteria; *in vitro* investigation of the production of cytokines; effective stimulation of immune activities and allergic inhibitory effects.	Jonganurakkun et al. (2008)
	Oenococcus oeni	*O. oeni* 9115; female BALB/c mice with 2, 4, 6-trinitrobenzene sulfonic acid-induced experimental colitis; $n = 20$; significant decrease in severity of colitis. Several *O. oeni* strains were able to modulate the immune response of immunocompetent cells *in vitro.*	Foligné et al. (2010)
Enterococci	*E. durans*	*E. durans* LAB18s; *in vitro* study; antimicrobial activity, antioxidant ability, evidenced in both culture supernatants and intracellular extracts; resistance to acidic conditions (pH 3) and bile salts.	Pieniz et al. (2014)
	E. faecium	*E. faecium* MMRA; *in vitro* study; genes, coding enterocins A, B, P and X; high survival rates under stress caused by acidic pHs (2-5) or bile salts (0.3%), and a high adhesive potential.	Rehaiem et al. (2014)
	E. faecalis	*E. faecalis* UGRA10; *in vitro* study; production of AS-48 enterocin; ability to form biofilms and to adhere to Caco 2 and HeLa 229 cells.	Cebrián et al. (2012)
	E. lactis	*E. lactis* IITRHR1 was administered by gavage; male Wistar rats with acetaminophen-induced hepatotoxicity; $n = 42$; pretreatment with the bacterium lowered the level of biomarkers of hepatotoxicity in serum; significant increase in the level of antioxidants; modulation of key apoptotic/anti-apoptotic proteins (cytochrome-c, Bcl2, Bax, expression of caspases).	Sharma et al. (2012)
Yeasts	*Saccharomyces boulardii*	Granulated *S. boulardii*; double-blind randomized placebo-controlled clinical trial; $n = 200$, children with acute diarrhea; significant decrease in severity of symptoms and duration of hospital stay.	Kurugol and Koturoglu (2005), Kelesidis and Pothoulakis (2012)

Bifidobacterium sp. strains can reduce the side effects of *H. pylori* eradication therapy (Canducci et al., 2002).

Pediococci are also related to the LAB group and are utilized in industrial fermentations of foods and silage (Raccach, 2014). Pediocin-producing *Pediococcus* sp. strains are of potential interest to food safety (Raccach, 2014), with three of them potentially possessing probiotic properties – *Pediococcus pentosaceus*, *P. parvulus,* and *P. acidilactici*. Osmanagaoglu et al. (2010) comprehensively studied the potential of a human *P. pentosaceus* isolate for probiotic use, and reported

that the strain produced an anti-*Listerial* bacteriocin, had excellent autoaggregation characteristics and was also able to co-aggregate with *Salmonella enterica* serotype typhimurium and enterotoxigenic *Escherichia coli* (Osmanagaoglu et al., 2010). Antagonistic activity against *Listeria monocytogenes* was also discovered in *P. acidilactici* (Guerra and Pastrana, 2002). Clinical trials employing another strain of *Pediococcus* sp. revealed that the administration of *P. parvulus* decreased the serum cholesterol levels and increased counts of fecal *Bifidobacterium* sp. (Mårtensson et al., 2005).

Another group of LAB promoted as probiotics are enterococci, which reportedly help in the maintenance of normal intestinal microflora and stimulate the immune system (Bhardwaj et al., 2008). Studies of potential probiotic properties of *E. faecium* showed its efficacy in reducing the recovery period of acute diarrhea (Benyacoub et al., 2003). Another study by Pieniz et al. (2014) showed that *E. durans* possessed antimicrobial activity and antioxidant ability and was resistant to simulated gastric juice and bile salts. Though enterococci have probiotic potential, they are considered opportunistic pathogens for humans as they might cause nosocomial infection and are also known to possess resistance to vancomycin (Tambyah et al., 2004). Due to these controversial properties, the use of enterococci as probiotics remains under debate.

Bifidobacteria

Bifidobacteria are major constituents of the GIT microbiota of animals and humans. They are Gram-positive, non-motile anaerobic saccharolytic bacteria (Gomes and Malcata, 1999). In the gut environment, bifidobacteria have a commensal relationship with their hosts, and contribute to host nutrition by utilizing complex carbohydrates, which are important sources of carbon and energy, but are not degraded in the stomach or intestine (Biavati, 1994). These substances include plant-derived dietary fiber and diet-related carbohydrates, such as starch, galactan, sucrose, amylopectin, and pullulan (Ventura et al., 2007, 2012). The capacity of bifidobacteria to metabolize non-digestible host dietary carbohydrates (prebiotics) can be used for selective stimulation of certain strains colonizing the intestinal tract. Bifidobacteria used as probiotics include strains belonging to species of *Bifidobacterium lactis, B. bifidum, B. animalis, B. thermophilum, B. breve, B. longum, B. infantis*, and *B. adolescentis* (**Table 1**). These bacteria have been shown to inhibit the adherence of enterotoxigenic *E. coli*, enteropathogenic *E. coli,* and *C. difficile* to intestinal epithelial cells, an important trait for use of these bacteria as probiotics (Tsai et al., 2008). Additional beneficial effects of bifidobacterial strains include the prevention or alleviation of infectious diarrhea and the improvement of inflammatory bowel disease symptoms (Sanz, 2007). Bifidobacteria have also been shown to modulate the host's immune response against other indigenous microflora (e.g., *B. adolescentis* down-regulates humoral immunity to *Bacteroides thetaiotaomicron*; Scharek et al., 2000). Some bifidobacterial strains suppress *H. pylori*-induced genes in human epithelial cells (Shirasawa et al., 2010) while other *Bifidobacterium* sp. cells and culture supernatants exerted inhibitory effects against *Streptococcus mutans* and *Streptococcus sobrinus*, important etiological agents in human dental caries (Lee et al., 2011).

Yeasts

Saccharomyces boulardii is one of the best-studied probiotic species, with a long history of successful use in treatment of multiple gastrointestinal disorders. The administration of this probiotic in lyophilized form was found effective in cases of diarrhea by decreasing the duration of the disease, regardless of its cause (McFarland, 2007; Dinleyici et al., 2012; Shan et al., 2013). It has also been reported that *S. boulardii* prevented and treated relapses of inflammatory bowel disease, including moderate cases of ulcerative colitis (Guslandi et al., 2000; Guslandi et al., 2003; Choi et al., 2011). Interesting results have also been reported by Lim et al. (2015), suggesting that yeasts can enhance the growth of other probiotics under acidic conditions: *Saccharomyces cerevisiae* EC-1118 was found to significantly enhance the viability of the probiotic strain *Lactobacillus rhamnosus* HN001 at pH 2.5–4.0. The use of *S. boulardii* in reduction of *C. difficile* infection relapse is still under debate due to controversial results of clinical trials (Flatley et al., 2015). Among other yeasts species, *Torulaspora delbrueckii*, *Debaromyces hansenii*, *Yarrowia lipolytica, Kluyveromyces lactis, Kluyveromyces marxianus, and Kluyveromyces lodderae* have shown strong antagonistic effect against pathogenic bacteria and high acid tolerance (Kumura et al., 2004; Psani and Kotzekidou, 2006; Chen et al., 2010). Despite an excellent record of safe use, yeasts may still be the cause of localized infections in immunocompromised patients (Thygesen et al., 2012).

Akkermansia muciniphila

Another recently described microorganism with possible probiotic potential is *Akkermansia muciniphila* – a mucin-degrading bacterium that resides within intestinal mucus layers (Derrien et al., 2004). According to several studies, obese patients have significantly lower amounts of this bacterium in their GIT (Collado et al., 2008; Karlsson et al., 2012). The genome sequence of *A. muciniphila* suggests the ability of this bacterium to metabolize a variety of complex carbohydrates, as well as synthesize multiple amino acids, vitamins, and cofactors (van Passel et al., 2011). Its influence on metabolic processes in the GIT is not fully investigated; however, it has already been shown that this bacterium may be a potential treatment for type II diabetes. Shin et al. (2014) have shown that oral administration of *A. muciniphila* to mice induced Foxp3 regulatory T cells in the visceral adipose tissue, which attenuated adipose tissue inflammation. Based on these results it has been suggested that pharmacological manipulation of the gut microbiota in favor of *A. muciniphila* might be beneficial in the treatment of diabetes.

Faecalibacterium prausnitzii and Other Clostridia

Another bacterium that has been demonstrated to have a considerable impact on human gastrointestinal microbiota is *Faecalibacterium prausnitzii* of the *Clostridium* sp. cluster IV.

This microorganism accounts for 5–15% of the total fecal microbiota, making it one of the most abundant butyrate-producing bacteria in the GIT (Hold et al., 2003; Flint et al., 2012). Since butyrate is a primary energy source for intestinal epithelial cells, it is essential for maintenance of epithelial barrier integrity. Multiple beneficial effects of butyrate for health also include reduction of cancer progression, protection against pathogens, and stimulation of the immune system (Macfarlane and Macfarlane, 2011). The reduction of *F. prausnitzii* counts in fecal and biopsy samples has been observed in multiple studies of inflammatory bowel disease (especially, ileal Crohn's disease and ulcerative colitis), suggesting that the presence of this species is important for normal GIT function (Wang et al., 2007; Swidsinski et al., 2008; Andoh et al., 2012). The first gnotobiotic rodent model with *F. prausnitzii* showed that it could influence gut physiology through the production of mucus *O*-glycans, thereby affecting the quality and quantity of produced mucus (Wrzosek et al., 2013). Though *F. prausnitzii* dysbiosis might be an important marker in the development of disease, routine diagnostic tools have not been developed mainly due to the extreme sensitivity of this species to oxygen.

Other bacteria of the class Clostridia might also find use as potential probiotics, since they are highly abundant in human GIT microbiota and may play an important role in metabolism and immune system function. Atarashi et al. (2013) have shown that a mixture of 17 strains of *Clostridium* sp., belonging to clusters IV, XIV, and XVIII, were able to suppress experimental colitis in mice through induction of interleukin-10-producing regulatory T cells. A similar mechanism of colitis suppression, via IL-10 production by induced macrophages, was observed using strain *C. butyricum* MIYAIRI 588 (Hayashi et al., 2013). According to another recent study, when mixed with *B. infantis, C. butyricum* was effective in treatment of experimentally-induced antibiotic-associated diarrhea in mice, and the beneficial effect of the mixture was superior to single strains (Ling et al., 2015). However, though clostridia have potential for use as probiotics, there is still not enough evidence to support their medical efficacy and safety for humans.

Use of Probiotics in Prevention and Treatment of Antibiotic-Associated Diseases

Although most antibiotics are generally safe, some have the potential to cause life-threatening side effects. Antimicrobial side effects are adverse drug reactions involving one or more organ systems. Moreover, even a short-term course of antibiotics may have a long-term negative impact on the normal human gut microbiota (Jernberg et al., 2010). The most commonly used classes of antibiotics include penicillins, cephalosporins, aminoglycosides, fluoroquinolones, macrolides, and tetracyclines; each of these compounds can cause their own specific side-effects (Cunha, 2001). In fact, most traditionally used antibiotics are able to cause health problems in the GIT, and are commonly related to disturbances in microflora composition caused by survival and spread of resistant strains. For instance, penicillins, which are known for having the least-frequent and -severe side effects, may cause diarrhea, and nausea, vomiting, and upset stomach. Fluoroquinolones are also considered relatively safe, but may similarly induce nausea, vomiting, diarrhea, and abdominal pain (Bertino and Fish, 2000). Side-effects of macrolides include GIT-associated nausea, vomiting, and diarrhea, whereas adverse effects of the tetracyclines depend on the concentration of the antibiotic in the affected organ. Their common side-effects include cramps or burning of the stomach, diarrhea, sore mouth, or tongue (Rubinstein, 2001). Research in this field is ongoing and has already provided evidence for efficacy of probiotic use for prevention of health problems emerging as a result of antibiotic use. Examples of such diseases are antibiotic-associated diarrhea (AAD) and *C. difficile*-associated diarrhea (CDAD; pseudomembranous colitis).

Antibiotic-associated diarrhea is defined as "otherwise unexplained diarrhea that occurs in association with the administration of antibiotics" (Friedman, 2012). However, mild cases of *C. difficile* infection are sometimes also considered as the cause of AAD (Kelly et al., 1994). The disease comes as one of the most frequent side effects of antibiotic use: 5–39% of patients, depending on the type of antibiotic (e.g., certain β-lactam antibiotics are more likely to cause diarrheal side-effects than cephalosporins) and is associated with increased length and cost of hospitalization (Videlock and Cremonini, 2012). There are several mechanisms of antibiotic effect on humans that can result in AAD. These include osmotic diarrhea, caused by suppression of anaerobic bacteria and a reduction in carbohydrate metabolism, disruption of protective effect of commensal bacteria and reduction of colonic mucosal resistance to pathogenic opportunistic bacteria. Full restoration of the normal gut microbiota may take several weeks or even months (Friedman, 2012; Kaier, 2012).

Many studies have been conducted assessing the efficacy of probiotics in the treatment of AAD and have provided data supporting the usage of both single-strain and mixed-probiotics for diarrhea treatment (Surawicz, 2003; Szajewska et al., 2006; McFarland, 2009). A meta-analysis by Hempel et al. (2012) revealed 82 studies that provided evidence of probiotic efficiency in treatment of AAD. Microorganisms used in these studies included the genera *Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus,* and *Bacillus.* According to Friedman (2012), several mechanisms of action of probiotics contribute to the prevention and treatment of diarrhea: enhancing mucosal barrier function by secreting mucins, increasing tight junctions in epithelial cells, providing colonization resistance, producing bacteriocins, increasing production of secretory lgA, producing balanced T-helper cell response, increasing production of IL-10 and transforming growth factor beta. Collectively, these factors contribute to the restoration of a normal gastrointestinal balance following damage by antibiotics (Friedman, 2012).

Clostridium difficile-associated diarrhea or pseudo-membranous colitisis is an inflammation of the intestine

walls caused by toxins produced by *C. difficile*. CDAD is one of the most common hospital-acquired infections and is a frequent cause of morbidity and mortality among elderly hospitalized patients. Complications include shock, need for colectomy, toxic megacolon, and in severe cases, perforation of the colon wall. *C. difficile* colonizes the GIT after the alteration of normal gut flora by antibiotic therapy (Bergogne-Bérézin, 2000; Ndegwa and Nkansah, 2008). Extremely high rates of CDAD have been reported in Quebec from 2002 to 2005, totaling 14000 cases (a 4.5-fold increased incidence compared with 1991), with evidence suggesting the emergence of a highly-virulent strain of *C. difficile* (Pepin et al., 2004, 2005).

Several studies have shown that probiotics aid in prevention and treatment of CDAD. Gao et al. (2010) reported lower risk of disease occurrence after intake of a preparation based on two *Lactobacillus* strains. *S. boulardii* has also been successfully used for treatment of CDAD (McFarland et al., 1994). However, a large multi-center study is needed to build sufficient evidence in support of probiotic use as a treatment for *C. difficile*-associated infections.

Problems Associated with Transfer of Antibiotic Resistance Determinants

Many probiotic strains have naturally acquired resistance toward one or several antimicrobial agents (**Table 2**). Though intrinsic resistance of probiotic bacteria to certain antibiotics might offer benefits for their use in the prevention and treatment of AAD, the issue of possible transfer of resistance determinants has been raised (Pflughoeft and Versalovic, 2012), particularly for strains that carry plasmids.

TABLE 2 | Intrinsic antibiotic resistance of several widely used probiotic species.

Probiotic species	Antibiotic resistance	Reference
B. longum JDM301	Ciprofloxacin, amikacin, gentamicin, streptomycin	Wei et al. (2012)
B. longum[2]	Kanamycin	Temmerman et al. (2003)
B. lactis[2]	Kanamycin	Temmerman et al. (2003)
L. rhamnosus[1] *L. casei*[1] *L. paracasei*[1] *L. plantarum*[1] *L. acidophilus*[1]	Vancomycin, teicoplanin, bacitracin, aminoglycosides	Charteris et al. (1998), Danielsen and Wind (2003)
L. reuteri[2]	Kanamycin, tetracycin, penicillin, vancomycin	Temmerman et al. (2003)
L. casei[2]	Kanamycin, vancomycin	Temmerman et al. (2003)
L. acidophilus[2]	Kanamycin	Temmerman et al. (2003)
E. faecalis[1]	Beta-lactams Vancomycin	Yamaguchi et al. (2013) Werner et al. (2008)
B. adolescentis[2] *B. animalis*[2] *B. longum*[2] *B. bifidum*[2]	Kanamycin, neomycin, streptomycin, nalidixic acid	Kheadr et al. (2004)
P. pentosaceus	Vancomycin, teicoplanin	Biavasco et al. (1997)
P. acidilactici	Vancomycin	Temmerman et al. (2003)

[1]*Antibiotic resistance is indicated for more than 50% of isolates/strains used in the study, which were related to the species.*
[2]*Antibiotic resistance is indicated for 100% isolates/strains used in the study, which were related to the species.*

Courvalin (2006) specified two distinct types of acquired antibiotic resistance in bacteria: (i) initially non-transferred resistance that occurred as a result of one or several mutations in indigenous gene(s), and (ii) transferred resistance, acquired from a different organism by horizontal gene transfer. Antibiotic resistance (both intrinsic and acquired) can occur as a result of three major mechanisms: (i) altering the outer- and/or inner membrane permeability and transport activity, which leads to lower accumulation of the antibiotic within the cell, (ii) using enzymes to detoxify the antibiotic, and (iii) modifying the antibiotic target site (Guardabassi and Courvalin, 2006). The gene responsible for acquisition of antibiotic resistance often resides on a plasmid or transposon, which might be easily transferred (Bennett, 2008). In fact, transposon-mediated transfer of genetic material between species was recently described as the most frequent mechanism contributing to the spread of antibiotic resistance in bacteria (Wozniak and Waldor, 2010).

Multiple studies have already shown that antibiotic resistance can be transferred between different bacterial species that reside in the human GIT. For instance, it has been reported that both *Lactobacillus lactis* and *Streptococcus thermophilus* are able to transfer erythromycin resistance [*erm(B)* gene, located on a plasmid] to *L. monocytogenes* under *in vitro* conditions (Toomey et al., 2009). Another study provided the evidence of *in vivo* transfer of ampicillin resistance between two strains of *E. coli* co-residing in human gut: it was demonstrated that a plasmid carrying a β-lactamase gene had been transferred from an ampicillin- resistant *E. coli* strain to an initially susceptible strain (Karami et al., 2007). Devirgiliis et al. (2009) reported the transfer of a *tet*(M) gene (tetracycline resistance; located on broad host range Tn*916* transposon) from *L. paracasei* to *E. faecalis in vitro*. In another set of experiments, the erythromycin resistance pLFE1 plasmid of *L. plantarum* strain M345 was successfully transferred to five different species: *L. rhamnosus*, *Lc. lactis*, *Listeria innocua*, *E. faecalis*, and *L. monocytogenes* (Feld et al., 2009). These and other examples raise a safety concern; strains to be used as probiotics should be carefully selected, and only those free of transferrable antibiotic-resistance determinants ought to be considered safe (Radulovic et al., 2012).

In Vitro and *In Vivo* Systems Used to Study Probiotic Effects

Novel probiotic-based strategies for therapeutic and prophylactic use against multiple GIT diseases are gaining popularity worldwide. Their effectiveness has been predicted by numerous animal model studies and proven by extensive research involving humans. However, the initial step in confirming probiotic effects is the extensive characterization of a bacterial strain to be used as a probiotic, which is usually performed under *in vitro*

conditions by studying bacterial acid resistance, bile resistance, carbon source utilization, and aggregative properties, or *ex vivo* for their ability to adhere to mammalian cells (Kotikalapudi et al., 2010; Wood et al., 2012). Similarly, probiotic delivery methods, such as lyophilization or encapsulation, are also tested for their protective potential *in vitro* under simulated gastric conditions (Klemmer et al., 2011; Wood et al., 2012; Khan et al., 2013; Wang et al., 2014, 2015a). The most popular materials used for encapsulation of bacteria are alginate, carrageenans and gums, since they are easy to process, resistant to low pH and freezing, and are generally recognized as safe (Gbassi and Vandamme, 2012). We have recently reported the efficient delivery of *B. adolescentis,* encapsulated for this purpose in an alginate-pea protein protective matrix, into the lower gut of rats (Varankovich et al., 2015).

Apart from basic synthetic gastric juice solutions (low pH, 37°C), more complex systems have been developed, such as SHIME (Simulator of the Human Intestinal Microbial Ecosystem), designed to simulate different parts of the human GIT (Cook et al., 2012). Probiotic strains and methods for their delivery, preselected *in vitro,* are subsequently tested in animal models.

Traditionally used animal models include mice and rats. Larger animals like rabbits, dogs, and pigs are generally considered to have more common features with the physiology and microflora of the human GIT (Kararli, 1995). However, rodents are cheap, standardized, and have short life-cycles; thus, their extensive use in large-scale research. Investigation of probiotic effects on animal microflora may be approached by: (i) examining the quantitative and qualitative characteristics of bacterial microflora in animals using cultivation and/or molecular biology techniques, such as real-time polymerase chain reaction (qPCR), next-generation sequencing (NGS), and fluorescence *in situ* hybridization (FISH), or (ii) evaluating treatment efficiency indirectly by using it to cure an artificially induced disease.

Distribution of specific species of microorganisms is still being studied in healthy humans and compared with those of patients with various gastrointestinal diseases. Perturbations of microbiota, even in case of alterations in numbers of a single species (i.e., *A. muciniphila*), might be a cause (and an indicator) of the development of disease (Karlsson et al., 2012). In this case, probiotic treatment might be useful in restoring microbiota balance in the gut. An interesting example of quantitative/qualitative analysis of animal gut microbiota after probiotic administration can be found in the study by Wang et al. (2015b): 454 pyrosequencing of fecal bacterial 16S rRNA genes in obese vs. lean mice showed that the probiotic strains shifted the overall structure of the gut microbiota of obese animals toward that of lean mice fed a normal diet, with significant changes observed in 83 operational taxonomic units. Due to complicated analyses required to understand specific mechanisms of disease development, as well as the mode of action of a certain probiotic microorganism, the use of disease models is generally more widespread.

Rodent Models of GIT Diseases

Generally, in order to establish a disease model, mice are infected with the pathogen or irritant either one time or continuously (Pawlowski et al., 2010; Bhinder et al., 2013). Subsequently, animals are treated with probiotics with concomitant monitoring of the disease symptoms and evaluation of changes in the gut microflora. Following this approach, Verdú et al. (2008) infected mice with *H. pylori* for 4–6 months to investigate the effect of probiotic therapy on upper gastrointestinal dysfunction induced by chronic *H. pylori* infection. The authors reported that with probiotic treatment delayed gastric emptying in mice normalized significantly faster post-eradication, compared to control groups, where the dysfunction was observed during 2 months after pathogen administration was ceased. Mice and rats have also been used to evaluate the efficacy of probiotics for the treatment of *Salmonella* and *E. coli* O157:H7 infections (Asahara et al., 2001, 2004), inflammatory bowel disease (Shiba et al., 2003) and immune suppression (Lollo et al., 2012). Asahara et al. (2001) showed that intestinal growth and subsequent extra-intestinal translocation of orally-infected *Salmonella typhimurium* in mice were inhibited during administration of probiotic *B. breve*. Later, the same group reported *B. breve* was also effective in protecting mice against Shiga toxic-producing *E. coli* 0157:H7 (Asahara et al., 2004). Extrapolation of results achieved in animal studies and *in vitro* experiments to humans remains a difficult challenge. Many factors, such as differences in physiology and microflora composition of respective gastrointestinal systems, must be considered before interpreting the outcome.

The majority of *in vivo* experiments investigating the effects of probiotics on pathogenic bacterial populations use gnotobiotic mice (usually with human microflora systems in their GIT; Bernet-Camard et al., 1997; Aiba et al., 1998; Gill et al., 2001; Pawlowski et al., 2010). For instance, in a study by Shiba et al. (2003), probiotic *B. infantis* 1222 was found to significantly suppress the systemic antibody response raised by *Bacteroides vulgates,* a representative pathogenic *Bacteroides* sp. species, in a gnotobiotic mice model of inflammatory bowel disease. The use of conventional mice as a model for investigating human diseases is more problematic due to significant differences in animal and human gut microflora. Nevertheless, it is possible to use murine-specific organisms as models for the study of human pathogens. For instance, Ge et al. (2001) used *H. hepaticus* infection as an animal model for examining the pathogenesis of gastrointestinal diseases in humans caused by *H. pylori.* More recently, Bhinder et al. (2013) described the *Citrobacter rodentium* mouse model for the study of pathogen and host contributions during infectious colitis. *C. rodentium* is a murine-specific bacterial pathogen, closely related to enteropathogenic and enterohaemorrhagic strains of *E. coli* (Borenstein et al., 2008). Several *C. rodentium* infection studies involving mice models have shown probiotics to reduce the severity of symptoms and prevent death caused by the pathogenic agent (Chen et al., 2005; Gareau et al., 2010; Mackos et al., 2013). Chen et al. (2005) successfully treated *C. rodentium*-induced murine colitis with probiotic *L. acidophilus*. Gareau et al. (2010) similarly reported that *L. rhamnosus*, combined with *L. helveticus*, were

effective in prevention and treatment of the same disease state in mice. Later, another group showed that *L. reuteri* was able to attenuate the severity of murine colitis caused by *C. rodentium* (Mackos et al., 2013). Further investigation of host–pathogen and probiotic–pathogen interactions will likely provide better insight into treatment of *C. rodentium* infection in mice, and possibly *E. coli* infections in humans. However, confirmation of probiotic benefits and possible side effects will ultimately require human trials.

Human Clinical Trials

Human studies generally take the form of randomized clinical trials involving participants with some type of intestinal disorder. After assessment of eligibility and recruitment, participants

TABLE 3 | Some of the major human trials of probiotics for the treatment of gastrointestinal diseases.

Probiotic strain	Disease	Number of participants	Reported outcome	Reference
Lactobacillus rhamnosus GG	*H. pylori* infection	60	Significant reduction ($p = 0.04$) of diarrhea, nausea and taste disturbances in the treatment group.	Armuzzi et al. (2001)
	Antibiotic-associated diarrhea in children	188	Significant reduction of the incidence of antibiotic-associated diarrhea in children treated with oral antibiotics for common childhood infections.	Vanderhoof et al. (1999)
		167	The treatment effect on the incidence of diarrhea (95% confidence interval) was −11% (−21−0%).	Arvola et al. (1999)
B. bifidum	Irritable bowel syndrome	122	Overall responder rates (decrease in symptoms severity) were 57% in the treatment group, but only 21% in the placebo group ($P = 0.0001$).	Guglielmetti et al. (2011)
B. infantis		362	The improvement in overall symptom assessment exceeded the placebo by more than 20% ($p < 0.02$).	Whorwell et al. (2006)
S. cerevisiae		179	The proportion of responders reporting improvement in abdominal pain/discomfort was significantly higher ($p = 0.04$) in the treated group than the placebo group (63% vs. 47%, OR = 1.88, 95%, CI: 0.99–3.57).	Pineton de Chamburn et al. (2015)
VSL#3*	Pouchitis	40	Three patients (15%) in the treatment group had relapses of the disease within the 9-months follow-up period, compared with 20 (100%) in the placebo group ($P < 0.001$).	Gionchetti et al. (2000)
		40	Two of the 20 patients (10%) in the treatment group had an episode of acute pouchitis compared with 8 of the 20 patients (40%) treated with placebo (log-rank test, $z = 2.273$; $P < 0.05$).	Gionchetti et al., 2003
		34	Treatment of patients with mild to moderate stages of disease, not responding to conventional therapy, with probiotic resulted in a combined induction of remission/response rate of 77% with no adverse events.	Bibiloni et al. (2005)
	Ulcerative colitis	124	The efficacy of probiotic was significant (recurrence rate 34.6%, compared with 64.7% on placebo; $p = 0.04$) in patients with recurrent CDD, but not in patients with initial CDD (recurrence rate 19.3% compared with 24.2% on placebo; $p = 0.86$).	McFarland et al. (1994)
Saccharomyces boulardii	*Clostridium difficile*-associated diarrhea (CDD)	168	A significant decrease in recurrence of CDD was observed only in patients treated with high-dose vancomycin (2 g/day) and probiotic (16.7%) compared with those who received high-dose vancomycin and placebo (50%; $p = 0.05$).	Surawicz et al. (2000)
		211	The mean (+/−SD) duration of diarrhea was 1.69 days (0.6) in patients given probiotic, compared with 2.81 days (0.9) in those given placebo.	Buydens and Debeuckelaere (1996)
Enterococcus faecium SF68	Antibiotic-associated diarrhea	123	The probiotic was shown to be effective in reducing the incidence of antibiotic-associated diarrhea (AAD) in comparison with placebo (8.7% compared with 27.2%, respectively).	Wunderlich et al. (1989)
Mixture of lactobacilli, bifidobacteria and streprococci	Travelers' diarrhea	94	Prophylaxis with the probiotic significantly decreased the frequency of diarrhea from 71 to 43% ($p = 0.019$).	Black et al. (1989)
Mixture of *B. infantis*, *B. bifidum*, *B. longum* and *L. acidophilus*	Necrotizing enterocolitis in newborns	186	Enteral administration of the probiotic in neonatal intensive care setup significantly reduced morbidity due to necrotising enterocolitis in very low birth weight newborn.	Samanta et al. (2009)

**A mixture of Lactobacillus casei, L. plantarum, L. acidophilus, L. delbrueckii subsp. bulgaricus, B. longum, B. breve, B. infantis, and Streptococcus salivarius sp. Thermophiles.*

are given either probiotic treatment or a placebo as a control. Results of these experiments have provided enough evidence for considering probiotics an efficient treatment for multiple GIT-associated diseases, such as acute gastroenteritis (Huang et al., 2002), irritable bowel syndrome (Nikfar et al., 2008) and necrotizing enterocolitis (Alfaleh and Anabrees, 2014). Some trials showing the efficacy of bacteria of interest in the treatment of specific gastrointestinal disorders are listed in **Table 3**. In one recent trial aimed to assess the efficiency of *S. cerevisiae* in treatment of irritable bowel syndrome, 179 adults, diagnosed with this condition, were randomized to receive once-daily 500 mg of *S. cerevisiae* or placebo for 8 weeks. Cardinal symptoms (abdominal pain/discomfort, bloating/distension, bowel movement difficulty) were recorded daily after a 2-week run-in period. The results showed that abdominal pain/discomfort scores were significantly reduced during probiotic intake (Pineton de Chamburn et al., 2015). A major trial involving 362 participants was conducted by Whorwell et al. (2006) in order to study the effect of *B. infantis* on symptoms of irritable bowel syndrome: probiotic administration lead to improvements in the majority of symptoms by more than 20%, compared to placebo. Another human clinical trial proved the efficacy of *Lactobacillus GG* in treatment of *H. pylori* infection: daily administration of the probiotic led to significant reduction in disease symptoms (diarrhea, nausea, and taste disturbances; Armuzzi et al., 2001). In general, data from multiple lines of research involving humans suggests that probiotic bacteria suppress gastrointestinal pathogens by simple competition by prevailing in numbers, and by producing antibacterial factors (bacteriocins and small organic molecules, such as fatty acids). Though more details into the mechanisms of action of probiotics on gut microbiota are essential, the large base of evidence already collected has proven the beneficial role in prevention and treatment of various GIT diseases in humans.

Conclusion

Many strains of genera *Lactobacillus* and *Bifidobacterium*, as well as some enterococci and yeasts, have been shown to possess probiotic properties with potential for prophylaxis and treatment of a range of gastrointestinal disorders. The effectiveness of probiotic bacteria in the treatment of these conditions is supported by many clinical trials involving patients of all ages and probiotic organisms chosen based on laboratory research trials. Notably, most of the work in the probiotic field has been conducted *in vitro*, as it is an essential step in the investigation of bacterial growth, metabolite production, ability to form biofilms, compete with pathogens, co-aggregate, and produce antimicrobials. All of these characteristics are important factors for identification of potential probiotic strains that possess desirable properties along with the ability to establish itself in the human gut. Intrinsic antibiotic resistance and transferability of genetic determinants are two additional factors to account for at the initial stage of a probiotic study. Novel putative probiotic species, such as *A. muciniphila*, are yet to be tested in both animal and human trials; however, the results achieved to date suggest that they might be beneficial in treatment or diagnosis of GIT diseases.

Acknowledgment

Financial support for this project was provided by the Saskatchewan Ministry of Agriculture Development Fund.

References

Aiba, Y., Suzuki, N., Kabir, A. M., Takagi, A., and Koga, Y. (1998). Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. *Am. J. Gastroenterol.* 93, 2097–2101. doi: 10.1111/j.1572-0241.1998.00600.x

AlFaleh, K., and Anabrees, J. (2014). Probiotics for prevention of necrotizing enterocolitis in preterm infants. *Cochrane Database Syst. Rev.* 2014:CD005496. doi: 10.1002/14651858.CD005496.pub4

Andoh, A., Kuzuoka, H., Tsujikawa, T., Nakamura, S., Hirai, F., Suzuki, Y., et al. (2012). Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease. *J. Gastroenterol.* 47, 1298–1307. doi: 10.1007/s00535-012-0605-0

Armuzzi, A., Cremonini, F., Bartolozzi, F., Canducci, F., Candelli, M., Ojetti, V., et al. (2001). The effect of oral administration of *Lactobacillus* GG on antibiotic-associated gastrointestinal side-effects during *Helicobacter pylori* eradication therapy. *Aliment. Pharmacol. Ther.* 15, 163–169. doi: 10.1046/j.1365-2036.2001.00923.x

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., et al. (2013). Enterotypes of the human gut microbiome. *Nature* 473, 174–180. doi: 10.1038/nature09944

Arvola, T., Laiho, K., Torkkeli, S., Mykkanen, H., Salminen, S., Maunula, L., et al. (1999). Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. *Pediatrics* 104, e64. doi: 10.1542/peds.104.5.e64

Asahara, T., Nomoto, K., Shimizu, K., Watanuki, M., and Tanaka, R. (2001). Increased resistance of mice to *Salmonella enterica* serovar *Typhimurium* infection by synbiotic administration of Bifidobacteria and transgalactosylated oligosaccharides. *J. Appl. Microbiol.* 91, 985–996. doi: 10.1046/j.1365-2672.2001.01461.x

Asahara, T., Shimizu, K., Nomoto, K., Asahara, T., Shimizu, K., and Nomoto, K. (2004). Probiotic Bifidobacteria protect mice from lethal infection with shiga toxin-producing *Escherichia coli* O157: H7. *Infect. Immun.* 72, 2240–2247. doi: 10.1128/IAI.72.4.2240-2247.2004

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of clostridia strains from the human microbiota. *Nature* 500, 232–236. doi: 10.1038/nature12331

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L., et al. (2011). Probiotics and health: an evidence-based review. *Pharmacol. Res.* 63, 366–376. doi: 10.1016/j.phrs.2011.02.006

Basu, S., Paul, D. K., Ganguly, S., Chatterjee, M., and Chandra, P. K. (2009). Efficacy of high-dose *Lactobacillus rhamnosus* GG in controlling acute watery diarrhea in Indian children: a randomized controlled trial. *J. Clin. Gastroenterol.* 43, 208–213. doi: 10.1097/MCG.0b013e31815a5780

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153, S347–S357. doi: 10.1038/sj.bjp.0707607

Benyacoub, J., Czarnecki-Maulden, G. L., Cavadini, C., Sauthier, T., Anderson, R. E., Schiffrin, E. J., et al. (2003). Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. *J. Nutr.* 133, 1158–1162.

Bergogne-Bérézin, E. (2000). Treatment and prevention of antibiotic associated diarrhea. *Int. J. Antimicrob. Agents* 16, 521–526. doi: 10.1016/S0924-8579(00)00293-4

Bernet-Camard, M. F., Lievin, V., Brassart, D., Neeser, J. R., Servin, A. L., and Hudault, S. (1997). The human *Lactobacillus acidophilus* strain LA1 secretes a nonbacteriocin antibacterial substance(s) active in vitro and in vivo. *Appl. Environ. Microbiol.* 63, 2747–2753.

Bertino, J., and Fish, D. (2000). The safety profile of the fluoroquinolones. *Clin. Ther.* 22, 798–817. doi: 10.1016/S0149-2918(00)80053-3

Bhardwaj, A., Malik, R. K., and Chauhan, P. (2008). Functional and safety aspects of enterococci in dairy foods. *Indian J. Microbiol.* 48, 317–325. doi: 10.1007/s12088-008-0041-2

Bhinder, G., Sham, H. P., Chan, J. M., Morampudi, V., Jacobson, K., and Vallance, B. A. (2013). The *Citrobacter rodentium* mouse model: studying pathogen and host contributions to infectious colitis. *J. Vis. Exp.* 72:e50222. doi: 10.3791/50222

Biavasco, F., Vignaroli, C., Lupidi, R., Manso, E., Facinelli, B., and Varaldo, P. E. (1997). In vitro antibacterial activity of LY333328, a new semisynthetic glycopeptide. *Antimicrob. Agents Chemother.* 41, 2165–2172.

Biavati, B. (1994). Degradation of complex carbohydrates. *Int. J. Food Microb.* 24, 199–210. doi: 10.1016/0168-1605(94)90119-8

Bibiloni, R., Fedorak, R. N., Tannock, G. W., Madsen, K. L., Gionchetti, P., Campieri, M., et al. (2005). VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am. J. Gastroenterol.* 100, 1539–1546. doi: 10.1111/j.1572-0241.2005.41794.x

Black, F. T., Andersen, P. L., Ørskov, J., Ørskov, F., Gaarslev, K., and Laulund, S. (1989). Prophylactic efficacy of lactobacilli on traveler's diarrhea. *Trav. Med.* 7, 333–335. doi: 10.1007/978-3-642-73772-5_70

Borenstein, D., McBee, M. E., and Schauer, D. B. (2008). Utility of the *Citrobacter rodentium* infection model in laboratory mice. *Curr. Open. Gastroenterol.* 24, 32–37. doi: 10.1097/MOG.0b013e3282f2b0fb

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., et al. (2011). Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16050–16055. doi: 10.1073/pnas.1102999108

Buydens, P., and Debeuckelaere, S. (1996). Efficacy of SF 68 in the treatment of acute diarrhea. A placebo-controlled trial. *Scand J. Gastroenterol.* 31, 887–891. doi: 10.3109/00365529609051997

Caballero-Franco, C., Keller, K., De Simone, C., and Chadee, K. (2007). The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am. J. Physiol. Gastroint. Liver Physiol.* 292, 315–322. doi: 10.1152/ajpgi.00265.2006

Canducci, F., Cremonini, F., Armuzzi, A., Di Caro, S., Gabrielli, M., Santarelli, L., et al. (2002). Probiotics and *Helicobacter pylori* eradication. *Digest Liver Dis.* 34, S81–S83. doi: 10.1016/S1590-8658(02)80172-4

Cebrián, R., Baños, A., Valdivia, E., Pérez-Pulido, R., Martínez-Bueno, M., and Maqueda, M. (2012). Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis* UGRA10, a new AS-48-producer strain. *Food Microbiol.* 30, 59–67. doi: 10.1016/j.fm.2011.12.002

Charteris, W. P., Kelly, P. M., Morelli, L., and Collins, J. K. (1998). Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *J. Food Prot.* 61, 1636–1643.

Chen, C.-C., Louie, S., Shi, H. N., and Walker, W. A. (2005). Preinoculation with the probiotic *Lactobacillus acidophilus* early in life effectively inhibits murine *Citrobacter rodentium* colitis. *Pediatr. Res.* 58, 1185–1191. doi: 10.1203/01.pdr.0000183660.39116.83

Chen, L.-S., Ma, Y., Maubois, J.-L., He, S.-H., Chen, L.-J., and Li, H.-M. (2010). Screening for the potential probiotic yeast strains from raw milk to assimilate cholesterol. *Dairy Sci. Technol.* 90, 537–548. doi: 10.1051/dst/2010001

Choi, C. H., Jo, S. Y., Park, H. J., Chang, S. K., Byeon, J. S., and Myung, S. J. (2011). A randomized, double-blind, placebo-controlled multicenter trial of *Saccharomyces boulardii* in irritable bowel syndrome: effect on quality of life. *J. Clin. Gastroenterol.* 45, 679–683. doi: 10.1097/MCG.0b013e318204593e

Collado, M. C., Isolauri, E., Laitinen, K., and Salminen, S. (2008). Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. *Am. J. Clin. Nutr.* 88, 894–899.

Collado, M. C., Meriluoto, J., and Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Lett. Appl. Microbiol.* 45, 454–460. doi: 10.1111/j.1472-765X.2007.02212.x

Cook, M. T., Tzortzis, G., Charalampopoulos, D., and Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *J. Control. Release* 162, 56–67. doi: 10.1016/j.jconrel.2012.06.003

Courvalin, P. (2006). Antibiotic resistance: the pros and cons of probiotics. *Dig. Liver Dis.* 38(Suppl. 2), S261–S265. doi: 10.1016/S1590-8658(07)60006-1

Cruchet, S., Obregon, M. C., Salazar, G., Diaz, E., and Gotteland, M. (2003). Effect of the ingestion of a dietary product containing *Lactobacillus johnsonii* La1 on *Helicobacter pylori* colonization in children. *Nutrition* 19, 716–721. doi: 10.1016/S0899-9007(03)00109-6

Cunha, B. A. (2001). Antibiotic therapy, Part II. *Int. Med. Clin. North Am.* 85, 149–185. doi: 10.1016/S0025-7125(05)70309-6

Danielsen, M., and Wind, A. (2003). Susceptibility of *Lactobacillus* spp. *to* antimicrobial agents. *Int. J. Food Microbiol.* 82, 1–11. doi: 10.1016/S0168-1605(02)00254-4

Derrien, M., Vaughan, E. E., Plugge, C. M., and de Vos, W. M. (2004). *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int. J. Syst. Evol. Microbiol.* 54, 1469–1476. doi: 10.1099/ijs.0.02873-0

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., and Dinan, T. G. (2008). The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. *J. Psychiatr. Res.* 43, 164–174. doi: 10.1016/j.jpsychires.2008.03.009

Devirgiliis, C., Coppola, D., Barile, S., Colonna, B., and Perozzi, G. (2009). Characterization of the Tn916 conjugative transposon in a food-borne strain of *Lactobacillus paracasei*. *Appl. Environ. Microbiol.* 75, 3866–3871. doi: 10.1128/AEM.00589-09

DiBaise, J. K., Frank, D. N., and Mathur, R. (2012). Impact of the gut microbiota on the development of obesity: current concepts. *Am. J. Gastroenterol. Suppl.* 1, 22–27. doi: 10.1038/ajgsup.2012.5

Dinan, T. G., Stanton, C., and Cryan, J. F. (2013). Psychobiotics: a novel class of psychotropic. *Biol. Psych.* 74, 720–726. doi: 10.1016/j.biopsych.2013.05.001

Dinleyici, E. C., Eren, M., Ozen, M., Yargic, Z. A., and Vandenplas, Y. (2012). Effectiveness and safety of *Saccharomyces boulardii* for acute infectious diarrhea. *Expert Opin. Biol. Ther.* 12, 395–410. doi: 10.1517/14712598.2012.664129

Doré, J., and Corthier, G. (2010). The human intestinal microbiota. *Gastroentérol. Clin. Biol.* 34, S7–S15. doi: 10.1016/S0399-8320(10)70002-6

Duca, F., Sakar, Y., Lepage, P., Devime, F., Langelier, B., Doré, J., et al. (2014). Replication of obesity and associated signaling pathways through transfer of microbiota from obese-prone rats. *Diabetes Metab. Res. Rev.* 63, 1624–1636. doi: 10.2337/db13-1526

Dusko Ehrlich, S., and MetaHIT Consortium. (2011). "MetaHIT: the European Union Project on metagenomics of the human intestinal tract," in *Metagenomics of the Human Body*, Chap. 15. ed. K. Nelson (Berlin: Springer).

FAO/WHO. (2001). *Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health, and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria* (Córdoba: Food and Agriculture Organization of the United Nations, World Health Organization).

Feld, L., Bielak, E., Hammer, K., and Wilcks, A. (2009). Characterization of a small erythromycin resistance plasmid pLFE1 from the food-isolate *Lactobacillus plantarum* M345. *Plasmid* 61, 159–170. doi: 10.1016/j.plasmid.2009.01.002

Flatley, E. A., Wilde, A. M., and Nailor, M. D. (2015). *Saccharomyces boulardii* for the prevention of hospital onset *Clostridium difficile* infection. *J. Gastrointestin. Liver Dis.* 24, 21–24. doi: 10.15403/jgld.2014.1121.fly

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

Foligné, B., Dewulf, J., Breton, J., Claisse, O., Lonvaud-Funel, A., and Pot, B. (2010). Probiotic properties of non-conventional lactic acid bacteria: immunomodulation by *Oenococcus oeni*. *Int. J. Food Microbiol.* 140, 136–145. doi: 10.1016/j.ijfoodmicro.2010.04.007

Foligne, B., Zoumpopoulou, G., Dewulf, J., Ben Younes, A., Chareyre, F., Sirard, J.-C., et al. (2007). A key role of dendritic cells in probiotic functionality. *PLoS ONE* 2:e313. doi: 10.1371/journal.pone.0000313

Fooks, L. J., Fuller, R., and Gibson, G. R. (1999). Prebiotics, probiotics and human gut microbiology. *Int. Dairy J.* 9, 53–61. doi: 10.1016/S0958-6946(99)00044-8

Friedman, G. (2012). Preface: clinical applications of probiotics in gastroenterology: questions and answers. *Gastroenterol. Clin. North Am.* 41, 763–779. doi: 10.1016/j.gtc.2012.08.002

Gao, X. W., Mubasher, M., Fang, C. Y., Reifer, C., and Miller, L. E. (2010). Dose-response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and *Clostridium* difficile-associated diarrhea prophylaxis in adult patients. *Am. J. Gastroenterol.* 105, 1636–1641. doi: 10.1038/ajg.2010.11

Gareau, M. G., Wine, E., Reardon, C., and Sherman, P. M. (2010). Probiotics prevent death caused by *Citrobacter rodentium* infection in neonatal mice. *J. Inf. Dis.* 201, 81–91. doi: 10.1086/648614

Gbassi, G. K., and Vandamme, T. (2012). Probiotic encapsulation technology: from microencapsulation to release into the gut. *Pharmaceutics* 4, 149–163. doi: 10.3390/pharmaceutics4010149

Ge, Z., White, D. A., Whary, M. T., and Fox, J. G. (2001). Fluorogenic PCR-Based quantitative detection of a murine pathogen *Helicobacter hepaticus*. *J. Clin. Microbiol.* 39, 2598–2602. doi: 10.1128/JCM.39.7.2598-2602.2001

Gill, H. S., Shu, Q., Lin, H., Rutherfurd, K. J., and Cross, M. L. (2001). Protection against translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. *Med. Microbiol. Immunol.* 190, 97–9104.

Gionchetti, P., Rizzello, F., Helwig, U., Venturi, A., Lammers, K. M., Brigidi, P., et al. (2003). Prophylactis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 124, 1202–1209. doi: 10.1016/S0016-5085(03)00171-9

Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., et al. (2000). Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 119, 305–309. doi: 10.1053/gast.2000.9370

Gomes, A. M. P., and Malcata, F. X. (1999). *Bifidobacterium* spp. *and Lactobacillus acidophilus:* biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends Food Sci. Tech.* 10, 139–157. doi: 10.1016/S0924-2244(99)00033-3

Guardabassi, L., and Courvalin, P. (2006). "Antibiotics: Mode of action and mechanisms of resistance," in *Antimicrobial Resistance in Bacteria of Animal Origin,* eds F. Aarestrup and H. K. Wegener (Washington, DC: American Society for Microbiology), 1–18.

Guerra, N. P., and Pastrana, L. (2002). Modelling the influence of pH on the kinetics of both nisin and pediocin production and characterization of their functional properties. *Proc. Biochem.* 37, 1005–1015. doi: 10.1016/S0032-9592(01)00312-0

Guglielmetti, S., Mora, D., Gschwender, M., and Popp, K. (2011). Randomised clinical trial: *Bifidobacterium bifidum* MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life – a double-blind, placebo-controlled study. *Alim. Pharmacol. Therapeu.* 33, 1123–1132. doi: 10.1111/j.1365-2036.2011.04633.x

Guo, X. H., Kim, J. M., Nam, H. M., Park, S. Y., and Kim, J. M. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. *Anaerobe* 16, 321–326. doi: 10.1016/j.anaerobe.2010.03.006

Guslandi, M., Giollo, P., and Testoni, P. A. (2003). A pilot trial of *Saccharomyces boulardii* in ulcerative colitis. *Eur. J. Gastroenterol. Hepatol.* 15, 697–698. doi: 10.1097/00042737-200306000-00017

Guslandi, M., Mezzi, G., Sorghi, M., and Testoni, P. A. (2000). *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Dig. Dis. Sci.* 45, 1462–1464. doi: 10.1023/A:1005588911207

Hamilton-Miller, J. M. T. (2003). The role of probiotics in the treatment and prevention of *Helicobacter pylori* infection. *Int. J. Antimicrob. Agents* 22, 360–366. doi: 10.1016/S0924-8579(03)00153-5

Hayashi, A., Sato, T., Kamada, N., Mikami, Y., Matsuoka, K., and Hisamatsu, T. (2013). A single strain of *Clostridium butyricum* induces intestinal IL-10-producing macrophages to suppress acute experimental colitis in mice. *Cell Host Microbe* 13, 711–722. doi: 10.1016/j.chom.2013.05.013

Hempel, S., Maher, A. R., Wang, Z., Miles, J. N. V., Shanman, R., Johnsen, B., et al. (2012). Probiotics for the prevention and treatment of antibiotic-associated diarrhea. A systematic review and meta-analysis. *J. Am. Med. Assoc.* 307, 1959–1969. doi: 10.1001/jama.2012.3507

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

Hold, G. L., Schwiertz, A., Aminov, R. I., Blaut, M., and Flint, H. J. (2003). Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. *Appl. Environ. Microbiol.* 69, 4320–4324. doi: 10.1128/AEM.69.7.4320-4324.2003

Holzapfel, W. H., and Schillinger, U. (2002). Introduction to pre- and probiotics. *Food Res. Int.* 35, 109–116. doi: 10.1016/S0963-9969(01)00171-5

Huang, J. S., Bousvaros, A., Lee, J. W., Diaz, A., and Davidson, E. J. (2002). Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. *Diges Dis. Sci.* 47, 2625–2634. doi: 10.1023/A:1020501202369

Jernberg, C., Löfmark, S., Edlund, C., and Jansson, J. K. (2010). Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 156(Pt 11), 3216–3223. doi: 10.1099/mic.0.040618-0

Jonganurakkun, B., Wang, Q., Xu, S. H., Tada, Y., Minamida, K., Yasokawa, D., et al. (2008). *Pediococcus pentosaceus* NB-17 for probiotic use. *J. Biosci. Bioeng.* 106, 69–73. doi: 10.1263/jbb.106.69

Jungersen, M., Wind, A., Johansen, E., Christensen, J., Stuer-Lauridsen, B., and Eskesen, D. (2014). The science behind the probiotic strain *Bifidobacterium animalis* subsp. lactis BB-12. *Microorganisms* 2, 92–110. doi: 10.3390/microorganisms2020092

Kaier, K. (2012). Economic implications of the dynamic relationship between antibiotic use and hospital-acquired infections. *Val. Health* 15, 87–93. doi: 10.1016/j.jval.2011.09.005

Karami, N., Martner, A., Enne, V. I., Swerkersson, S., Adlerberth, I., and Wold, A. E. (2007). Transfer of an ampicillin resistance gene between two *Escherichia coli* strains in the bowel microbiota of an infant treated with antibiotics. *J. Antimicrob. Chemother.* 60, 1142–1145. doi: 10.1093/jac/dkm327

Kararli, T. T. (1995). Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* 16, 351–380. doi: 10.1002/bdd.2510160502

Karlsson, C. L., Onnerfält, J., Xu, J., Molin, G., Ahrné, S., and Thorngren-Jerneck, K. (2012). The microbiota of the gut in preschool children with normal and excessive body weight. *Obesity* 20, 2257–2261. doi: 10.1038/oby.2012.110

Kelesidis, T., and Pothoulakis, C. (2012). Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. *Therapeu. Adv. Gastroenterol.* 5, 111–125. doi: 10.1177/1756283X11428502

Kelly, C. P., Pothoulakis, C., and Lamont, J. T. (1994). *Clostridium difficile* colitis. *N. Engl. J. Med.* 330, 257–262. doi: 10.1056/NEJM199401273300406

Khan, N. H., Korber, D. R., Low, N. H., and Nickerson, M. T. (2013). Development of extrusion-based legume protein isolate-alginate capsules for the protection and delivery of the acid sensitive probiotic, *Bifidobacterium adolescentis*. *Food Res. Int.* 54, 730–737. doi: 10.1016/j.foodres.2013.08.017

Kheadr, E., Bernoussi, N., Lacroix, C., and Fliss, I. (2004). Comparison of the sensitivity of commercial strains and infant isolates of bifidobacteria to antibiotics and bacteriocins. *Int. Dairy J.* 14, 1041–1053. doi: 10.1016/j.idairyj.2004.04.010

Klemmer, K. J., Korber, D. R., Low, N. H., and Nickerson, M. T. (2011). Pea protein-based capsules for probiotic and prebiotic delivery. *Int. J. Food Sci. Tech.* 46, 2248–2256. doi: 10.1111/j.1365-2621.2011.02743.x

Konieczna, P., Ferstl, R., Ziegler, M., Frei, R., Nehrbass, D., Lauener, R. P., et al. (2013). Immunomodulation by *Bifidobacterium infantis* 35624 in the murine lamina propria requires retinoic acid-dependent and independent mechanisms. *PLoS ONE* 8:e62617. doi: 10.1371/journal.pone.0062617

Kotikalapudi, B. L., Low, N. H., Nickerson, M. T., and Korber, D. R. (2010). *In vitro* characterization of probiotic survival, adherence and antimicrobial resistance: candidate selection for encapsulation in a pea protein isolate–alginate delivery system. *Int. J. Probiotics Prebiotics* 5, 1–12.

Kotzampassi, K., and Giamarellos-Bourboulis, E. J. (2012). Probiotics for infectious diseases: more drugs, less dietary supplementation. *Int. J. Antimicrob. Agents* 40, 288–296. doi: 10.1016/j.ijantimicag.2012.06.006

Kumura, H., Tanoue, Y., Tsukahara, M., Tanaka, T., and Shimazaki, K. (2004). Screening of dairy yeast strains for probiotic applications. *J. Dairy Sci.* 87, 4050–4056. doi: 10.3168/jds.S0022-0302(04)73546-8

Kurugol, Z., and Koturoglu, G. (2005). Effects of *Saccharomyces boulardii* in children with acute diarrhoea. *Acta Paediatr.* 94, 44–47. doi: 10.1080/08035250410022521

Lee, D. K., Park, S. Y., An, H. M., Kim, J. R., Kim, M. J., Lee, S. W., et al. (2011). Antimicrobial activity of *Bifidobacterium* spp. isolated from healthy adult Koreans against cariogenic microflora. *Arch. Oral. Biol.* 56, 1047–54. doi: 10.1016/j.archoralbio.2011.03.002

Lee, J.-Y., Chu, S.-H., Jeon, J. Y., Lee, M.-K., Park, J.-H., Lee, D.-C., et al. (2014a). Effects of 12 weeks of probiotic supplementation on quality of life in colorectal cancer survivors: a double-blind, randomized, placebo-controlled trial. *Dig. Liver Dis.* 46, 1126–1132. doi: 10.1016/j.dld.2014.09.004

Lee, D. K., Park, J. E., Kim, M. J., Seo, J. G., Lee, J. H., and Ha, N. J. (2014b). Probiotic bacteria, *B. longum* and *L. acidophilus* inhibit infection by rotavirus in vitro and decrease the duration of diarrhea in pediatric patients. *Clin. Res. Hepatol. Gastroenterol.* 39, 237–244. doi: 10.1016/j.clinre.2014.09.006

Lim, P. L., Toh, M., and Liu, S. Q. (2015). *Saccharomyces cerevisiae* EC-1118 enhances the survivability of probiotic *Lactobacillus rhamnosus* HN001 in an acidic environment. *Appl. Microbiol. Biotechnol.* doi: 10.1007/s00253-015-6560-y [Epub ahead of print].

Ling, Z., Liu, X., Cheng, Y., Luo, Y., Yuan, L., Li, L., et al. (2015). *Clostridium butyricum* combined with *Bifidobacterium infantis* probiotic mixture restores fecal microbiota and attenuates systemic inflammation in mice with antibiotic-associated diarrhea. *Biomed Res. Int.* 2015:582048. doi: 10.1155/2015/582048 [Epub ahead of print].

Lollo, P. C. B., Cruz, G., Morato, P. N., Moura, C. S., Carvalho-Silva, L. B., Oliveira, C. F., et al. (2012). Probiotic cheese attenuates exercise-induced immune suppression in Wistar rats. *J. Dairy Sci.* 95, 3549–3558. doi: 10.3168/jds.2011-5124

Macfarlane, G. T., and Macfarlane, S. (2011). Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. *J. Clin. Gastroenterol.* 45, S120–S127. doi: 10.1097/mcg.0b013e31822fecfe

Mackos, A. R., Eubank, T. D., Parry, N. M. A., and Bailey, M. T. (2013). Probiotic *Lactobacillus reuteri* attenuates the stressor-enhanced severity of *Citrobacter rodentium* infection. *Infect. Immun.* 81, 3253–3263. doi: 10.1128/IAI.00278-13

Mangin, I., Bouhnik, Y., Bisetti, N., and Decaris, B. (1999). Molecular monitoring of human intestinal Bifidobacterium strain diversity. *Res. Microbiol.* 150, 343–350. doi: 10.1016/S0923-2508(99)80060-6

Mårtensson, O., Biörklund, M., Lambo, A. M., Dueñas-Chasco, M., Irastorza, A., Holst, O., et al. (2005). Fermented, ropy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. *Nutr. Res.* 25, 429–442. doi: 10.1016/j.nutres.2005.03.004

McFarland, L. V. (2007). Meta-analysis of probiotics for the prevention of traveler's diarrhea. *Travel. Med. Infect. Dis.* 5, 97–105. doi: 10.1016/j.tmaid.2005.10.003

McFarland, L. V. (2009). Evidence-based review of probiotics for antibiotic-associated diarrhea and *Clostridium difficile* infections. *Anaerobe* 15, 274–280. doi: 10.1016/j.anaerobe.2009.09.002

McFarland, L. V., Surawicz, C. M., Greenberg, R. N., Fekety, R., Elmer, G. W., Moyer, K. A., et al. (1994). A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. *J. Am. Med. Assoc.* 271, 1913–1918. doi: 10.1001/jama.1994.03510480037031

Mikelsaar, M. (2011). Human microbial ecology: *Lactobacilli*, probiotics, selective decontamination. *Anaerobe* 17, 463–467. doi: 10.1016/j.anaerobe.2011.07.005

Naseribafrouei, A., Hestad, K., Avershina, E., Sekelja, M., Linløkken, A., Wilson, R., et al. (2014). Correlation between the human fecal microbiota and depression. *Neurogastroenterol. Motil.* 26, 1155–1162. doi: 10.1111/nmo.12378

Ndegwa, S., and Nkansah, E. (2008). *Vancomycin for C. difficile Pseudomembranous colitis: Guidelines and a Clinical Effectiveness Review*. Ottawa, ON: CAN: Canadian Agency for Drugs and Technologies in Health, 2008.

NIH HMP Working Group, Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., et al. (2009). The NIH human microbiome project. *Genome Res.* 19, 2317–2323. doi: 10.1101/gr.096651.109

Nikfar, S., Rahimi, R., Rahimi, F., Derakhshani, S., and Abdollahi, M. (2008). Efficacy of probiotics in irritable bowel syndrome: a meta-analysis of randomized, controlled trials. *Dis. Colon. Rectum* 5, 1775–1780. doi: 10.1007/s10350-008-9335-z

Omar, J. M., Chan, Y., Jones, M. L., Prakash, S., and Jones, P. J. H. (2013). *Lactobacillus fermentum* and *Lactobacillus amylovorus* as probiotics alter body adiposity and gut microflora in healthy persons. *J. Funct. Foods* 5, 116–123. doi: 10.1016/j.jff.2012.09.001

Osmanagaoglu, O., Kiran, F., and Ataoglu, H. (2010). Evaluation of in vitro probiotic potential of *Pediococcus pentosaceus* OZF isolated from human breast milk. *Probiotics Antimicrobl. Prot.* 2, 162–174. doi: 10.1007/s12602-010-9050-7

Owen, L., Reinders, M., Narramore, R., Marsh, A. M. R., Gar Lui, F., Baron, R., et al. (2014). A double blind, placebo controlled, randomised pilot trial examining the effects of probiotic administration on mood and cognitive function. *Proceed Nutr. Soc.* 73:e29. doi: 10.1017/S0029665114000433

Pawlowski, S. W., Calabrese, G., Kolling, G. L., Freire, R., Alcantara Warren, C., Liu, B., et al. (2010). Murine Model of *Clostridium difficile* infection with aged gnotobiotic C57BL/6 Mice and a BI/NAP1 strain. *J. Infect. Dis.* 202, 1708–1712. doi: 10.1086/657086

Pepin, J., Valiquette, L., Alary, M. E., Villemure, P., Pelletier, A., Forget, K., et al. (2004). *Clostridium difficile* - associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* 171, 466–472. doi: 10.1503/cmaj.1041104

Pepin, J., Valiquette, L., and Cossette, B. (2005). Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* 173, 1037–1042. doi: 10.1503/cmaj.050978

Pflughoeft, K. J., and Versalovic, J. (2012). Human microbiome in health and disease. *Annu. Rev. Pathol.* 7, 99–122. doi: 10.1146/annurev-pathol-011811-132421

Pieniz, S., Andreazza, R., Anghinoni, T., Camargo, F., and Brandelli, A. (2014). Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s. *Food Control* 37, 251–256. doi: 10.1016/j.foodcont.2013.09.055

Pineton de Chambrun, G., Neut, C., Chau, A., Cazaubiel, M., Pelerin, F., Justen, P., et al. (2015). A randomized clinical trial of *Saccharomyces cerevisiae* versus placebo in the irritable bowel syndrome. *Dig. Liver Dis.* 47, 119–124. doi: 10.1016/j.dld.2014.11.007

Pitkala, K. H., Strandberg, T. E., Finne Soveri, U. H., Ouwehand, A. C., Poussa, T., and Salminen, S. (2007). Fermented cereal with specific bifidobacteria normalizes bowel movements in elderly nursing home residents. *A randomized controlled trial. J. Nutr. Health Agents* 11, 305–311.

Psani, M., and Kotzekidou, P. (2006). Technological characteristics of yeast strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World J. Microbiol. Biotechnol.* 22, 1329–1336. doi: 10.1007/s11274-006-9180-y

Raccach, M. (2014). "Pediococcus," in *Encyclopedia of Food Microbiology*, 2nd Edn, eds C. A. Batt and M. L. Tortorello (London: Academic Press Inc.), 1–5. doi: 10.1016/B978-0-12-384730-0.00247-0

Radulovic, Z., Petrovic, T., and Bulajic, S. (2012). "Antibiotic susceptibility of probiotic bacteria, antibiotic resistant bacteria," *A Continuous Challenge in the New Millennium*, ed. M. Pana (Belgrade: University of Belgrade, Faculty of Agriculture, Republic of Serbia), 550–576.

Rehaiem, A., Belgacem, Z., Ben Edalatian, M. R., Martínez, B., Rodríguez, A., Manai, M., et al. (2014). Assessment of potential probiotic properties and multiple bacteriocin encoding-genes of the technological performing strain *Enterococcus faecium* MMRA. *Food Control.* 37, 343–350. doi: 10.1016/j.foodcont.2013.09.044

Rubinstein, E. (2001). Comparative safety of the different macrolides. *Int. J. Antimicrob. Agents* 18(Suppl. 1), 71–76. doi: 10.1016/S0924-8579(01)00397-1

Samanta, M., Sarkar, M., Ghosh, P., Ghosh, J. K., Sinha, M. K., and Chatterjee, S. (2009). Prophylactic probiotics for prevention of necrotizing enterocolitis in very low birth weight newborns. *J. Trop. Pediatr.* 55, 128–131. doi: 10.1093/tropej/fmn091

Sanz, Y. (2007). Ecological and functional implications of the acid-adaptation ability of *Bifidobacterium*: a way of selecting improved probiotic strains. *Int. Dairy J.* 17, 1284–1289. doi: 10.1016/j.idairyj.2007.01.016

Sapkota, A. R., Lefferts, L. Y., Mckenzie, S., and Walker, P. (2007). What do we feed to food-production animals? A review of animal feed ingredients and their potential impacts on human health. *Environ. Health Perspect.* 115, 663–670. doi: 10.1289/ehp.9760

Sarker, S. A., Sultana, S., Fuchs, G. J., Alam, N. H., Azim, T., Brüssow, H., et al. (2005). *Lactobacillus paracasei* strain ST11 has no effect on rotavirus but ameliorates the outcome of nonrotavirus diarrhea in children from Bangladesh. *Pediatrics* 116:e221–e228. doi: 10.1542/peds.2004-2334

Scharek, L., Hartmann, L., Heinevetter, L., and Blaut, M. (2000). *Bifidobacterium adolescentis* modulates the specific immune response to another human gut bacterium, *Bacteroides thetaiotaomicron*, in gnotobiotic rats. *Immunobiology* 202, 429–441. doi: 10.1016/S0171-2985(00)80102-3

Shan, L. S., Hou, P., Wang, Z. J., Liu, F. R., Chen, N., Shu, L. H., et al. (2013). Prevention and treatment of diarrhoea with *Saccharomyces boulardii* in children with acute lower respiratory tract infections. *Benef. Microbes* 4, 329–334. doi: 10.3920/BM2013.0008

Sharma, S., Chaturvedi, J., Chaudhari, B. P., Singh, R. L., and Kakkar, P. (2012). Probiotic *Enterococcus lactis* IITRHR1 protects against acetaminophen-induced hepatotoxicity. *Nutrition* 28, 173–181. doi: 10.1016/j.nut.2011.02.012

Shiba, T., Aiba, Y., Ishikawa, H., Ushiyama, A., Takagi, A., Mine, T., et al. (2003). The suppressive effect of bifidobacteria on *Bacteroides vulgatus*, a putative pathogenic microbe in inflammatory bowel disease. *Microbiol. Immunol.* 47, 371–378. doi: 10.1111/j.1348-0421.2003.tb03368.x

Shimakawa, Y., Matsubara, S., Yuki, N., Ikeda, M., and Ishikawa, F. (2003). Evaluation of *Bifidobacterium breve* strain Yakult-fermented soymilk as a probiotic food. *Int. J. Food Microbiol.* 81, 131–136. doi: 10.1016/S0168-1605(02)00224-6

Shin, N. R., Lee, J. C., Lee, H. Y., Kim, M. S., Whon, T. W., Lee, M. S., et al. (2014). An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* 63, 727–735. doi: 10.1136/gutjnl-2012-303839

Shirasawa, Y., Shibahara-Sone, H., Lino, T., and Ishikawa, F. (2010). *Bifidobacterium bifidum* BF-1 suppresses *Helicobacter pylori*-induced genes in human epithelial cells. *J. Dairy Sci.* 93, 4526–4534. doi: 10.3168/jds.2010-3274

Shornikova, A. V., Casas, I. A., Isolauri, E., Mykkanen, H., and Vesikari, T. (1997). *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. *J. Ped. Gastroenterol. Nutr.* 24, 399–404. doi: 10.1097/00005176-199704000-00008

Sinn, D. H., Song, J. H., Kim, H. J., Lee, J. H., Son, H. J., Chang, D. K., et al. (2008). Therapeutic effect of *Lactobacillus acidophilus*-SDC 2012, 2013 in patients with irritable bowel syndrome. *Digest Dis. Sci.* 53, 2714–2718. doi: 10.1007/s10620-007-0196-4

Surawicz, C. M. (2003). Probiotics, antibiotic-associated diarrhoea and *Clostridium difficile* diarrhoea in humans. *Best Pract. Res. Clin. Gastroenterol.* 17, 775–783. doi: 10.1016/S1521-6918(03)00054-4

Surawicz, C. M., Mcfarland, L. V., Greenberg, R. N., Rubin, M., Fekety, R., Mulligan, M. E., et al. (2000). The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with *Saccharomyces boulardii*. *Clin. Infect. Dis.* 31, 1012–1017. doi: 10.1086/318130

Swidsinski, A., Loening-Baucke, V., Vaneechoutte, M., and Doerffel, Y. (2008). Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. *Infl. Bowel. Dis.* 14, 147–161. doi: 10.1002/ibd.20330

Szajewska, H., Ruszczynski, M., and Radzikowski, A. (2006). Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. *J. Pediatr.* 149, 367–372. doi: 10.1016/j.jpeds.2006.04.053

Tambyah, P. A., Marx, J. A., and Maki, D. G. (2004). Nosocomial infection with vancomycin-dependent enterococci. *Emerg. Infect. Dis.* 10, 1277–1281. doi: 10.3201/eid1007.030993

Temmerman, R., Pot, B., Huys, G., and Swings, J. (2003). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int. J. Food Microbiol.* 81, 1–10. doi: 10.1016/S0168-1605(02)00162-9

Thygesen, J. B., Glerup, H., and Tarp, B. (2012). *Saccharomycesboulardii* fungemia caused by treatment with a probioticum. *BMJ Case Rep.* 2012. doi: 10.1136/bcr.06.2011.4412

Toomey, N., Monaghan, A., Fanning, S., and Bolton, D. J. (2009). Assessment of antimicrobial resistance transfer between lactic acid bacteria and potential foodborne pathogens using in vitro methods and mating in a food matrix. *Foodborne Pathog. Dis.* 6, 925–933. doi: 10.1089/fpd.2009.0278

Tsai, C.-C., Lai, C.-H., Yu, B., and Tsen, H.-Y. (2008). Use of specific primers based on the 16S-23S internal transcribed spacer (ITS) region for the screening *Bifidobacterium adolescentis* in yogurt products and human stool samples. *Anaerobe* 14, 219–223. doi: 10.1016/j.anaerobe.2008.05.001

Tuohy, K. M., Conterno, L., Gasperotti, M., and Viola, R. (2012). Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. *J. Agric. Food Chem.* 60, 8776–8782. doi: 10.1021/jf2053959

Turpin, W., Humblot, C., Thomas, M., and Guyot, J.-P. (2010). *Lactobacilli* as multifaceted probiotics with poorly disclosed molecular mechanisms. *Int. J. Food Microbiol.* 143, 87–102. doi: 10.1016/j.ijfoodmicro.2010.07.032

Turroni, F., Van Sinderen, D., and Ventura, M. (2011). Genomics and ecological overview of the genus *Bifidobacterium*. *Int. J. Food Microbiol.* 149, 37–44. doi: 10.1016/j.ijfoodmicro.2010.12.010

van Passel, M. W., 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

Vanderhoof, J. A., Whitney, D. B., Antonson, D. L., Hanner, T. L., Lupo, J. V., and Young, R. J. (1999). *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children. *J. Pediatr.* 135, 564–568. doi: 10.1016/S0022-3476(99)70053-3

Varankovich, N. V., Khan, N. H., Nickerson, M. T., Kalmokoff, M., and Korber, D. R. (2015). Evaluation of pea protein–polysaccharide matrices for encapsulation of acid-sensitive bacteria. *Food Res. Int.* 70, 118–124. doi: 10.1016/j.foodres.2015.01.028

Ventura, M., O'Connell Motherway, M., Leahy, S., Moreno-Munoz, J. A., Fitzgerald Gerald, F., and van Sinderen, D. (2007). From bacterial genome to functionality; case *Bifidobacteria*. *Int. J. Food Microbiol.* 120, 2–12. doi: 10.1016/j.ijfoodmicro.2007.06.011

Ventura, M., Turroni, F., Motherway, M. O. C., Macsharry, J., and Van Sinderen, D. (2012). Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends Microbiol.* 20, 467–476. doi: 10.1016/j.tim.2012.07.002

Verdú, E. F., Bercik, P., and Collins, S. M. (2009). Effect of probiotics on gastrointestinal function: evidence from animal models. *Therap. Adv. Gastroenterol.* 2(Suppl. 4), 31–35. doi: 10.1177/1756283X09337645

Verdú, E. F., Bercik, P., Huang, X. X., Lu, J., Al-Mutawaly, N., Sakai, H., et al. (2008). The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295, G664–G670. doi: 10.1152/ajpgi.90316.2008

Videlock, E. J., and Cremonini, F. (2012). Meta-analysis: probiotics in antibiotic-associated diarrhoea. *Aliment. Pharmacol. Ther.* 35, 1355–1369. doi: 10.1111/j.1365-2036.2012.05104.x

Wallace, T. C., Guarner, F., Madsen, K., Cabana, M. D., Gibson, G., Hentges, E., et al. (2011). Human gut microbiota and its relationship to health and disease. *Nutr. Rev.* 69, 392–403. doi: 10.1111/j.1753-4887.2011.00402.x

Wang, J., Korber, D. R., Low, N. H., and Nickerson, M. T. (2014). Entrapment, survival and release of Bifidobacterium adolescentis within chickpea protein-based microcapsules. *Food Res. Int.* 55, 20–27. doi: 10.1016/j.foodres.2013.09.018

Wang, J., Korber, D. R., Low, N. H., and Nickerson, M. T. (2015a). Encapsulation of *Bifidobacterium adolescentis* with legume proteins and its survival under stimulated gastric conditions and during storage within commercial fruit juices. *Food Sci. Biotechnol.* 24, 383–391. doi: 10.1007/s10068-015-0051-x

Wang, J., Tang, H., Zhang, C., Zhao, Y., Derrien, M., Rocher, E., et al. (2015b). Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice. *ISME J.* 9, 1–15. doi: 10.1038/ismej.2014.99

Wang, M., Molin, G., Ahrné, S., Adawi, D., and Jeppsson, B. (2007). High proportions of proinflammatory bacteria on the colonic mucosa in a young patient with ulcerative colitis as revealed by cloning and sequencing of 16S rRNA genes. *Dig. Dis. Sci.* 52, 620–627. doi: 10.1007/s10620-006-9461-1

Wei, Y.-X., Zhang, Z.-Y., Liu, C., Malakar, P. K., and Guo, X.-K. (2012). Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences. *World J. Gastroenterol.* 18, 479–488. doi: 10.3748/wjg.v18.i5.479

Wen, K., Li, G., Bui, T., Liu, F., Li, Y., Kocher, J., et al. (2012). High dose and low dose *Lactobacillus acidophilus* exerted differential immune modulating effects on T cell immune responses induced by an oral human rotavirus vaccine in gnotobiotic pigs. *Vaccine* 30, 1198–1207. doi: 10.1016/j.vaccine.2011.11.107

Werner, G., Coque, T. M., Hammerum, A. M., Hope, R., Hryniewicz, W., Johnson, A., et al. (2008). Emergence and spread of vancomycin resistance among enterococci in Europe. *Euro. Surveill.* 13, Pii: 19046.

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

Wood, K. A., Korber, D. R., Nickerson, M. T., and Low, N. H. (2012). Oligosaccharide utilization by and encapsulation of Bifidobacterium adolescentis in alginate and/or protein capsules. *Int. J. Probiotics Prebiotics* 7, 153–164.

Wozniak, R. A., and Waldor, M. K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat. Rev. Microbiol.* 8, 552–563. doi: 10.1038/nrmicro2382

Wrzosek, L. M. S., Noordine, M. L., Bouet, S., Joncquel Chevalier-Curt, M., Robert, V., Philippe, C., 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.* 2013:11. doi: 10.1186/1741-7007-11-61

Wunderlich, P. F., Braun, L., Fumagalli, I., D'Apuzzo, V., Heim, F., Karly, M., et al. (1989). Double-blind report on the efficacy of lactic acid-producing *Enterococcus* SF68 in the prevention of antibiotic-associated diarrhoea and in the treatment of acute diarrhoea. *J. Int. Med. Res.* 17, 333–338.

Yamaguchi, T., Miura, Y., and Matsumoto, T. (2013). Antimicrobial susceptibility of *Enterococcus* strains used in clinical practice as probiotics. *J. Infect. Chemother.* 19, 1109–1115. doi: 10.1007/s10156-013-0633-6

Conflict of Interest Statement: 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: 28 July 2015
doi: 10.3389/fmicb.2015.00762

Reutericyclin producing *Lactobacillus reuteri* modulates development of fecal microbiota in weanling pigs

Yan Yang[1], *Xin Zhao*[1], *Minh H. A. Le*[1], *Ruurd T. Zijlstra*[1] *and Michael G. Gänzle*[1,2]*

[1] *Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada,* [2] *School of Food and Pharmaceutical Engineering, Hubei University of Technology, Wuhan, China*

Lactobacillus reuteri is used as probiotic culture in food and feed applications; however, strain specific properties of *L. reuteri* that mediate probiotic activity remain unknown. This study aimed to determine effects of feed fermentation with exopolysaccharide and reutericyclin producing *L. reuteri* on the transition of the gut microbiome of piglets after weaning. The reutericyclin and reuteran producing *L. reuteri* TMW1.656 was compared to the reutericyclin negative and levan producing *L. reuteri* LTH5794 and unfermented controls. Both strains were fermented at conditions supporting exopolysaccharide formation, or at conditions not supporting exopolysaccharide formation. Fecal microbiota were characterized by partial sequencing of 16S rRNA genes, and by quantitative PCR targeting clostridial toxins. The transition to solid food resulted in a transient increase of *Proteobacteria* to 12% of total bacteria, and increased bacterial diversity by increasing the abundance of anaerobic fiber fermenting *Firmicutes*. Three weeks after weaning, *Prevotella* and *Lactobacillus* were among the dominant bacterial genera. Feed fermentation with *L. reuteri* affected the abundance of few bacterial taxa and particularly reduced the abundance of *Enterobacteriaceae* ($P < 0.05$) when compared to unfermented controls. Reutericyclin producing *L. reuteri* increased the abundance of *Dialister* spp. and *Mitsuokella* spp. ($P < 0.05$) but did not influence the abundance of clostridial toxins in the feces. In conclusion, data on the contribution of specific metabolic activities of *L. reuteri* to probiotic activity will facilitate the strain selection for probiotic applications in food and feed.

Keywords: enterterotoxigenic *Escherichia coli*, ETEC, pigs, feed fermentation, reutericyclin, exopolysaccharides, *Lactobacillus reuteri*, probiotic

OPEN ACCESS

Edited by:
Maria De Angelis,
University of Bari Aldo Moro, Italy

Reviewed by:
Zhao Chen,
Clemson University, USA
Amit Kumar Tyagi,
The University of Texas MD Anderson Cancer Center, USA

***Correspondence:**
Michael G. Gänzle,
Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Ag/For Centre, Edmonton, AB T6G 2P5, Canada
mgaenzle@ualberta.ca

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

Received: *01 June 2015*
Accepted: *13 July 2015*
Published: *28 July 2015*

Citation:
Yang Y, Zhao X, Le MHA, Zijlstra RT and Gänzle MG (2015) Reutericyclin producing Lactobacillus reuteri modulates development of fecal microbiota in weanling pigs. Front. Microbiol. 6:762. doi: 10.3389/fmicb.2015.00762

Introduction

Lactobacillus reuteri is a host-specific intestinal symbiont of humans and vertebrate animals (Walter, 2008; Frese et al., 2011). *L. reuteri* is commercially applied in cereal fermentations (Brandt, 2014) and as probiotic culture (Tuohy et al., 2003). Strain- or lineage specific metabolic traits of *L. reuteri* mediate host-specific colonization (Frese et al., 2014; Wilson et al., 2014), and contribute to its competitiveness in cereal fermentations and improved bread quality (Galle et al., 2012; Lin and Gänzle, 2014; Zhao et al., 2015). Strain specific properties of *L. reuteri* that mediate probiotic activity, however, remain unknown.

Metabolic traits that were suggested to mediate probiotic activity of *L. reuteri* include acid resistance (Teixeira et al., 2014), histamine decarboxylation (Spinler et al., 2014), exopolysaccharide production (Chen et al., 2014), and antimicrobial activity against pathogens (Gänzle, 2004; Rea et al., 2014). Reuteran and levan from *L. reuteri* prevented adhesion of enterotoxigenic *Escherichia coli* (ETEC) *in vitro*, and reduced mucosa-adherent ETEC in a swine model (Wang et al., 2010; Chen et al., 2014). The production of reuterin and reutericyclin by *L. reuteri* were proposed to provide protection against *Salmonella* Typhimurium and *Clostridium difficile*, respectively (Hurdle et al., 2011; De Weirdt et al., 2012). However, only two studies performed in rodent models demonstrate that antimicrobial compounds from lactic acid bacteria are active *in vivo*. Bacteriocin production by probiotic lactic acid bacteria reduced infection by *Listeria monocytogenes* (Corr et al., 2007), and reduced colonization by vancomycin resistant enterococci (Millette et al., 2008). Evidence for activity of antimicrobial metabolites of probiotics against autochtonous microbiota is inconclusive. The bacteriocin producing *L. salivarius* Abp118 altered the gut microbiome of swine and mice when compared to controls that did not receive probiotics. Changes induced by the bacteriocin producing strain, however, were not different from those induced by a bacteriocin-negative derivative of the same strain (Riboulet-Bisson et al., 2012).

Probiotic applications of *L. reuteri* and related organisms specifically targeted piglets (Konstantinov et al., 2008). Gut microbiota of pigs undergo a transition after weaning (Konstantinov et al., 2006; Lallès et al., 2007). The microbiome of suckling pigs is dominated by lactobacilli (Konstantinov et al., 2006) while strict anaerobic *Firmicutes* and *Bacteroidetes* dominate the microbiome of adult pigs (Lamendella et al., 2011; Riboulet-Bisson et al., 2012). The abundance of lactobacilli decreases after weaning (Konstantinov et al., 2006), providing opportunity for overgrowth of pathogens. Probiotics may decrease the abundance of pathogens (Zhang et al., 2010; Bednorz et al., 2013). Supplementation with *L. amylovorus* reduced levels of ETEC in the intestine but did not alter the hindgut microbiome (Konstantinov et al., 2008; Su et al., 2008).

This study aimed to determine the effect of feed fermentation with *L. reuteri* on the development of the gut microbiome in weanling piglets. The experimental design aimed to determine the contribution of viable *L. reuteri*, exopolysaccharide formation by *L. reuteri*, and reutericyclin formation by *L. reuteri* on the evolution of the gut microbiome in weanling piglets. The fecal microbiome was characterized by high throughput sequencing of 16S rRNA genes, and by quantitative PCR (qPCR) specifically targeting *C. difficile*, *C. perfringens*, and toxins produced by these organisms.

Materials and Methods

Feed Fermentation and Diet Preparation

Wheat flour was provided by University of Alberta Swine Research and Technology Centre, mixed with an equal amount of tap water, and inoculated with approximately 10^7 CFU g^{-1} of the levan-producing *L. reuteri* LTH5794 or the reuteran-producing *L. reuteri* TMW1.656. A more detailed account of the feed fermentation and the control experiments ensuring the identity of fermentation microbiota with the inoculum is provided by Yang et al. (2015). Feed fermentation was carried out with addition of 10% (w/w flour) sucrose to support levan or reuteran formation during fermentation, or addition of 5% (w/w flour) glucose and 5% (w/w flour) fructose, which do not support reuteran or levan formation by *L. reuteri* but result in a formation of comparable levels of lactic and acetic acids. A chemically acidified control was prepared with 5% (w/w flour) fructose, 5% glucose (w/w flour), and addition of lactic acid (80%) and glacial acidic acid in a ratio of 4:1 (v/v) to acidify the feed to a pH of 3.8 (**Table 1**). Basal diets were mixed with 20–50% fermented or acidified wheat to produce the experimental feeds. Control diet was obtained by a mixture of basal diet and unfermented wheat. All diets were formulated to meet or exceed nutrient recommendation of National Research Council Canada (NRC) (2012) for 5–10 kg pigs. Titanium dioxide (TiO_2) was added to each of the test diets as an indigestible marker.

Animals and Experimental Design

This animal trial was approved by the University of Alberta Animal Care and Use Committee under the guidelines of the Canadian Council on Animal Care and was conducted at the University of Alberta Swine Research and Technology Centre, Edmonton, AB, Canada. A total of 36 crossbred castrated weaning male pigs (∼21 d of age) were selected and housed in a temperature-controlled room (28 ± 2.5°C). Pigs were divided into six consecutive and similar blocks with six pigs per block and one pig per pen (0.5 × 1.22 m). The experiment was designed to indicate whether the bacterial metabolites lactic and acetic acid, reuteran, levan, or reutericyclin influence the evolution of gut microbiota (**Table 1**). One pig per block was assigned to one of the six diets for a total of six observations per diet (Yang et al., 2015). Pigs were offered *ad libitum* food and water intake allowing for adequate growth. Meals in mash form were provided in equal amount twice daily (at 8 a.m. and 4 p.m.). Fresh fecal samples were collected from the pen floor in a sterile plastic bag at weaning, and 1, 2, or 3 weeks after weaning. A total of 137 samples obtained were collected and stored at −20°C. Frozen

TABLE 1 | Experimental diets used in this study.

Components	Control	Chem. acid	*L. reuteri*			
			TMW1.656		LTH 5794	
			Sucrose	Glu + Fru	Sucrose	Glu + Fru
Acids	−	+	+	+	+	+
L. reuteri	−	−	+	+	+	+
Reutericyclin	−	−	+	+	−	−
Reuteran	−	−	+	−	−	−
Levan	−	−	−	−	+	−

+ means component present in the diet, − means component not present in the diet.

samples were thawed, mixed aseptically by spatula and 2–3 g subsamples were stored at −80°C.

DNA Extraction

Bacterial DNA was extracted from fecal samples using QIAamp® DNA stool Mini kit (50) (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's instructions. Fecal DNA was quantified by Nano-Drop spectrophotometer system ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA). DNA quality was assessed by determining the ratio of absorbance at 260 and 280 nm. Only DNA samples that had 260:280 nm ratios higher than 1.8 were used for further analysis.

PCR Primers and Probes

Primers and probes used in this study are listed in **Table 2**. Sequences of genes coding for clostridial toxins (α-, β-, β2-, entero-, and ι-toxin) collected from GeneBank (http://www.ncbi.nlm.nih.gov/genbank). The sequence data were aligned with CLUSTAL-W (Thompson et al., 1994) to identify conserved sequences. Primers and probes were designed to target the conserved toxin sequences. The specificity of primer sequences was checked by Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.).

Quantification of Clostridia and their Toxins by qPCR

Quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using methodology described earlier (Metzler-Zebeli et al., 2010). To obtain positive controls for primers and probes targeting clostridial toxins, gBlocks® Gene Fragments were designed and

TABLE 2 | Oligonucleotide sequences of the primers and probes used in this study.

Target group/gene	Primer/probe	Oligonucleotide sequence (5′-3′)*	T_A (°C)[a]	Product size (bp)	References
Clostridium cluster I	Cl F	GTGAAATGCGTAGAGATTAGGAA	58	665	Le Bourhis et al., 2005
	Cl R	GATYYGCGATTACTAGYAACTC			
Clostridium cluster XI	CXI F	ACGGTACTTGAGGAGGA	58	139	Schwab et al., 2011, this study
	CXI R	GAGCCGTAGCCTTTCACT			
Total bacteria	HDA F	ACTCCTACGGGAGGCAGCAGT	62	198	Walter et al., 2000
	HDA R	GTATTACCGCGGCTGCTGGCAC			
C. perfringens α toxin (*cpa*)	CPα F	CTTGGAGAGGCTATGCACTATTT	60	90	This study
	CPα P	6FAM-CCATATCATCCTGCTAATGTTACTGCCGT-TAMRA			
	CPα R	CTTAACATGTCCTGCGCTATCA			
C. perfringens β toxin (*cpb*)	CPβ F	TCAAACAACCCTGTATATGGAAATG	60	149	This study
	CPβ P	6FAM-ACGGAAGATATACTAATGTTCCTGCAACTG-TAMRA			
	CPβ R	GGAGCAGTTAGAACTACAGACAT			
C. perfringens β-2 toxin (*cpb2*)	CPβ2 F	TGCAACTTCAGGTTCAAGAGA	60	121	This study
	CPβ2 P	6FAM-ACCATTTGAGAAGCTTTAACATCATCTCCC-TAMRA			
	CPβ2 R	TTGTCTAGCAGAATCAGGGTTT			
C. perfringens enterotoxin (*cpe*)	CPe F	AGCTGCTGCTACAGAAAGATTA	60	101	This study
	CPe P	6FAM-CTGATGCATTAAACTCAAATCCAGCTGGT-TAMRA			
	CPe R	GAGTCCAAGGGTATGAGTTAGAAG			
C. perfringens ι toxin (*iap*)	CPia F	CGTGGAGGATATACCGCAAT	60	116	This study
	CPia P	6FAM-TGGTCCTTTAAATAATCCTAATCCA-TAMRA			
	CPia R	GGTGTGAGCTTTAATGCGTTT			
C. perfringens ε toxin (*etx*)	CP etx F	AGCTTTTCCTAGGGATGGTTA	58	112	Messelhäußer et al., 2007
	CP etx R	AACTGCACTATAATTTCCTTTTCC			
C. difficile toxin B (*tcdB*)	CD tcdB F	GAAAGTCCAAGTTTACGCTCAAT	56	176	van den Berg et al., 2006
	CD tcdB P	6FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA			
	CD tcdB R	GCTGCACCTAAACTTACACCA			

*Y = T/C. F, Forward; R, Reverse; P, Probe.

[a]Annealing temperature.

synthesized by Integrated DNA Technologies. Standard curves for quantification of toxin genes were generated with 10-fold serial dilutions of purified PCR amplicons, which were amplified from gBlocks® Gene Fragments with the same primer pair and probe. For quantification of eubacteria and *Clostridium* clusters, standard curves were generated with amplicons that were amplified from serial dilutions of fecal DNA. The concentration of amplicons was determined by Nano-Drop spectrophotometer system ND-1000.

Fecal DNA was diluted to a concentration of 100 mg/L and analyzed in duplicate in a MicroAmp Fast Optical 96-well reaction plate sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). Genes coding for 16S rRNA and the ε-toxin were amplified with the Quanti Fast SYBR Green master mix (Applied Biosystems). Taqman Fast master mix (Applied Biosystems) was used for detection of other toxins with probes. qPCR reaction contained 12.5 μL master mix, 4 μL of 10 μM primer solution in water, 2 μL of template DNA, and 6.5 μL nuclease-free water. Amplification of target sequences was achieved in 40 PCR cycles with primer annealing temperatures as shown in **Table 2**. Specific amplification of the target DNA was verified by melting curve analysis where applicable and by determination of the size of the amplicons by agarose gel electrophoresis.

Sequencing of 16S rRNA Sequence Tags and Sequence Data Analysis

High throughput sequencing of 16S rRNA sequence tags was performed by the University of Minnesota Genomics Center (Minneapolis, MN, USA) on a Illumina MiSeq. The V1–V3 regions of the 16S rRNA gene was amplified using primers Meta_V1_27F (TCGTCGGCAGCGTCAGATG TGTATAAGAGACAG **AGAGTTTGATCMTGGCTCAG**) and Meta_V3_534R (GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAG **ATTACCGCGGCTGCTGG**). The bold part of each primer is complementary to the eukaryotic 16S sequences while upstream sequences corresponded to Illumina adapters that are required for sequencing and multiplexing. Paired-end sequencing was performed according to the manufacturer's instructions.

The QIIME pipeline (MacQIIME 1.8.0 20140103 OS10.6) (Caporaso et al., 2010) was used to analyze the sequences of 16S rRNA genes. PANDAseq (Masella et al., 2012) was used for quality filtering and assembly of the two ends of each read into contigs. Pairs with miscalled or uncalled bases in the overlapping region were discarded. Operational Taxonomic Units (OTUs) were generated using the UPARSE workflow (Edgar, 2013). Briefly, all sequences were merged into a single file, and the library name was used for multiplexing. To minimize computing time, sequences were dereplicated and sorted by abundance. Unique sequences in the data set were discarded. Sequences were clustered into OTUs by USEARCH (Edgar, 2010) using the Greengenes reference database (release October 2013), and a 97% similarity threshold. UCHIME (Edgar, 2010; Edgar et al., 2011) was used for filtering of chimeric sequences. OTUs with abundance below 0.005% of the total number of sequences were discarded (Bokulich et al., 2013).

Downstream analyses including taxonomy assignments, and alpha and beta diversity estimations were conducted using the QIIME workflow core_diversity_analysis.py, with a sampling depth of 7939 (Navas-Molina et al., 2013). This analysis was conducted with default parameters: taxonomy was assigned using Ribosomal Database Project (RDP) Classifier V2 (Wang et al., 2007), alpha diversity was estimated by Phylogenetic Diversity (PD) Whole Tree, Chao 1 and Observed Species indices (Colwell et al., 2012), beta-diversity was estimated through UniFrac distances (Vázquez-Baeza et al., 2013).

Statistical Methods

Data analyses of relative abundance and qPCR results were performed in SAS, (version 9.3, SAS Institute, 2012). The gene copy numbers of *Clostridium* cluster I and XI were converted into percentage of total bacteria gene counts for analysis. Mixed Procedure (Proc MIXED) was used based on randomized complete block design with repeated measurement. In the model, diet and week and diet × week were considered as fixed effects, while block was considered as random effect and pigs were considered as experimental unit. Comparisons of treatments were determined by contrast of target groups (SAS version 9.3). For relative abundance analysis, data obtained at weaning were used as covariate when comparing the difference between combination groups to assess the effects of *L. reuteri* and its metabolites exopolysaccharides and reutericyclin.

To test hypotheses, $p < 0.05$ was considered significant, after Bonferroni-adjustment. Normality of all variables was tested by Kolmogorov-Smirnoff test (Young, 1977). Results are presented as means ± standard deviation. Alpha- and beta- diversity were analyzed in MacQIIME v1.8.

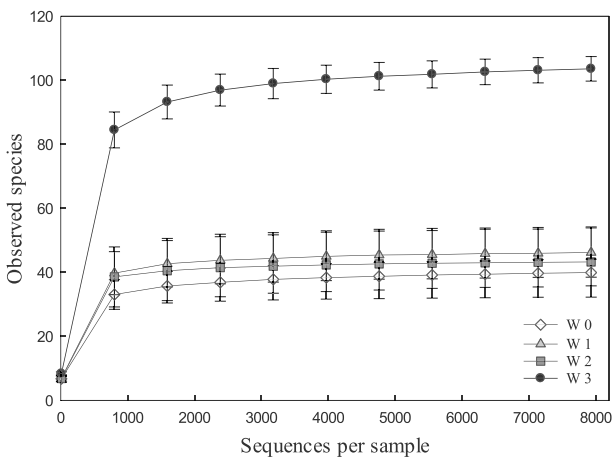


FIGURE 1 | Rarefaction curves indicating the effect of the number of partial sequences of 16S rRNA genes that were analyzed on the number of OUT's in fecal microbiota of pigs. Rarefaction curves were calculated in QIIME with the sample depth of 7939 sequences per sample from 137 fecal samples obtained at weaning (week 0, $n = 29$), or at week 1 ($n = 36$), week 2 ($n = 36$), and week 3 ($n = 36$) after weaning.

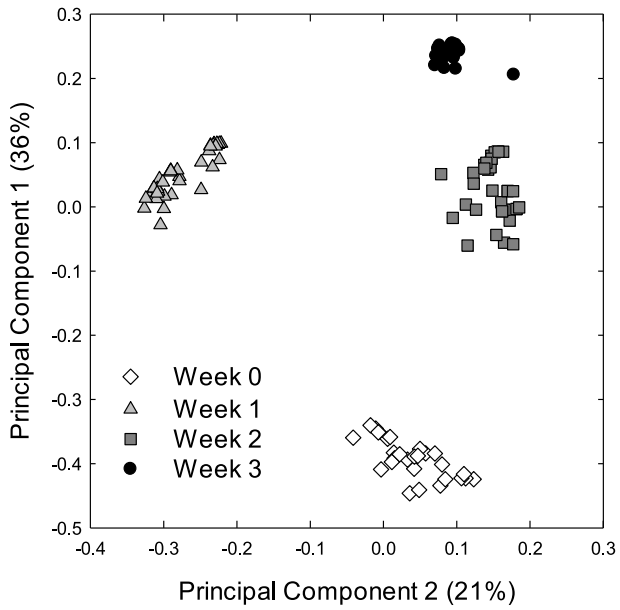


FIGURE 2 | Principle coordinate analysis (PCA) of the bacterial microbiota of piglets. The PCA plot was generated using the unweighted UniFrac distance metric. Each dot represents an individual sample collected at the start of weaning week 0 ($n = 29$) or after week 1 ($n = 36$), week 2 ($n = 36$), and week 3 ($n = 36$) of weaning.

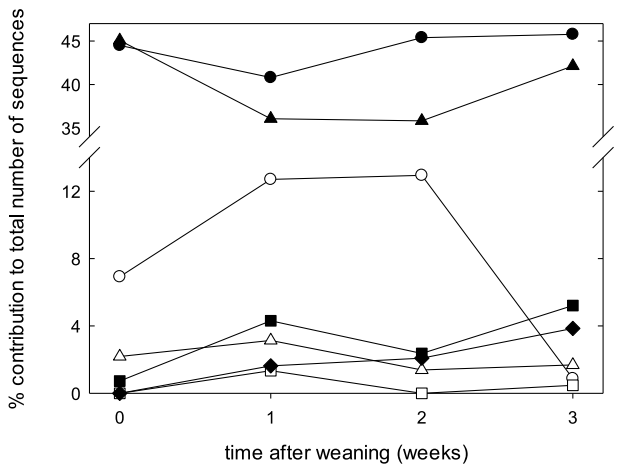


FIGURE 3 | Composition of fecal microbiota at the phylum level at weaning, and during the first 3 weeks after weaning. Data represent the median proportions of each phylum as determined by the RDP (Ribosomal Database Project) classifier. The phyla are represented by symbols as follows: ●, *Bacteroidetes;* ▲, *Firmicutes;* ❍, *Proteobacteria;* △, *Spirochaetes*; ■, *Tenericutes;* ❑, *Planctomycetes;* ♦, unassigned.

Results

Effects of Diet on Animal Health

All pigs remained healthy during the experiment period and diarrhea or any clinical signs of disease were not observed. Prior analyses of samples obtained in the same study reported the strain-specific quantification of *L. reuteri* and ETEC but not the overall composition of the hindgut microbiome (Yang et al., 2015).

Diversity of the Fecal Microbiome of Piglets

During the feeding trial, 137 fecal samples were collected and a total of 5,292,722 sequences with a minimum of 7939 sequences per sample were obtained. A total of 7434 OTUs were identified, representing 6 phyla, 27 families, 42 genera, and 49 species. Phyla in fecal samples and their abundance included *Bacteroidetes* (40.8–45.8%), *Firmicutes* (35.8–45.1%), *Proteobacteria* (0.9–12.9%), *Tenericutes* (0.7–5.2%), *Spirochaetes* (1.4–3.1%), and *Planctomycetes* (0–1.3%).

Alpha and beta diversity analyses revealed that the effects of treatments were small when compared to the differences occurring over time. In Alpha diversity metrics (within sample diversity), species richness of samples taken at week 3 was significantly higher ($p < 0.001$) than the diversity of samples taken earlier in the experiment (**Figure 1**) but differences between diets were not significant ($p > 0.05$). Beta diversity (between sample diversity) also demonstrated that samples taken at different times differed ($p < 0.0001$) (**Figure 2**). The distances between samples within each week were always smaller than the between week comparisons (**Figure 2**).

Transition of Bacteria over Time and Prevalent Bacterial Genera

The fecal microbiome was characterized by analysis of the relative abundance of bacterial taxa at the phylum level (**Figure 3**) and at the genus level (**Table 3**). The proportion of *Bacteriodetes* and *Firmicutes* remained unchanged over the study period. Major shifts were observed in the *Proteobacteria, Tenericutes*, and *Planctomycetes* (**Figure 3**). *Proteobacteria* peaked at week 1 and 2 and decreased again at week 3; *Tenericutes* and *Planctomycetes* increased over time (**Figure 3**). Within the *Bacteroidetes*, *Prevotella* and the unassigned S24-7 genus increased while *Bacteroides* and other unknown genera decreased (**Table 3**). The overall increase of microbial diversity (**Figure 1**) was largely attributed to increased abundance and diversity of bacterial taxa in the *Firmicutes*. Changes in the *Proteobacteria* were mainly attributable to *Enterobacteriaceae* (compare **Table 3** and **Figure 3**).

Clostridia Clusters and Toxins Quantified by qPCR

To determine effects of feed fermentation on the *Clostridium* cluster I and XI, these organisms and toxins produced by *C. difficile* and *C. perfringens* were quantified using qPCR (**Table 4** and data not shown). Both *Clostridium* clusters were relatively abundant in fecal samples; changes over time or changes within diets, however, were not significant ($p > 0.05$; data not shown). The α- and β-2 toxins from *C. perfringens* were detected in samples collected at week 0 and in a few samples from week 1 but not in samples taken at later times. Differences in the abundance of toxins in samples from animals fed different diets were not significant ($p > 0.05$). The abundance of other clostridial toxins, namely the β-, entero-, ι-, and ε-toxin of *C. perfringens* and the *C. difficile* toxin B, was below

TABLE 3 | Relative abundance (%) of bacterial genera in fecal microbiota of pigs at weaning (week 0) and at week 1, 2, or 3 after weaning, determined by Illumina sequencing of 16S rRNA tags.

Genus	Week 0	Week 1	Week 2	Week 3
BACTEROIDETES				
Bacteroides	13 ± 2.6	ND	ND	ND
Parabacteroides	2.2 ± 4.3^{ab}	3.1 ± 2.6^{a}	1.7 ± 0.8^{b}	1.2 ± 0.43^{b}
Prevotella	8.1 ± 0.71^{a}	9.6 ± 1.1^{ab}	14 ± 1.0^{b}	20 ± 2.8^{c}
[F: S24-7]	3.9 ± 2.6^{a}	10 ± 5.5^{b}	15 ± 6.9^{c}	15 ± 0.45^{c}
CF231	ND	ND	ND	0.47 ± 0.39
[F: Paraprevotellaceae]	ND	2.3 ± 1.0^{a}	0.6 ± 0.07^{b}	1.3 ± 0.7^{b}
Other (O: Bacteroidales)	11 ± 22	ND	ND	ND
[O: Bacteroidales]	1.7 ± 0.04^{a}	ND	1.7 ± 0.04^{a}	3.2 ± 1.5^{b}
[F: P-2534-18B5]	5.4 ± 0.01^{a}	16 ± 32^{b}	14 ± 10^{b}	3.9 ± 3.1^{a}
FIRMICUTES				
Lactobacillus	8.2 ± 15^{a}	12 ± 1.3^{ab}	15 ± 5.1^{b}	11 ± 11^{ab}
Other (O: Clostridiales)	ND	1.1 ± 0.2^{a}	ND	0.2 ± 0.04^{a}
[O: Clostridiales]	4.0 ± 3.6^{ab}	4.4 ± 11^{a}	1.8 ± 1.7^{b}	6.5 ± 4.1^{a}
[F: Christensenellaceae]	2.0 ± 0.14^{a}	2.9 ± 11^{a}	1.6 ± 1.2^{a}	0.59 ± 0.51^{a}
[F: Clostridiaceae]	ND	ND	ND	0.46 ± 0.02
Blautia	ND	ND	ND	0.47 ± 0.08
Coprococcus	ND	0.9 ± 1.7^{a}	2.4 ± 0.43^{a}	0.86 ± 0.57^{a}
Lachnospira	ND	ND	1.1 ± 0.16^{a}	0.82 ± 0.07^{a}
Roseburia	1.3 ± 1.7^{a}	ND	0.3 ± 0.02^{b}	1.1 ± 0.32^{a}
Other (F: Lachnospiraceae)	2.6 ± 0.32^{a}	ND	0.47 ± 0^{b}	ND
[F: Lachnospiraceae]	3.8 ± 1.6^{a}	4.6 ± 3.7^{a}	2.2 ± 1.6^{b}	2.9 ± 0.74^{ab}
Faecalibacterium	ND	ND	1.0 ± 0.29^{a}	0.7 ± 0.09^{b}
Oscillospira	1.4 ± 0.89^{a}	ND	0.41 ± 0.12^{b}	1.5 ± 0.22^{a}
[F: Ruminococcaceae]	18 ± 7.0^{a}	6.7 ± 0.4^{b}	4.93 ± 2.7^{b}	5.7 ± 0.75^{b}
Dialister	ND	ND	ND	1.1 ± 0.01
Megasphaera	ND	0.8 ± 0.04^{a}	2.6 ± 0.01^{ab}	3.7 ± 0.03^{b}
Mitsuokella	ND	ND	ND	1.1 ± 0
Bulleidia	ND	ND	1.1 ± 0.08^{a}	0.84 ± 0.11^{a}
Catenibacterium	ND	ND	0.52 ± 0^{a}	0.46 ± 0.06^{a}
Eubacterium	0.48 ± 0.28^{a}	ND	ND	0.41 ± 0.01^{a}
p-75-a5	2.0 ± 1.3^{a}	1.6 ± 1.5^{a}	2.0 ± 0.92^{a}	1.1 ± 0.66^{a}
PLANCTOMYCETES				
[F: Pirellulaceae]	ND	1.4 ± 0.2^{a}	ND	0.5 ± 0.68^{b}
PROTEOBACTERIA				
Desulfovibrio	1.36 ± 0.32	ND	ND	ND
Succinivibrio	ND	3.1 ± 0.27^{a}	ND	0.38 ± 0.63^{b}
[F: Enterobacteriaceae]	5.0 ± 2.7^{ab}	9.1 ± 15^{a}	9 ± 16^{a}	0.48 ± 0.03^{b}
SPIROCHAETES				
Treponema	2.1 ± 0.85^{ab}	3.5 ± 1.2^{a}	1.6 ± 5.4^{b}	1.9 ± 0.52^{b}
TENERICUTES				
[O: RF39]	1.1 ± 0.26^{a}	4.7 ± 2.8^{b}	2.3 ± 1.4^{a}	5.4 ± 1.1^{b}
Unassigned	ND	1.8 ± 0.28^{a}	2.3 ± 0.36^{a}	4.0 ± 2.6^{b}
Total	97.96	99.61	99.6	100

Data are presented as means ± standard deviation of 29 (week 0) or 36 (weeks 1, 2, and 3) observations.
Data in the same row that do not share a common superscript are significantly different ($P < 0.05$). ND, not detected.
Unassigned genera are presented with upper level of family (F) or order (O) in square brackets. "Unassigned" means a good hit to a particular sequence, but that sequence has a poorly defined taxonomy itself at the genus level. "Other" means the assignment is ambiguous.

TABLE 4 | Quantification of alpha and beta-2 toxins of *C. perfringens* in fecal samples collected at weaning (week 0) and at week 1, 2, 3 after weaning.

Diets	α toxin log(copy number/g)			β-2 toxin log(copy number/g)		
	Week 0	Week 1	Weeks 2 and 3	Week 0	Week 1	Weeks 2 and 3
Control	6.9 ± 1.4 (6/6)[a]	4.2 ± 0.5 (6/6)	ND[b]	6.2 ± 1.2 (6/6)	3.9 ± 0.5 (3/6)	ND
Chem. Acid	6.3 ± 0.8 (6/6)	3.8 ± 0.2 (2/6)	ND	5.6 ± 0.8 (6/6)	3.6 (1/6)	ND
TMW1.656 sucrose	6.6 ± 1.1 (4/4)	4.2 ± 0.3 (3/6)	ND	5.8 ± 0.9 (4/4)	3.7 ± 0.2 (3/6)	ND
TMW1.656 Glu+Fru	6.7 ± 1.1 (2/2)	3.9 ± 0.1 (4/6)	ND	5.6 ± 0.3 (2/2)	3.6 (1/6)	ND
LTH5794 sucrose	7.0 ± 0.4 (5/5)	4.2 ± 0.3 (4/6)	ND	6.3 ± 0.3 (5/5)	3.8 ± 0.1 (2/6)	ND
LTH5794 GluFru	6.6 ± 1.2 (5/5)	3.8 ± 0.5 (4/6)	ND	5.9 ± 1.2 (5/5)	3.8 ± 0 (2/6)	ND

Data are presented as log(copy number/g feces) and reported as means ± standard deviation of positive samples.

[a] *Data are reported as means ± standard deviation of positive samples. The number in brackets indicates the (number of positive samples/number of samples analyzed).*

[b] *ND, not detected [below the detection limit of 3.6 log(copy number/g)].*

the detection limit of 3.6 log(copy number/g) in all samples (**Table 4**).

Effects of Treatments on Bacteria Species

Analysis of diet-induced changes accounted for the individual differences between animals in the same group by using data from each pig at week 0 as covariate (**Table 5**). To analyse the effect of specific feed components or metabolites that were present in several diets, diets were grouped as follows: Diets containing *L. reuteri* (groups 3, 4, 5, and 6) or not (group 1 and 2); diets containing reutericyclin (groups 3 and 4) or not (groups 1, 2, 5, and 6), and diets containing exopolysaccharides (groups 3 and 5) or not (groups 1, 2, 4, and 6). Moreover, the impact of feed fermented with *L. reuteri* TMW1.656 was compared to *L. reuteri* LTH5794 (**Table 5**).

Significant differences between individual diets pertain to few bacterial taxa in the phyla *Bacteroidetes* and *Firmicutes* (**Table 5**). Any *L. reuteri* strain altered the abundance of 6 bacterial taxa when compared to the control diets (**Table 5**). These changes particularly included a reduced number of the family *Enterobacteriacae*. Diets containing reutericyclin significantly changed the abundance of a *Mitsuokella* species and a family in the phylum *Bacteroidetes* (**Table 5**); these differences were also significant when the reutericyclin negative strain *L. reuteri* LTH5794 was compared to the reutericyclin positive *L. reuteri* TMW1.656 (**Table 5**). The presence or absence of exopolysaccharides had no significant ($p > 0.05$) influence on any bacterial taxon.

Discussion

The study investigated the effect of *L. reuteri* fermented diets on the development of intestinal microbiota of pigs after weaning. The experimental design aimed to determine the contribution of specific metabolites, i.e., reuteran, levan, and reutericyclin, on the evolution of the intestinal microbiome. The present study is the first employing high throughput sequencing to document the transition of the microbiome of piglets. In contrast, the development of the infant microbiota after birth is well documented (Koenig et al., 2011; La Rosa et al., 2014). The infant microbiome is characterized by low diversity and stability. Important determinants of the infant gut microbiome include the mode of delivery, type of feeding, antibiotic use, and the gestational age of the mother (Penders et al., 2006; Koenig et al., 2011). From birth to weaning, both human infants and piglets experience a succession of *Lactobacillus* spp. in the gut due to the consumption of milk (Tannock et al., 1990; Roger et al., 2010). The gradual adaptation of the diet in infants typically avoids major problems that are associated with a shifting microbiome. The sudden change of diet after weaning, however, often causes dysbiosis and diarrheal diseases in piglets, and is thus a major concern in pig production (Lallès et al., 2007).

Bacteroidetes and *Firmicutes* dominated the intestinal microbiome in piglets, in keeping with past studies on the swine microbiome (Leser et al., 2002; Lamendella et al., 2011; Riboulet-Bisson et al., 2012). The present study additionally documents that strict anaerobes such as *Ruminococcaceae, Bacteroides,* and *Prevotella* were the dominant bacteria at weaning. After weaning, *Lactobacillus* and *Prevotella* spp. replaced *Ruminococcaceae* and *Bacteroides* as the most abundant bacterial genera. Dietary changes modulate the gut microbiome of pigs (Lu et al., 2014). Bacteria belonging to *Bacteroides-Prevotella-Porphyromonas* play an important role in fiber degradation, and are stimulated by fermentable non-starch polysaccharides in pig diets (Metzler-Zebeli et al., 2010; Ivarsson et al., 2014). Human studies have linked the diversity of different plant fibers in whole grains to an increased diversity of the gut microbiome, particularly in the genera *Roseburia*, *Bifidobacterium, Eubacterium,* and *Dialister* (Martínez et al., 2013). Accordingly, low carbohydrate diets resulted in a substantial and diet-dependent reduction of *Firmicutes* (Duncan et al., 2008). The increase of bacterial diversity after weaning was particularly attributable to an increased diversity in the phylym *Firmicutes* and may thus be linked to the presence of whole wheat in the piglets' diet, which accounted for 20% of the diet after weaning and for 50% diet after week 1 (Yang et al., 2015).

The strains of *L. reuteri* used in the present study are rodent-lineage allochthones to the pig intestine (Su et al., 2012; Frese et al., 2014). Major changes in the intestinal microbiota were attributable to the presence of probiotic *L. reuteri* and its metabolites. The most prominent change attributable to the presence of *L. reuteri* was the reduced

TABLE 5 | Effects of diets and reutericyclin producing *L. reuteri* on the composition of fecal microbiota of pigs 3 weeks after weaning, as determined by Illumina sequencing of 16S rRNA tags.

Variables		Effects of diets (relative abundance%)					Effects of *L. reuteri* (*P*-value)		
Species	**Control**	**Chem. acidified**	**LTH5794 sucrose**	**LTH5794 Glu + Fru**	**THW1.656 sucrose**	**TMW1.656 Glu+Fru**	**TMW1.656 vs LTH5794**	**TMW1.656 vs other diets**	***L. reuteri* vs no bacteria**
BACTEROIDETES									
[G: *Prevotella*]	6.63 ± 2.99^{B}	12.8 ± 6.71^{A}	6.23 ± 2.22^{B}	8.23 ± 4.56^{AB}	6.06 ± 2.58^{B}	5.05 ± 3.3^{B}	0.68	0.27	0.07
Copri	10.4 ± 8.44^{AB}	5.62 ± 3.54^{A}	14.2 ± 5.88^{AB}	8.59 ± 6.46^{AB}	16.9 ± 7.38^{B}	11.1 ± 6.56^{AB}	0.87	0.46	0.16
[F: *S24-7*]	19.4 ± 7.42	14.0 ± 1.47	16.4 ± 2.64	16.5 ± 3.27	11.7 ± 3.93	13.88 ± 6.1	0.09	0.06	0.22
[G: *CF231*]	0.48 ± 0.31	0.95 ± 0.43	0.22 ± 0.19	0.46 ± 0.46	0.3 ± 0.23	0.38 ± 0.21	0.96	0.31	0.02
[F: *p-2534-18B5*]	5.18 ± 4.91	2.5 ± 1.56	4.16 ± 3.66	3.3 ± 3.59	3.47 ± 6.01	4.72 ± 6.03	<0.0001	<0.0001	<0.0001
FIRMICUTES									
[O: *Clostridiales*]	4.96 ± 2.08	4.26 ± 1.48	6.68 ± 2.67	8.89 ± 3.96	6.76 ± 2.53	7.22 ± 3.88	0.64	0.47	0.02
[G: *Coprococcus*]	0.14 ± 0.25	0.2 ± 0.3	1.52 ± 1.4	1.21 ± 1.73	1.36 ± 3.25	0.73 ± 0.65	0.049	0.35	0.07
[G: *Oscillospira*]	2.58 ± 1.08	1.4 ± 0.45	1.46 ± 0.45	1.29 ± 0.38	1.16 ± 0.69	1.08 ± 0.51	0.88	0.43	0.03
[G: *Dialister*]	0.19 ± 0.37^{A}	0.03 ± 0.06^{A}	0.02 ± 0.03^{A}	0.38 ± 0.86^{A}	4.37 ± 7.08^{B}	1.72 ± 3.08^{AB}	0.10	0.06	0.20
[G: *Mitsuokella*]	0.35 ± 0.35^{AB}	0.07 ± 0.12^{A}	0.16 ± 0.2^{A}	0.15 ± 0.24^{A}	3.74 ± 5.43^{C}	2.2 ± 0.86^{BC}	0.0001	<0.0001	0.01
PROTEOBACTERIA									
[F: *Enterobacteriaceae*]	1.22 ± 1.45	0.89 ± 1.11	0.42 ± 0.59	0.05 ± 0.05	0.21 ± 0.42	0.09 ± 0.13	0.83	0.21	0.01

Effects of diets were analyzed based on the relative abundance of bacteria groups. To analyse effects of reutericyclin producing L. reuteri, data obtained at weaning (week 0) of each pig sample are used as covariate when P-value was calculated. Data are presented as means ± standard deviation of 29 (week 0) or 36 (weeks 1, 2, and 3) observations and significant effects are shown.

Data in the same row that do not share a common superscript are significantly different ($P < 0.05$).

Data indicating the effects of L. reuteri or reutericyclin are presented as P-value. $P < 0.05$ indicates statistically significant difference. ND means not detected. Unassigned species are presented as square brackets with upper level of genus (G), family (F), or order (O).

abundance of *Enterobacteriaceae*. This result conforms to prior reports obtained with diverse probiotic cultures (De Angelis et al., 2007; Konstantinov et al., 2008; Bednorz et al., 2013; Valdovska et al., 2014) and indicates that successful competition with *Enterobacteriaceae* is not a specific property of *L. reuteri*. Remarkably, this study also demonstrated that the abundance of several members of the *Firmicutes* and *Bacteroidetes* was influenced by *L. reuteri*.

Exopolysaccharides did not influence the composition of gut microbiota. Levan and reuteran are not digested by pancreatic digestive enzymes and selectively fermented by hindgut microbiota (Korakli et al., 2002; van Bueren et al., 2015). However, the exopolysaccharides levels in the feed used in this study, 1–3 g/kg feed (Yang et al., 2015), are low when compared to other studies reporting prebiotic intervention (Valdovska et al., 2014). Fermented feed containing reuteran, however, specifically reduced the abundance of enterotoxigenic *E. coli* (ETEC) in weanling piglets (Yang et al., 2015). The lack of any effect of reuteran on the overall composition of the gut microbiome (this study) coupled to the specific reduction of ETEC colonization (Yang et al., 2015) supports the hypothesis that effects of reuteran are mediated by a specific reduction of ETEC adhesion rather than a prebiotic effect (Chen et al., 2014).

Bioinformatic analyses of the metagenome of intestinal microbiota suggested that the ecology of colonic microbiota is shaped by competition for substrates rather than the production of antimicrobial compounds (Walter and Ley, 2011; Zheng et al., 2015). Accordingly, bacteriocin producing *L. salivarius* did not induce significant changes in the gut microbiome of pigs when compared to an isogenic bacteriocin-negative strain (Riboulet-Bisson et al., 2012). Medication of grower pigs with in feed antibiotics (Chlortetracycline, sulfamethazine, and penicillin), however, caused much more substantial changes of the colonic microbiome than was observed in this study for reutericyclin (Looft et al., 2014). Reutericyclin is a unique antimicrobial compound with broad spectrum of activity against Gram-positive bacteria (Gänzle et al., 2000; Gänzle, 2004; Hurdle et al., 2011; Lin et al., 2015). Reutericyclin is produced during growth of *L. reuteri* in wheat sourdough (Gänzle and Vogel, 2002), suggesting that feed fermentation with *L. reuteri* TMW1.656 delivers active concentrations of reutericyclin to the swine gut. We hypothesized that reutericyclin may reduce the abundance of the *Clostridium* clusters I and XI. The pathogenic species *C. perfringens* (cluster I) and *C. difficile* (cluster XI), are sensitive to reutericyclin (Hurdle et al., 2011; Hofstetter et al., 2013). However, neither the abundance of *Clostridium* cluster I and XI nor the abundance of genes coding for clostridial toxins was influenced by reutericyclin-producing *L. reuteri*. The abundance of *Dialister* and *Mitsuokella* increased upon feeding of the reutericyclin producing *L. reuteri* TMW1.656, possibly as a consequence of the inhibition of reutericyclin-sensitive competitors.

In conclusion, the present study monitored the transition of fecal microbiota of weanling piglets, and determined the impact of *L. reuteri* and its metabolites on this transition of intestinal microbiota. After weaning, bacterial diversity increased, mainly due to an increase of bacterial taxa in the phylum Firmicutes. Weaning was also associated by a transient increase of *Enterobacteriaceae*, which corresponds to the susceptibility of weanling piglets to infection by enteric pathogens. The gut microbiome of weanling piglets was not influenced by the inclusion of organic acids in the diet; however, the presence of viable *L. reuteri*, reutericyclin, and reuteran all affected the gut microbiome. Probiotic *L. reuteri* altered the abundance of several bacterial taxa, notably *Enterobacteriaceae* including *E. coli*. The reutericyclin producing strain significantly increased the abundance of two strict anaerobic members of the *Firmicutes*, while reuteran affected the colonization with ETEC but none of the numerically dominant members of fecal microbiota (Yang et al., 2015, this study). Data on the contribution of specific metabolic activities of *L. reuteri* to probiotic activity will facilitate the strain selection for probiotic feed applications in animal production. The study also novel opens novel avenues to reduce the incidence of childhood diarrhea in developing countries (Thapar and Sanderson, 2004) by application of probiotic cultures, or by food fermentations with probiotic *L. reuteri* (Sekwati-Monang and Gänzle, 2011).

Acknowledgments

The Alberta Livestock and Meat Agency is acknowledged for funding (Grant No. 2013R002R). MG acknowledges the Canada Research Chair program for funding. YY is grateful to Dr. Juan Jovel and Dr. Petya T. Koleva for the help in sequencing data analysis.

References

Bednorz, C., Guenther, S., Oelgeschläger, K., Kinnemann, B., Pieper, R., Hartmann, S., et al. (2013). Feeding the probiotic *Enterococcus faecium* strain NCIMB 10415 to piglets specifically reduces the number of *Escherichia coli* pathotypes that adhere to the gut mucosa. *Appl. Environ. Microbiol.* 79, 7896–7790. doi: 10.1128/aem.03138-13

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Brandt, M. J. (2014). Starter cultures for cereal based foods. *Food Microbiol.* 37, 41–43. doi: 10.1016/j.fm.2013.06.007

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

Chen, X. Y., Woodward, A., Zijlstra, R. T., and Gänzle, M. G. (2014). Exopolysaccharides synthesized by *Lactobacillus reuteri* protect against enterotoxigenic *Escherichia coli* in piglets. *Appl. Environ. Microbiol.* 80, 5752–5760. doi: 10.1128/AEM.01782-14

Colwell, R. K., Chao, A., Gotelli, N. J., Lin, S. Y., Mao, C. X., Chazdon, R. L., et al. (2012). Models and estimators linking individual-based and sample-based rarefaction, extrapolation and comparison of assemblages. *J. Plant Ecol.* 5, 3–21. doi: 10.1093/jpe/rtr044

Corr, S. C., Li, Y., Riedel, C. U., O'Toole, P. W., Hill, C., and Gahan, C. G. M. (2007). Bacteriocin production as a mechanism for the anti-infective activity of *Lactobacillus salivarius* UCC118. *Proc. Nat. Acad. Sci. U.S.A.* 104, 7617–7621. doi: 10.1073/pnas.0700440104

De Angelis, M., Siragusa, S., Caputo, L., Ragni, A., Burzigotti, R., and Gobbetti, M. (2007). Survival and persistence of *Lactobacillus plantarum* 4.1 and *Lactobacillus reuteri* 3S7 in the gastrointestinal tract of pigs. *Vet. Microbiol.* 123, 133–144. doi: 10.1016/j.vetmic.2007.02.022

De Weirdt, R., Crabbé, A., Roos, S., Vollenweider, S., Lacroix, C., van Pijkeren, J. P., et al. (2012). Glycerol supplementation enhances *L. reuteri*'s protective effect against *S. Typhimurium* colonization in a 3-D model of colonic epithelium. *PLoS ONE* 7:e37116. doi: 10.1371/journal.pone.0037116

Duncan, S. H., Lobley, G. E., Holtrop, G., Ince, J., Johnstone, A. M., Louis, P., et al. (2008). Human colonic microbiota associated with diet, obesity and weight loss. *Int. J. Obes.* 32, 1720–1724. doi: 10.1038/ijo.2008.155

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. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

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

Frese, S. A., Benson, A. K., Tannock, G. W., Loach, D. M., Kim, J., Zhang, M., et al. (2011). The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. *PLoS Genet.* 7:e1001314. doi: 10.1371/journal.pgen.1001314

Frese, S. A., Mackenzie, D. A., Peterson, D. A., Schmaltz, R., Fangman, T., Zhou, Y., et al. (2014). Molecular characterization of host-specific biofilm formation in a vertebrate gut symbiont. *PLoS Genet.* 9:e1004057. doi: 10.1371/journal.pgen.1004057

Galle, S., Schwab, C., Dal Bello, F., Coffey, A., Gänzle, M. G., and Arendt, E. K. (2012). Influence of *in-situ* synthesized exopolysaccharides on the quality of gluten-free sorghum sourdough bread. *Int. J. Food Microbiol.* 155, 105–112. doi: 10.1016/j.ijfoodmicro.2012.01.009

Gänzle, M. G. (2004). Reutericyclin: biological activity, mode of action, and potential applications. *Appl. Microbiol. Biotechnol.* 64, 326–332. doi: 10.1007/s00253-003-1536-8

Gänzle, M. G., Höltzel, A., Walter, J., Jung, G., and Hammes, W. P. (2000). Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. *Appl. Environ. Microbiol.* 66, 4325–4333. doi: 10.1128/AEM.66.10.4325-4333.2000

Gänzle, M. G., and Vogel, R. F. (2002). Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. *Int. J. Food Microbiol.* 80, 31–45. doi: 10.1016/S0168-1605(02)00146-0

Hofstetter, S., Gebhardt, D., Ho, L., Gänzle, M., and McMullen, M. L. (2013). Effects of nisin and reutericyclin on resistance of endospores of *Clostridium* spp. to heat and high pressure. *Food Microbiol.* 34, 46–51. doi: 10.1016/j.fm.2012.11.001

Hurdle, J. G., Heathcott, A. E., Yang, L., Yan, B., and Lee, R. E. (2011). Reutericyclin and related analogues kill stationary phase *Clostridium difficile* at achievable colonic concentrations. *J. Antimicrob. Chemother.* 66, 1773–1776. doi: 10.1093/jac/dkr201

Ivarsson, E., Roos, S., Liu, H. Y., and Lindberg, J. E. (2014). Fermentable non-starch polysaccharides increases the abundance of *Bacteroides-Prevotella-Porphyromonas* in ileal microbial community of growing pigs. *Animal* 8, 1777–1787. doi: 10.1017/S1751731114001827

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., et al. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proc. Natl. Acad. Sci. U.S.A.* 108(Suppl. 1), 4578–4585. doi: 10.1073/pnas.1000081107

Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., et al. (2006). Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8, 1191–1199. doi: 10.1111/j.1462-2920.2006.01009.x

Konstantinov, S. R., Smidt, H., Akkermans, A. D., Casini, L., Trevisi, P., Mazzoni, M., et al. (2008). Feeding of *Lactobacillus sobrius* reduces *Escherichia coli* F4 levels in the gut and promotes growth of infected piglets. *FEMS Microbiol. Ecol.* 66, 599–607. doi: 10.1111/j.1574-6941.2008.00517.x

Korakli, M., Gänzle, M. G., and Vogel, R. F. (2002). Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *J. Appl. Microbiol.* 92, 958–965. doi: 10.1046/j.1365-2672.2002.01607.x

Lallès, J. P., Bosi, P., Smidt, H., and Stokes, C. R. (2007). Weaning - a challenge to gut physiologists. *Livestock Sci.* 108, 82–93. doi: 10.1016/j.livsci.2007.01.091

Lamendella, R., Domingo, J. W., Ghosh, S., Martinson, J., and Oerther, D. B. (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol.* 11:103. doi: 10.1186/1471-2180-11-103

La Rosa, P. S., Warner, B. B., Zhou, Y., Weinstock, G. M., Sodergren, E., Hall-Moore, C. M., et al. (2014). Patterned progression of bacterial populations in the premature infant gut. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12522–12527. doi: 10.1073/pnas.1409497111

Le Bourhis, A. G., Saunier, K., Dore, J., Carlier, J. P., Chamba, J. F., Popoff, M. R., et al. (2005). Development and validation of PCR primers to assess the diversity of *Clostridium* spp. in cheese by temporal temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* 71, 29–38. doi: 10.1128/AEM.71.1.29-38.2005

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., and Møller, K. (2002). Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl. Environ. Microbiol.* 68, 673–690. doi: 10.1128/AEM.68.2.673-690.2002

Lin, X. B., and Gänzle, M. G. (2014). Effect of lineage-specific metabolic traits of *Lactobacillus reuteri* on sourdough microbial ecology. *Appl. Environ. Microbiol.* 80, 5782–5789. doi: 10.1128/AEM.01783-14

Lin, X. B., Lohans, C. T., Duar, R., Zheng, J., Vederas, J. C., Walter, J., et al. (2015). Genetic determinants of reutericyclin biosynthesis in *Lactobacillus reuteri*. *Appl. Environ. Microbiol.* 81, 2032–2041. doi: 10.1128/AEM.03691-14

Looft, T., Allen, H. K., Cantarel, B. L., Levine, U. Y., Bayles, D. O., Alt, D. P., et al. (2014). Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *ISME J.* 8, 1566–1576. doi: 10.1038/ismej.2014.12

Lu, X. M., Lu, P. Z., and Zhang, H. (2014). Bacterial communities in manures of piglets and adult pigs bred with different feeds revealed by 16S rDNA 454 pyrosequencing. *Appl. Microbiol. Biotechnol.* 9, 2657–2665. doi: 10.1007/s00253-013-5211-4

Martínez, I., Lattimer, J. M., Hubach, K. L., Case, J. A., Yang, J., Weber, C. G., et al. (2013). Gut microbiome composition is linked to whole grain-induced immunological improvements. *ISME J.* 7, 269–280. doi: 10.1038/ismej.2012.104

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., and Neufeld, J. D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13:31. doi: 10.1186/1471-2105-13-31

Messelhäußer, U., Zucker, R., Elmer-Englhard, D., Busch, U., Hörmansdorfer, S., Pudich, U., et al. (2007). Nachweis und Charakterisierung von *Clostridium perfringens* mittels real-time-PCR. *JVL* 2, 194–197. doi: 10.1007/s00003-007-0173-z

Metzler-Zebeli, B. U., Hooda, S., Pieper, R., Zijlstra, R. T., van Kessel, A. G., Mosenthin, R., et al. (2010). Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic *Escherichia coli* in the pig gastrointestinal tract. *Appl. Environ. Microbiol.* 76, 3692–3701. doi: 10.1128/AEM.00257-10

Millette, M., Cornut, G., Dupont, C., Shareck, F., Archambault, D., and Lacroix, M. (2008). Capacity of human nisin- and pediocin-producing lactic acid bacteria to reduce intestinal colonization by vancomycin-resistant enterococci. *Appl. Environ. Microbiol.* 74, 1997–2003. doi: 10.1128/AEM.02150-07

Navas-Molina, J. A., Peralta-Sanchez, J. M., Gonzalez, A., McMurdie, P. J., Vazquez-Baeza, Y., Xu, Z., et al. (2013). Advancing our understanding of the human microbiome using QIIME. *Meth. Enzymol.* 531, 371–444. doi: 10.1016/B978-0-12-407863-5.00019-8

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., et al. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 118, 511–521. doi: 10.1542/peds.2005-2824

Rea, M. C., Alemayehu, D., Casey, P. G., O'Connor, P. M., Lawlor, P. G., Walsh, M., et al. (2014). Bioavailability of the anti-clostridial bacteriocin thuricin CD in gastrointestinal tract. *Microbiology* 160, 439–445. doi: 10.1099/mic.0.068767-0

Riboulet-Bisson, E., Sturme, M. H., Jeffery, I. B., O'Donnell, M. M., Neville, B. A., Forde, B. M., et al. (2012). Effect of *Lactobacillus salivarius* bacteriocin Abp118 on the mouse and pig intestinal microbiota. *PLoS ONE* 7:e31113. doi: 10.1371/journal.pone.0031113

Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L., and McCartney, A. L. (2010). Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology* 156, 3329–3341. doi: 10.1099/mic.0.043224-0

Schwab, C., Cristescu, B., Northrup, J. M., Stenhouse, G. B., and Gänzle, M. G. (2011). Diet and environment shape fecal bacterial microbiota composition and enteric pathogen load of grizzly bears. *PLoS ONE* 6:e27905. doi: 10.1371/journal.pone.0027905

Sekwati-Monang, B., and Gänzle, M. G. (2011). Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana. *Int. J. Food Microbiol.* 150, 115–121. doi: 10.1016/j.ijfoodmicro.2011.07.021

Spinler, J. K., Sontakke, A., Hollister, E. B., Venable, S. F., Oh, P. L., Balderas, M. A., et al. (2014). From prediction to function using evolutionary genomics: human-specific ecotypes of *Lactobacillus reuteri* have diverse probiotic functions. *Genome Biol. Evol.* 6, 1772–1789. doi: 10.1093/gbe/evu137

Su, M. S., Oh, P. L., Walter, J., and Gänzle, M. G. (2012). Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis. *Appl. Environ. Microbiol.* 78, 6777–6780. doi: 10.1128/AEM.01678-12

Su, Y., Yao, W., Perez-Gutierrez, O. N., Smidt, H., and Zhu, W. Y. (2008). 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets after oral administration of *Lactobacillus sobrius* S1. *Anaerobe* 14, 78–86. doi: 10.1016/j.anaerobe.2007.12.004

Tannock, G. W., Fuller, R., and Pedersen, K. (1990). *Lactobacillus* succession in the piglet digestive tract demonstrated by plasmid profiling. *Appl. Environ. Microbiol.* 56, 1310–1316.

Teixeira, J. S., Seeras, A., Sanchez-Maldonado, A. F., Zhang, C., Su, M. S., and Gänzle, M. G. (2014). Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*. *Food Microbiol.* 42, 172–180. doi: 10.1016/j.fm.2014.03.015

Thapar, N., and Sanderson, I. R. (2004). Diarrhoea in children: an interface between developing and developed countries. *Lancet* 363, 641–653. doi: 10.1016/S0140-6736(04)15599-2

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Tuohy, K. M., Probert, H. M., Smejkal, C. W., and Gibson, G. R. (2003). Using probiotics and prebiotics to improve gut health. *Drug Discov. Today* 8, 692–700. doi: 10.1016/S1359-6446(03)02746-6

Valdovska, A., Jemeljanovs, A., Pilmane, M., Zitare, I., Konosonoka, I. H., and Lazdins, M. (2014). Alternative for improving gut microbiota: use of Jerusalem artichoke and probiotics in diet of weaned piglets. *Pol. J. Vet. Sci.* 17, 61–69. doi: 10.2478/pjvs-2014-0008

van Bueren, A. L., Saraf, A., Martens, E. C., and Dijkhuizen, L. (2015). Differential metabolism of exopolysaccharides from probiotic lactobacilli by the human gut symbiont *Bacteroides thetaiotaomicron*. *Appl. Environ. Microbiol.* 81, 3973–3983. doi: 10.1128/AEM.00149-15

van den Berg, R. J., Kuijper, E. J., van Coppenraet, L. E. S. B., and Claas, E. C. J. (2006). Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. *Clin. Microbiol. Infect.* 12, 184–186. doi: 10.1111/j.1469-0691.2005.01301.x

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., and Knight, R. (2013). EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* 2:16. doi: 10.1186/2047-217X-2-16

Walter, J. (2008). Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Appl. Environ. Microbiol.* 74, 4985–4996. doi: 10.1128/AEM.00753-08

Walter, J., and Ley, R. (2011). The human gut microbiome: ecology and recent evolutionary changes. *Annu. Rev. Microbiol.* 65, 411–429. doi: 10.1146/annurev-micro-090110-102830

Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., et al. (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* 66, 297–303. doi: 10.1128/AEM.66.1.297-303.2000

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, Y., Gänzle, M. G., and Schwab, C. (2010). Exopolysaccharide synthesized by *Lactobacillus reuteri* decreases the ability of enterotoxigenic *Escherichia coli* to bind to porcine erythrocytes. *Appl. Environ. Microbiol.* 76, 4863–4866. doi: 10.1128/AEM.03137-09

Wilson, C. M., Loach, D., Lawley, B., Bell, T., Sims, I. M., O'Toole, P. W., et al. (2014). *Lactobacillus reuteri* 100-23 modulates urea hydrolysis in the murine stomach. *Appl. Environ. Microbiol.* 80, 6104–6013. doi: 10.1128/AEM.01876-14

Yang, Y., Galle, S., Le, M. H. A., Zijlstra, R., and Gänzle, M. G. (2015). Feed fermentation with reuteran- and levan producing *Lactobacillus reuteri* reduced colonization of weanling pigs with enterotoxigenic *Escherichia coli*. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.01525-15. [Epub ahead of print].

Young, I. T. (1977). Proof without prejudice - use of Kolmogorov-Smirnov test for analysis of histograms from flow systems and other sources. *J. Histochem. Cytochem.* 25, 935–941. doi: 10.1177/25.7.894009

Zhang, L., Xu, Y. Q., Liu, H. Y., Lai, T., Ma, J. L., Wang, J. F., et al. (2010). Evaluation of *Lactobacillus rhamnosus* GG using an *Escherichia coli* K88 model of piglet diarrhoea: effects on diarrhoea incidence, faecal microflora and immune responses. *Vet. Microbiol.* 141, 142–148. doi: 10.1016/j.vetmic.2009.09.003

Zhao, C., Kinner, M., Wismer, W., and Gänzle, M. G. (2015). Effect of glutamate-accumulation during sourdough fermentation with *L.* reuteri on the taste of bread and sodium-reduced bread. *Cereal Chem.* 92, 224–230. doi: 10.1094/CCHEM-07-14-0149-R

Zheng, J., Gänzle, M. G., Lin, X. B., Ruan, L., and Sun, M. (2015). Diversity and dynamics of bacteriocins from the human microbiome. *Environ. Microbiol.* 17, 2133–2143. doi: 10.1111/1462-2920.12662

Conflict of Interest Statement: 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: 08 September 2015
doi: 10.3389/fmicb.2015.00944

Geriatric respondents and non-respondents to probiotic intervention can be differentiated by inherent gut microbiome composition

Suja Senan[1], Jashbhai B. Prajapati[2], Chaitanya G. Joshi[3], Sreeja V.[2], Manisha K. Gohel[4], Sunil Trivedi[5], Rupal M. Patel[5], Himanshu Pandya[6], Uday Shankar Singh[4], Ajay Phatak[7] and Hasmukh A. Patel[1]*

[1] Department of Dairy Science, South Dakota State University, Brookings, SD, USA, [2] Department of Dairy Microbiology, Anand Agricultural University, Anand, India, [3] Department of Animal Biotechnology, Anand Agricultural University, Anand, India, [4] Department of Community Medicine, H. M Patel Center for Medical Care and Education, Karamsad, India, [5] Department of Microbiology, H. M Patel Center for Medical Care and Education, Karamsad, India, [6] Department of Medicine, H. M Patel Center for Medical Care and Education, Karamsad, India, [7] Central Research Services, Charutar Arogya Mandal, Karamsad, India

OPEN ACCESS

Edited by:
Kate Howell,
University of Melbourne, Australia

Reviewed by:
Stella Maris Reginensi Rivera,
Universidad de la República Oriental del Uruguay, Uruguay
Amit Kumar Tyagi,
The University of Texas MD Anderson Cancer Center, USA

***Correspondence:**
Jashbhai B. Prajapati,
Department of Dairy Microbiology, SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat 388 110, India
prajapatijashbhai@yahoo.com, jbprajapati@aau.in

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

Received: 12 February 2015
Accepted: 14 August 2015
Published: 08 September 2015

Citation:
Senan S, Prajapati JB, Joshi CG, Sreeja V, Gohel MK, Trivedi S, Patel RM, Pandya H, Singh US, Phatak A and Patel HA (2015) Geriatric respondents and non-respondents to probiotic intervention can be differentiated by inherent gut microbiome composition. Front. Microbiol. 6:944. doi: 10.3389/fmicb.2015.00944

Scope: Probiotic interventions are known to have been shown to influence the composition of the intestinal microbiota in geriatrics. The growing concern is the apparent variation in response to identical strain dosage among human volunteers. One factor that governs this variation is the host gut microbiome. In this study, we attempted to define a core gut metagenome, which could act as a predisposition signature marker of inherent bacterial community that can help predict the success of a probiotic intervention.

Methods and results: To characterize the geriatric gut microbiome, we designed primers targeting the 16S rRNA hypervariable region V2–V3 followed by semiconductor sequencing using Ion Torrent PGM. Among respondents and non-respondents, the chief genera of phylum Firmicutes that showed significant differences are *Lactobacillus*, *Clostridium*, *Eubacterium*, and *Blautia* ($q < 0.002$), while in the genera of phylum Proteobacteria included *Shigella*, *Escherichia*, *Burkholderia* and *Camphylobacter* ($q < 0.002$).

Conclusion: We have identified potential microbial biomarkers and taxonomic patterns that correlate with a positive response to probiotic intervention in geriatric volunteers. Future work with larger cohorts of geriatrics with diverse dietary influences could reveal the potential of the signature patterns of microbiota for personalized nutrition.

Keywords: geriatric, gut, metagenome, probiotics, MTCC 5463

Abbreviations: µl, microliter; AAU, Anand Agricultural University; CFU, colony forming unit; dL, deciliter; dNTP, deoxynucleotide; ICMR, Indian Council of Medical Research; ISAPP, International Scientific Association for Probiotics and Prebiotics; mg, milligram; ml, milliliter; mM, millimolar; MTCC, microbial type culture collection; nt, nucleotide; PGM, Personal Genome Machine; pmol, picomol; QIIME, Quantitative Insights into Microbial Ecology; rRNA, ribosomal RNA; s, seconds; S, Svedberg unit; U, unit.

Introduction

An integrative study of the host and its surrounding environment is imperative to comprehend the complex biological system of the human body. As a part of the environment, the human host more than 100 trillion bacteria forming the "in-vironment" (de Wouters et al., 2000) made up of millions of microbial genes in the intestine (Lederberg, 2000). The indigenous microbial community plays an integral role in regulating the host's physiological, nutritional, and immunological processes (Hooper et al., 2001). The study of the diversity of the indigenous microbial community can explain the host microbe interaction (Gerritsen et al., 2011). The gut microbiota composition changes with age due to physiological reasons and increased use of medications (Bartosch et al., 2004; Mueller et al., 2006; Mariat et al., 2009; Zwielehner et al., 2009). The microbiota of elderly people showed a higher *Bacteroidetes*/*Firmicutes* ratio along with a high inter-individual variation in microbiota composition at the phylum level when compared with young adults (Claesson et al., 2011). Studies of the geriatric gut have shown a decline in the count and diversity among Bacteroidetes (Hopkins and Macfarlane, 2002; Woodmansey et al., 2004; Guigoz et al., 2008). Proteolytic bacteria increase on aging in the large bowel, leading to putrefaction (Hopkins et al., 2001). Such changes in intestinal microbiome cause prolonged intestinal transit time and fecal retention among geriatrics (Tiihonen et al., 2009). An understanding of the changes in the microbiome of the elderly has led to the possibility of correcting the dysbiosis by administering probiotics. Probiotics have been successful in increasing the levels of health-promoting bacteria in the fecal microbiota of elderly (Ahmed et al., 2007; Lahtinen et al., 2009; Matsumoto et al., 2009), improving the frequency of bowel movements (Pitkala et al., 2007), *Clostridium difficile*-associated diarrhea incidence (Ouwehand et al., 2009), and frequency of defecation (An et al., 2010).

The translation of the above-mentioned benefits of probiotics to the host cannot be guaranteed. This could be due to the individual differences with respect to diet, the structure and operations of the gut microbiota, nutrient and energy harvest, variations in human environmental exposures, microbial ecology, and genotype (Turnbaugh et al., 2009). In order to assure uniform outcomes of therapy among subjects, the International Scientific Association for Probiotics and Prebiotics (ISAPP) had come up with recommendations for conducting a well-defined trial (Reid et al., 2010). Briefly, they include (1) clearly define the end goal, (2) design the study by identifying precise parameters and defining the level of response that will be tested, (3) base the selection of the intervention on scientific investigations, and (4) carefully select the study cohort. Inter-individual diversity in responses toward probiotics could also be due to core gut microbiome patterns. Recently, role of microbial biomarkers for determining dietary responsiveness were identified in obese individuals (Korpela et al., 2014) and metabolic diseases (McOrist et al., 2011; Walker et al., 2011; Louis, 2012; Lampe et al., 2013), paving the way for personalized nutrition. This study takes up the challenge to identify the factors that differentiate a respondent from a non-respondent and utilize the findings to define the precise dose and response prognosis. This finding can help design probiotic supplements catering to a niche market defined by age, location, or disease state.

From an Indian perspective, gut metagenomics have been studied in malnourished children (Gupta et al., 2011), obese individuals (Patil et al., 2012), and children of varying nutritional status (Ghosh et al., 2014). It was for the first time in India that the present study was conducted to investigate the elderly gut metagenome to identify microbial biomarkers determining responsiveness of the host to a probiotic therapy. We hypothesized that by studying the baseline gut microbiota diversity of elderly subjects, we could identify a core gut microbiome signature pattern that is likely to positively influence the response of an individual to the probiotic strain. The strain under study, *Lactobacillus helveticus* MTCC 5463 is an indigenous potential probiotic with *in vitro*, *in vivo*, and *in silico* studies providing suggestive evidences of the strain's robustness in the gut and transit, adhesion, autoaggregation, colonization, antibacterial property, hypocholesterolemic, and immunomodulatory properties (Senan et al., 2015). The outcome of this study paves the way forward for tailored probiotic therapy.

Materials and Methods

Origin and Maintenance of Bacterial Strains

The indigenous probiotic strain *L. helveticus* MTCC 5463 (Prajapati et al., 2011) and starter culture *Streptococcus thermophilus* MTCC 5460 (Prajapati et al., 2013) were maintained by the Department of Dairy Microbiology, Anand Agricultural University, India at −80°C as 15% glycerol stocks and were routinely cultured in de Man, Rogosa, Sharpe (MRS) and M 17 medium, respectively (HiMedia India Ltd., India).

Product Preparation

The test product was a fermented probiotic drink (*Lassi*) with double toned milk fermented with culture containing *S. thermophilus* MTCC 5460 and *L. helveticus* MTCC 5463. The cultures were added at 0.1% each and incubated aerobically till an acidity of 0.8–0.9% lactic acid was obtained. Both the test and placebo products contained sugar and prebiotic honey in a standardized ratio. The control product was made in a similar manner without the addition of MTCC 5463. The shelf life of the fermented drink was 28 days at 4°C, corresponding to the lower level (10^9 CFU/ml) of strain MTCC 5463.

Participant Selection

Individuals ranging from 64 to 74 years were recruited. Initially, 112 subjects were enrolled in the trial, 36 had to withdraw because of antibiotic consumption. Volunteers were asked to sign the consent form before recruitment. Exclusion criteria included lactose intolerance, recent antibiotic treatment, frequent gastrointestinal disorders, or metabolic diseases. Participants included in the trial had no known allergies or intolerance to dairy foods. The trial had 80% power at a 5% 2-sided significance level to detect a >50% change in the primary outcome among subjects. No antibiotics or laxatives were taken 2 months before or during the study.

Intervention

Sixteen participants showing diversity in lactobacilli count and cholesterol levels were involved in the double-blind, crossover, placebo-controlled, and randomized-feeding trial. The trial was divided into five consecutive periods: a pre-feeding period (2 weeks), followed by a feeding period (4 weeks), a washout period (4 weeks), a second-feeding period (4 weeks), and a final washout period (2 weeks).

Collection and Analysis of the Blood Samples

Blood samples were taken from each volunteer immediately before and after each treatment period using EDTA-containing vacutainers. Total cholesterol (TC) was measured using enzyme-spectrophotometry kits and IgG, IgM, TNF-alpha, INF-gamma, and IL-2 by ELISA capture assay (Siemens Medical Solutions Diagnostics Ltd., India). All the tests were done at the Central Diagnostic Laboratory, Shri Krishna Medical College Karamsad, Anand, Gujarat, an NABL accredited and ISO 15189:2003 laboratory.

Selection of Respondents and Non-Respondents

A respondent was defined as a subject having an improvement in the levels of *L. helveticus* MTCC 5463 strain count in feces and a reduction in cholesterol levels. Similarly, a non-responder was defined as a subject who displayed an absence of decrease in cholesterol levels and increase in viability of the bacterial strain. Based on these criteria, we identified eight subjects each in respondents and non-respondents category.

Fecal Sample Collection

Single fecal samples were collected at the end of every 2 weeks. Participants were given 60 ml sterile stool container with a sterile plastic spoon (Polylab Plasticware, India) and were asked to fill the tube to the 30 ml mark with feces from the midstream defecation period. During the second-feeding period, there was a crossover of the feeding design. For every collection, the stool samples were immediately frozen at −20°C.

DNA Extraction from Stool Samples

DNA was extracted from feces using a QIAamp MiniPrep DNA extraction kit following the manufacturer's instructions. The DNA was stored at −20°C. Quality and purity of the isolated genomic DNA were confirmed by agarose gel electrophoresis and spectrophotometry on the NanoDrop 2000 device (Fisher Scientific, Schwerte, Germany). DNA concentration was estimated with the Qubit 2.0 instruments applying the Qubit dsDNA HS Assay (Life Technologies, Invitrogen division, Darmstadt, Germany).

Quantification of Lactobacilli in Stool

The subjects voided their feces into a 60-ml sterile stool container with a sterile plastic spoon (Polylab Plasticware, India) from the midstream defecation period. Within an hour of sample procurement, samples were diluted and homogenized to give a 10-fold dilution (wet weight/volume). Stool Lactobacilli content was determined by plating aliquots (1 ml) of each dilution on freshly prepared de Mann Rogosa Sharp agar (Himedia, India), incubated for 24–48 h at 37°C under anaerobic conditions. Stool *L. helveticus* MTCC 5463 content was determined by quantitative PCR (qPCR) using StepOne Real-Time PCR System (ABI/Thermo Fisher Scientific, Bangalore, India). Primers and 3′ minor groove binder (MGB) probes for accurate detection and quantification of *L. helveticus* MTCC 5463 in human fecal samples were developed with Primer Express v3.0 [Thermo Fisher Scientific (earlier Applied Biosystems), Bangalore, India]. The temperature profile of the qPCR consisted 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C, and 1 min at 60°C. Species specific primers and probe targeted on the bile salt hydrolase gene of *L. helveticus* MTCC 5463 (Accession number AEYL01000315; locus tag AAULH_13111 2049 bp). BLAST[1] and EMBL[2] database were used to ensure the specificity of the primers. Genomic DNA standards prepared with six different serial dilutions (2.68×10^6, 2.68×10^5, 2.68×10^4, 2.68×10^3, 2.68×10^2, and 2.68×10) being equivalent to ranges from 10^6 to 10^1 CFU/ml of target genome (MTCC 5463). The cycle threshold (C_T) was evaluated to create the standard curve. The amplification efficiencies were determined using the formula $E = [10^{(-1/\text{slope})} - 1]$.

16S Primers and Amplicon Library Generation

PCR amplification of the 16S rRNA hypervariable region V2–V3 was performed with a pool of 32 degenerated forward and 1 degenerated reverse primer targeting bacteria as described by Schmalenberger et al. (2001) with barcode sequences at the 5′ end of the primers. Primers were assessed for specificity using the SILVA 108 SSU Reference 16S rRNA gene database and BLASTN matches with corresponding 16S rRNA gene sequences. The addition of the barcodes to the primers resulted in an amplicon approximately 430 nt in length. The primers were diluted and pooled to equimolar quantities. For amplicon library preparation, 4 ng of each genomic DNA, 5 mM dNTPs, 2 mM $MgCl_2$ (Roche Diagnostics, USA), 1 U Platinum Taq DNA polymerase High Fidelity, and 10 pmol primer-mix were used per 25 µl amplification reaction. The PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 94°C for 15 s, 60°C for 45 s, 70°C for 30 s, and a final elongation step of 72°C for 10 min. Amplicon product purification was performed via gel electrophoresis on a 1.5% Tris Borat EDTA agarose gel-stained with ethidium bromide (EtBr) (Life Technologies). All positive PCR reactions were electrophoresized in agarose gels and products with the expected size were cut and purified with Qiagen Gel extraction kit (Qiagen, Düsseldorf, Germany). The exact fragment sizes were determined using HT DNA High Sensitivity LabChip Kit (Caliper Life Sciences GmbH, Mainz, Germany). Amplicon library concentration was estimated with the Qubit 2.0 instrument using the Qubit dsDNA HS assay (Life Technologies).

Emulsion PCR and Sequencing

The emulsion PCR was carried out applying the Ion XPress Template kit V2.0 (Life Technologies) as described in the appropriate user Guide (Part No. 4469004 Rev. B 07/2011) provided by the manufacturer. Quality and quantity of the enriched spheres were checked on the Guava easyCyte5 system (Millipore GmbH,

[1] http://www.ncbi.nlm.nih.gov/blast/
[2] http://www.ebi.ac.uk/embl/

Schwalbach am Taunus, Germany) as described in the appendix of the Ion Xpress Template Kit User Guide (Part Number 4467389 Rev. B, 05/2011). Sequencing of the amplicon libraries was carried out on the Ion Torrent Personal Genome Machine (PGM) system using the Ion Sequencing 200 kit (all Life Technologies) following the corresponding protocol (Part No. 4471999 Rev B, October 13, 2011). Quality check passed libraries were subjected to emulsion PCR using the Ion PGM 200 Xpress Template Kit (Life Technologies). After bead enrichment, beads were loaded onto Ion 316 chips and sequenced using an Ion Torrent PGM.

Sequence Analysis

The sequence data sets obtained were uploaded to the Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) server[3] and subsequently checked for low-quality reads. The sequence reads that passed the quality filtering step were then subjected to further analysis of the taxonomic annotation of the fecal DNA sequences using QIIME pipeline (Caporaso et al., 2010). To investigate the species diversity, we used rarefaction curves, and richness estimators like Chao1 in QIIME. Statistic comparison of samples organized into respondents and non-respondents for the deferentially abundant microbial diversity was studied using an alignment platform, STAMP.[4]

Results

We conducted a comparative analysis of respondents and non-respondents fecal microbiome to reveal differences and identified biomarkers that differentiate them.

Quantification of Lactobacilli Count Using Traditional Plating and qPCR

Traditional plate counts of lactobacilli at genus level on selective medium ranged from a baseline reading of 8.6 log CFU/g of wet fecal matter, which rose to 9.3 log CFU/g at the end of feeding period and a gradual decrease to 8.7 log CFU/g at the end of the placebo feeding. The qPCR primers targeted the bile salt hydrolase gene of MTCC 5463, which made the gene copy count a fraction of the plate count. On the other hand, the precise *L. helveticus* MTCC 5463 strain count from real-time PCR showed a complete absence of the strain before feeding. At the end of 30 days, the strain appeared in the feces of all subjects in the treated group, reaching a level as high as 8.32 to the lowest amount of 6.17 log gene copies/g fecal matter at end of feeding period.

Summary of Sequence Processing Data

Primers targeting the 16S rRNA gene V2–V3 region (Schmalenberger et al., 2001) precisely generated amplicons from members of the domain bacteria and did not hybridize to sequences of the domains Archaea and Eucarya. By using the ARB SILVA 108 SSU database, a high match of 84.5% at a maximum number of four mismatches was observed. The primer pair theoretically targeted all 16S rRNA gene sequences of the gut microbiota bacterial orders and generated a single amplicon. All 32 amplicons from the pool of test and placebo groups were mixed together at an equimolar ratio. After pooling and elution of the amplicons from the gel, we got one band for the probiotic fed group of 520 bp having a concentration of 1912.09 pg/μl and molarity of 5562.5 pmol/l. The placebo fed group gave an amplicon of 513 bp having a concentration of 1435.87 pg/μl and 4237.7 pmol/l molarity. The data sets for 16 subjects before probiotic feeding had reads ranging from 13,061 to 980,628 with a read length ranging from 201 to 251 bp and a total amount of 42,52,62,470 bases.

Inter-Individual Differences in Shifts in Phyla Abundance (%) Before and After Probiotic Feeding

The relative abundance of major genera in the elderly gut metagenome and high-level of inter-individual variation is shown in **Figure 1**. We presume that the inter-individual differences are indicative of a highly personal fecal microbiota profile, which determines the response of the host to probiotics. Host factors probably play a major effect in shaping the intestinal microbial ecosystem during an intervention. In the present study, we attempted to understand the core microbiome of respondents and non-respondents to probiotics.

Probiotic Feeding and Effect on Lipid Profile and Immunologic Parameters

In addition to TC (primary outcome), in this study, we also investigated the effect of probiotic intervention on lipid profile, beta glucouronidase activity, and immunological parameters. The mean β-glucuronidase activity was reduced in test group from 1.40 to 0.73 (microgram/min/mg of protein), while in case of placebo group, no effect on enzyme activity was observed. A significant immunomodulatory effect on the TNF-α and IL-2 levels in subjects among probiotic group compared to placebo group was observed. There was however no significant beneficiary effect found on IFN-γ, IgG, or IgM levels. Paired *t*-test showed that there were statistically significant differences in serum cholesterol, VLDL, TC/HDL, LDL/HDL in placebo group and in LDL, TC/HDL, and LDL/HDL in probiotic group. A significant ($p = 0.01$) decrease in the LDL value was seen in the probiotic group at the end of 30 days of feeding (**Table 1**).

Participant Diversification into Respondents and Non-Respondents

The primary outcome of this trial was a reduction in TC after 4 weeks of feeding probiotic MTCC 5463. We defined non-respondents as those subjects who experienced elevations in TC of $\geq$2.509 mg/dL, whereas respondents were the ones who showed no change in TC or $<$1.72 mg/dL TC in response to the probiotic intervention of 4 weeks. Among the 59 subjects who could complete the study, we classified a total of 16 subjects into respondents ($n = 8$) and non-respondents ($n = 8$) based on cholesterol levels and lactobacilli counts (**Figure 2**). There were no significant differences in the baseline characteristics of the two groups. This eliminates the influence of gender, weight, and age in influencing the response toward probiotic intervention. The abundance of *L. helveticus* MTCC 5463 was significant

[3]http://metagenomics.nmpdr.org/
[4]http://kiwi.cs.dal.ca/Software/STAMP

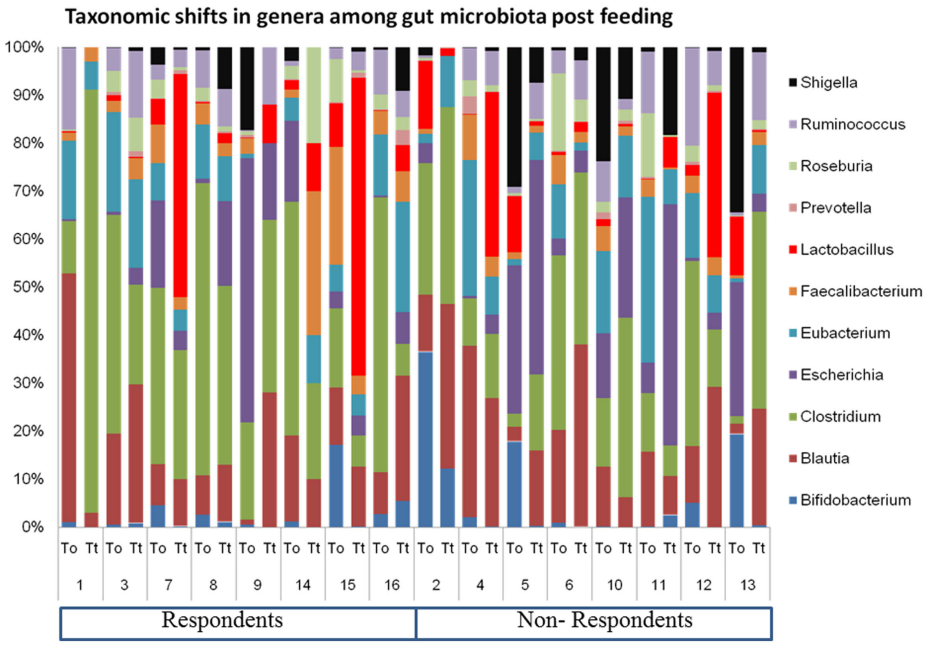


FIGURE 1 | Inter-individual variation in the geriatric gut microbiome pre- and post-probiotic feeding.

TABLE 1 | Effect of probiotic and placebo interventions on lipid profile in geriatric subjects.

Variables	Probiotic (means ± SD)	Placebo (means ± SD)
Total cholesterol (TC) (mg/dL)		
Baseline	161.67 ± 41.05	174.32 ± 49.99
Post-intervention	158.09 ± 42.63	167.09 ± 43.11
p-Value	0.12	<0.001
Triglyceride (mg/dL)		
Baseline	103.77 ± 49.84	116.38 ± 71.01
Post-intervention	104.00 ± 56.43	108.58 ± 70.74
p-Value	0.96	0.03
High density lipoprotein (HDL) (mg/dL)		
Baseline	46.21 ± 12.46	49.67 ± 15.97
Post-intervention	47.08 ± 13.97	48.77 ± 12.98
p-Value	0.24	0.34
Low density lipoprotein (LDL) (mg/dL)		
Baseline	98.48 ± 37.12	88.93 ± 38.37
Post-intervention	92.93 ± 35.79	84.56 ± 31.13
p-Value	0.01	0.09
Very low density lipoprotein (mg/dL)		
Baseline	21.63 ± 12.04	23.28 ± 14.20
Post-intervention	21.34 ± 11.97	21.71 ± 14.12
p-Value	0.74	0.03
Total cholesterol/HDL (mg/dL)		
Baseline	3.91 ± 1.22	3.77 ± 1.33
Post-intervention	3.74 ± 1.20	3.65 ± 1.23
p-Value	<0.001	0.04
LDL/HDL (mg/dL)		
Baseline	2.37 ± 0.96	2.23 ± 1.01
Post-intervention	2.21 ± 0.91	2.13 ± 0.96
p-Value	<0.001	0.04

($p < 0.05$) higher in the individuals with an increase in cholesterol levels, as compared to those with a decrease. The decrease in cholesterol levels among respondents was a maximum 14.19% with a 23.66% increase in lactobacilli count in feces. Among non-respondents, a maximum increase of 34.13% in cholesterol with a 9.31% decrease in lactobacilli count was observed. The increase in lactobacilli counts with a decrease in cholesterol in case of respondents indicated that the observed hypocholesterolemic effect of the strain was dependent on the number of lactobacilli in the gut.

Microbiome Diversity Estimates Associated with Respondents and Non-Respondents

The alpha diversity of the respondent group before probiotic feeding (30.8 ± 4.8) and after feeding (26.6 ± 3.6) was higher than non-respondents' measures for before (25.6 ± 4.5) and after probiotic feeding (24.6 ± 5.8). This indicates that bacterial richness is a factor that promotes responsiveness toward beneficial strains in the gut. To investigate differences in rarefaction measures, we rarified each sample at 33,000 reads and performed the two-sample *t*-test on the two groups. Respondents had significantly greater alpha diversity indices like phylogenetic distance ($p = 0.022$), Chao1 ($p = 0.019$), and Shannon index ($p = 0.00058$) than the non-respondents (**Figure 3**). A non-significant increase in observed species in case of non-respondents ($p = 0.27$) could be due to presence of distinct commensals that reflect the host's dietary and geographical differences. There was a non-significant abundance of *Clostridium*, *Shigella*, and *Listeria* among rural respondents. Poor sanitation and hygiene maintained in the rural households could have led to the distinct differences in gut microbiota.

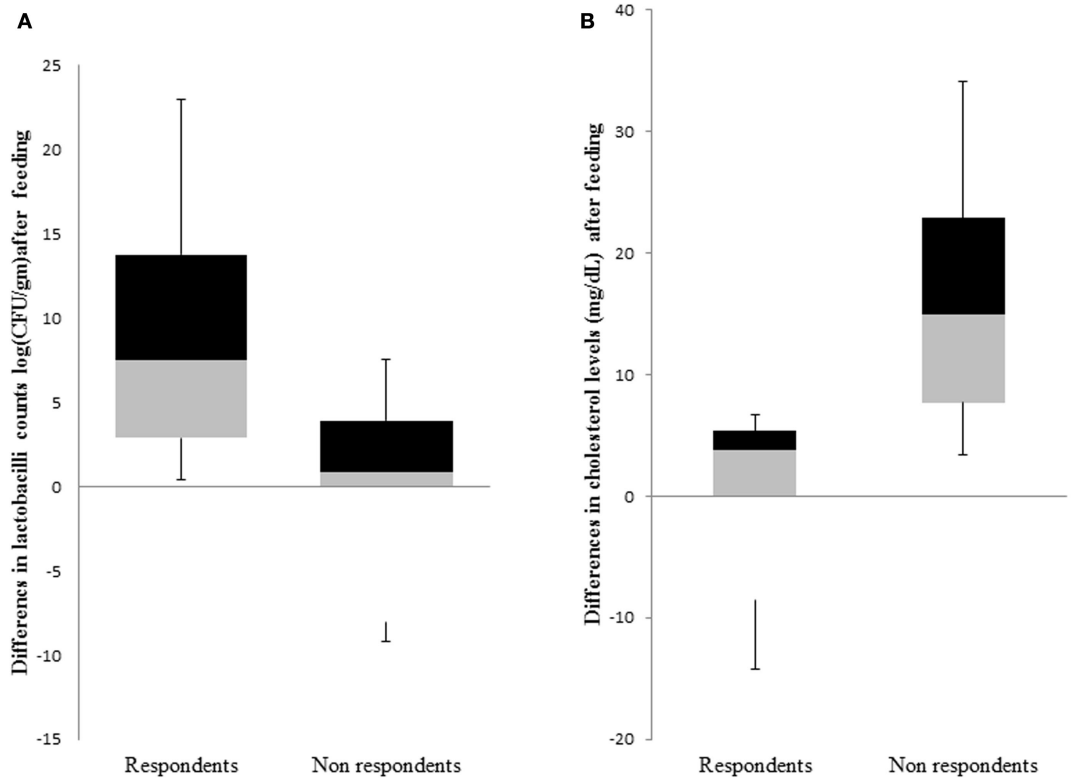


FIGURE 2 | Comparison of response groups with respect to (A) lactobacilli counts and (B) cholesterol levels. The middle line represents the median, the box represents the interquartile range, and the whiskers represent the total range.

Bacterial Taxa Populations Associated with Respondents and Non-Respondents

We performed a comparison of the microbiota between respondents and non-respondents to find specialized bacterial members within the abundant phyla *Firmicutes* and *Proteobacteria*. Respondents carried a lower proportion of *Clostridium* and a higher proportion of *Eubacterium* compared to the non-respondents (**Figure 4**). Surprisingly, although the non-respondents had a higher proportion of gut lactobacilli (31%) compared to 28% in respondents, a favorable reduction in cholesterol corresponding to the increase in strain MTCC 5463 was not observed. This could be due to competitive exclusion by a higher proportion of *Clostridium* (24%) in the gut on non-respondents compared to respondents (6%). The presence of *Listeria* in the non-respondents further emphasizes the need to investigate the association of gut microbiota, especially pathobionts with probiotic strain. Comparing the abundance in the genera of Proteobacteria group, it can be observed (**Figure 5**) that respondents carried a higher amount of *Burkholderia* (63%) and a lower amount of *Shigella* (7%) compared to non-respondents, who harbored lower count of *Burkholderia* (36%) and a higher amount of *Shigella* (31%), which must have affected the colonization of the probiotic strain. Non-respondents carried a higher amount of *Escherichia* and *Brucella* in the gut. *Shigella* seemed to have a symbiont asymptomatic existence in the host, showing no discomfort to the subjects. The higher amount of *Escherichia* and *Camphylobacter* could be the deciding biomarkers of non-responsiveness toward probiotic intervention.

Statistical Analysis of Metagenomic Data

A remarkable significant difference among the chief genera of Proteobacteria including *Shigella*, *Escherichia*, *Burkholderia*, and *Camphylobacter* ($q < 0.002$) was observed. The chief genera of Firmicutes that showed remarkable significant difference were *Lactobacillus*, *Clostridium*, *Eubacterium*, and *Blautia* ($q < 0.002$) (**Figure 6**). Although non-respondents carried a higher proportion of Lactobacilli, a favorable physiological function may not be translated to the host possibly due to an increase in *Clostidum*, *Shigella*, and *Eschericihia* with a decrease in *Blautia* and *Burkholderia*. We would like to add that the results of population wide samples taken at one time point for a study might not be able to display the entire variation that exists in that population over time and place.

Discussion

A primary beneficial effect of consuming a bile-salt-hydrolyzing *L. helveticus* MTCC 5463 strain is a reduction in serum cholesterol levels. In the clinical trials carried out to prove the hypocholesterolemic effect of the strain (Ashar and Prajapati, 2001; Prajapati et al., 2012) we could observe participants responding differently to the same treatment. Similar cases of inter-individual variability

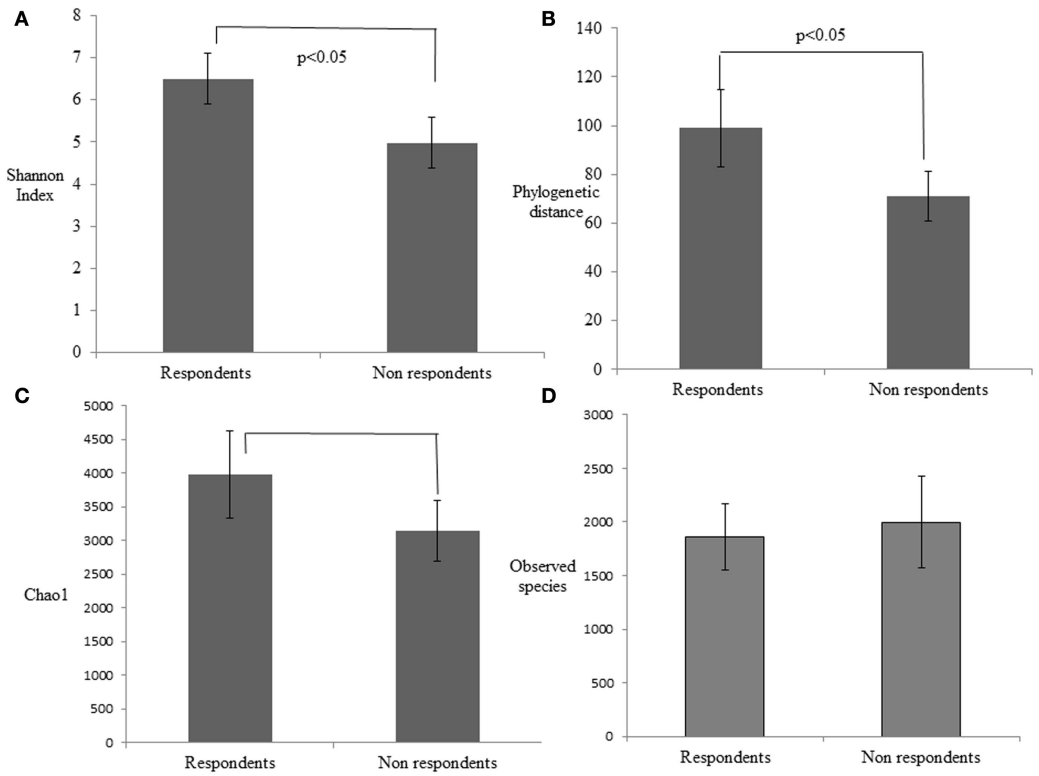


FIGURE 3 | Estimation of the phylogenetic diversity of the gut microbiota in the respondent and non-respondent groups using the (A) Shannon index, (B) phylogenetic distance, (C) Chao1, and (D) observed species. The values are means, and error bars indicate the 95% confidence intervals.

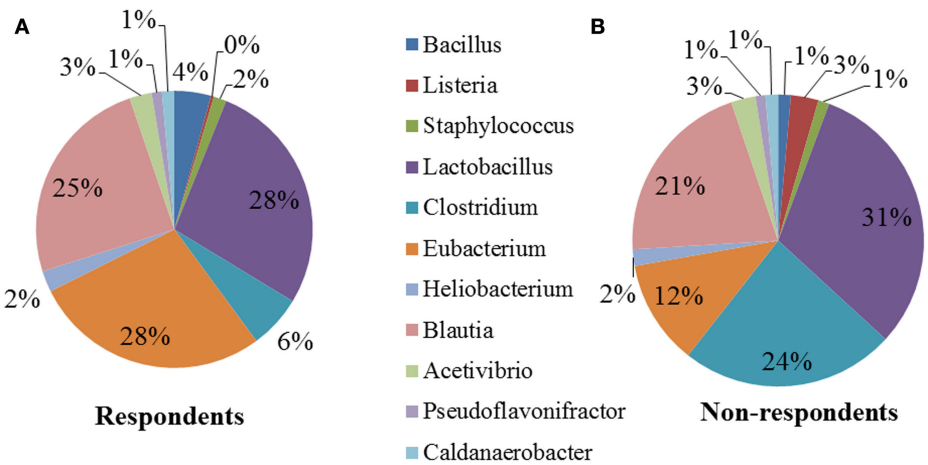


FIGURE 4 | Relative abundances of the dominant genera (Firmicutes) in (A) respondents and (B) non-respondents.

in response to probiotics (van Baarlen et al., 2011; Grzeskowiak et al., 2012; Arboleya et al., 2013) have been published. Previously, subjects had been classified as respondents and non-respondents based on a greater than or less than 10% change in cholesterol (Cox et al., 2014) but this classification was criticized as being impractical (Ding and Schloss, 2014). Classification using a fecal biomarker (Coen et al., 2009) or biomarkers of the host's basic metabolism (Naruszewicz et al., 2002; Herron et al., 2003; Ibrahim et al., 2010) has been suggested. We conducted a comparative analysis of fecal microbiomes of respondents and non-respondents to identify bacterial biomarkers. Results of such microbial profiling may serve as a clinically useful biomarker in geriatric care (Kostic et al., 2013).

Primer usage is one of the most critical factors affecting 16S rDNA analysis (Armougom and Raoult, 2009). There exists a possibility that amplification efficacy of the primers could have

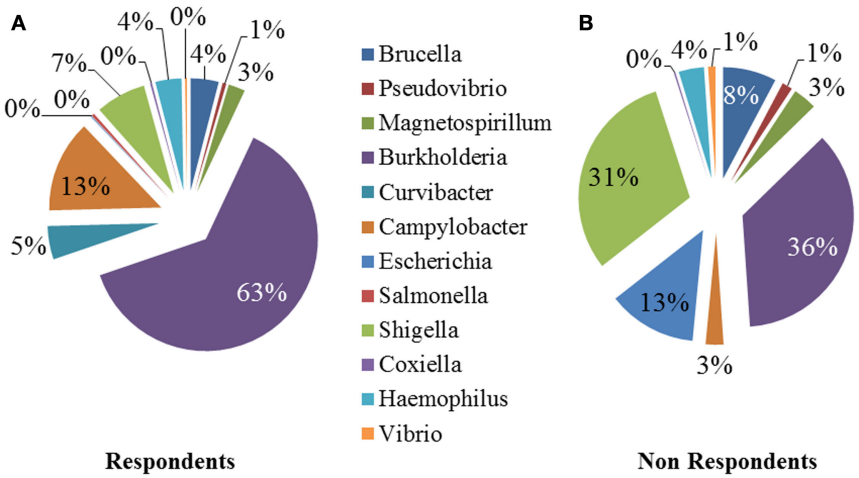


FIGURE 5 | Relative abundances of the dominant genera (Proteobacteria) in (A) respondents and (B) non-respondents.

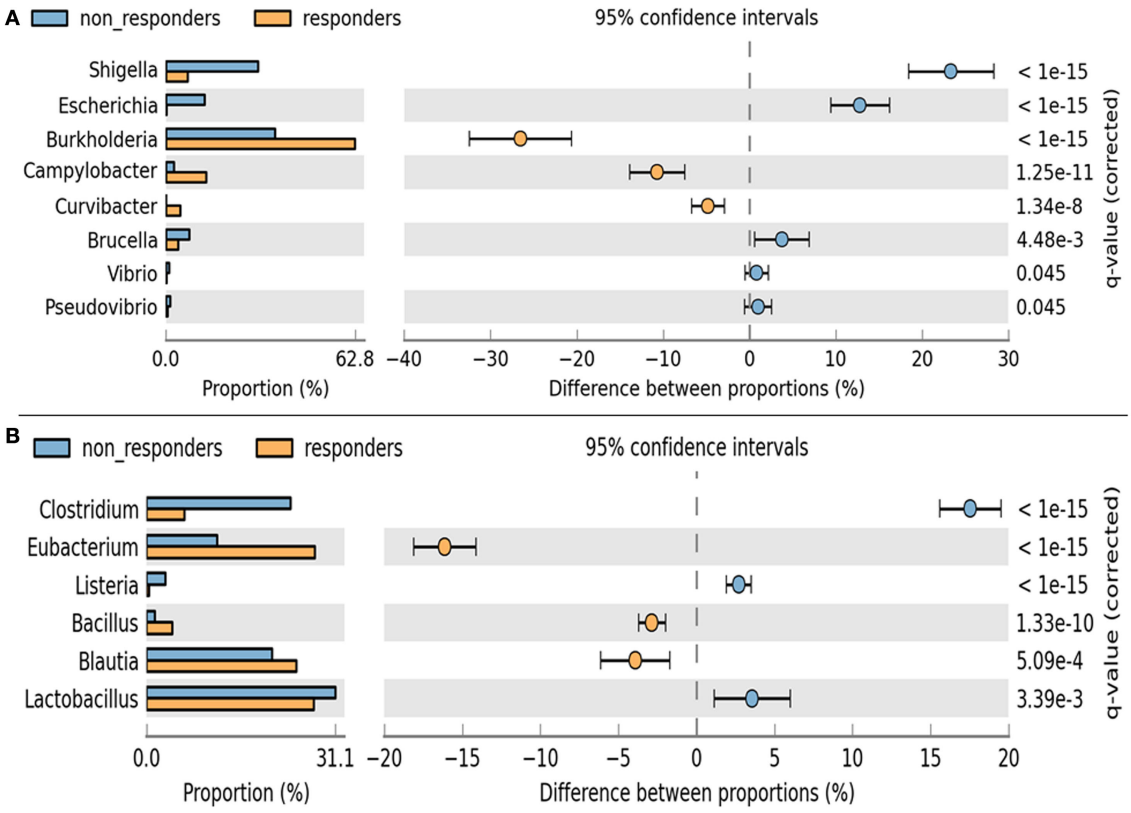


FIGURE 6 | Analysis of significance in abundance of (A) Firmicutes and (B) Proteobacteria among respondents and non-respondents using two-sample Fischer's exact test.

led to the underestimation of bacterial richness, in this study. We chose the Ion Torrent PGM platform due to its inherent low-cost per sequencing run that allowed us to perform the next-generation sequencing on site at the Veterinary faculty at AAU.

There were no significant differences in the age or weight of respondents and non-respondents, though respondents had a tendency to be older. An effect size of 0.8 among the groups further eliminated the influence of gender, weight, and age toward the expected response. Grouping of similar sequences as operational taxonomic units (OTUs) and quantifying the number of OTUs gave an approximation of species diversity in a sample (Sun et al., 2009; Schloss et al., 2014). Species diversity has been reported as a characteristic feature determining state of health or disease. Reduced colonic microbial diversity of dysbiosis is reported in

Crohn disease (Ott et al., 2004), ulcerative colitis (McLaughlin et al., 2010), antibiotic-associated diarrhea (Chang et al., 2008), and *Clostridium difficile* infection (Seto et al., 2014). A higher alpha diversity of gut bacteria among respondents compared to non-respondents seemed to support microbial integrity. Bacterial richness and evenness play an integral role in the success of a probiotic therapy as earlier observed in colitis (Kennedy et al., 2000).

A higher Chao's estimator and the Shannon index among respondents indicate higher saturation and unevenness in taxa abundance within samples. A non-significant increase in observed species in case of non-respondents suggested the presence of species that may have prevented the probiotic to establish and exert the functionality. We could identify distinct microbial diversity in rural subjects compared to the urban dwellers especially in the presence of *Clostridium*, *Shigella*, and *Listeria*, similar to Russian rural communities (Tyakht et al., 2013). Gram-negative bacteria were more abundant than Gram-positive bacteria in the rural population. *Shigella* and *Escherichia* were significantly under-represented in rural African children than urban European children (De Filippo et al., 2010). In the present study, we observed *Shigella* and *Escherichia* higher in geriatric rural dwellers, which reflected the age and geography-induced diversities in human gut microbiome.

Phyla associated with a healthy state in Indian geriatrics as suggested in this study are Firmicutes accounting for at least 50%, followed by Actinobacteria (20%) and Proteobacteria (10%). At the phylum level, the majority of the intestinal bacteria are known to belong to *Bacteroidetes* and *Firmicutes* (Eckburg et al., 2005). Surprisingly, none of the sequences from the present study were assigned to the Bacteroidetes. An under representation of Bacteroidetes could be due to inter-subject variability (Ley et al., 2006), variation due to adiposity (Frank et al., 2007; Wu et al., 2011), or suppression due to inflammatory bowel disease (Lazarevic et al., 2009). We could not ignore the possibility of loss of this phylum in fecal samples when stored for longer periods and MG-RAST-based classifications' sensitivity for Proteobacteria (Korpela et al., 2014). Antibiotic usage in elderly can again cause a decline in commensal anaerobes like *Bacteroides*, *Lactobacillus*, and *Bifidobacterium* (Macfarlane, 2014).

Responses of the host to individual bacterial strains are influenced by the baseline composition of the gut microbiota (Rajilić-Stojanović et al., 2015). The respondents showed a lower percentage of Firmicutes and non-respondents showed a comparative lower amount of Proteobacteria. Among Firmicutes, Clostridia were higher in case of non-respondents (24% compared to 6% in respondents). Although the volunteers were not consuming antibiotics during the trial, prior usage of antibiotics could have diminished the population of total and commensal bacteria (Biagi et al., 2010) leading to an overgrowth of *Clostridium* (Round and Mazmanian, 2009) in non-respondents. Antibiotics are readily available over the counter at pharmacies in India and inconsistent hospital standards toward antibiotic usage could have led to higher proportion of Clostridia in the gut. Respondents carried a higher proportion of *Eubacterium* (28% compared to 12% in non-respondents), which reflected a healthy state. An increased diversity of Eubacteria has been observed in the elderly (Hopkins and Macfarlane, 2002). Decrease in *Eubacterium* lead to decreased levels of SCFA, facilitating easier entry of *Enterobacteriaceae* into the intestinal mucosa due to an impaired secretion of mucins by the intestinal epithelial cells (Garrett et al., 2010). The outgrowth of anaerobic *Enterobacteriaceae* must have led to a competitive exclusion of aerotolerant MTCC 55463 strains in the host intestine.

Proteobacteria, recently defined as "pathobionts" (Morgan et al., 2004) are considered to be minor and opportunistic components of the human gut ecosystem. The majority of the sequences assigned to the Proteobacteria were Burkholderiales. A higher proportion of *Burkholderia* is a signature of good health as earlier observed in the healthy Indian child data set (Schultz et al., 2004). Non-respondents had a higher proportion of Proteobacteria, especially *Escherichia*/*Shigella* (indistinguishable as a 16S-based phylotype), previously implicated in intestinal inflammation (Guslandi et al., 2004). Although the volunteers were seemingly healthy with no complaints of gastrointestinal disturbances, a core structural and functional dysbiosis caused by an overgrowth of *Escherichia*/*Shigella* (Malchow, 1997) could have led to a lack of translation of functionality of MTCC 5463 to the host in spite of being present at a higher proportion in the non-respondents gut.

In this study, it was shown that consumption of probiotic yogurt did not significantly reduce TC levels, the intervention significantly reduced serum levels of LDL, TC/HDL ratio, and LDL/HDL ratio in geriatric volunteers. Many studies in literature support the beneficiary effect of probiotics on lipid profiles of subjects. There also exist some contradictory reports where eating probiotic yogurt did not change lipid profiles (Hatakka et al., 2008; Sadrzadeh-Yeganeh et al., 2010). This indicates that apart from the probiotic strain, the host gut microbiome has a big role to play on the response of the host to the probiotic strain.

STAMP analysis revealed proportions of distinct microbial biomarkers like *Shigella*, *Escherichia*, *Burkholderia*, *Camphylobacter*, *Lactobacillus*, *Clostridium*, *Eubacterium*, and *Blautia* that can help tailor a probiotic therapy to a niche population. The authors would like to strike an analogy to feeding probiotics to a host with imbalanced consortia in the gut to the likes of pouring water to a filled pitcher. Like the water flows out, the probiotic strains are lost in the feces and fail to colonize and translate the functionality to the host. Metatranscriptomic studies could furnish further information for comprehending the molecular basis of responsiveness toward a probiotic therapy because gene expression profiles are more individualized than DNA-level profiles and less variable than microbial composition. Geriatric care is critical in the aging global population and the role of gut metagenomics cannot be overstated in understanding its role in health and disease for the future development of personalized nutrition.

Conclusion

Globally today, the elderly populations are looking for natural means of sustaining digestive health. Compared to the growing awareness and market penetration of probiotics, there is a dearth of scientific evidence on how probiotics affect the

composition of gut microbiota. The well-documented probiotic *L. helveticus* MTCC 5463 was administered to geriatrics in a clinical trial, and a deep sequencing technology was employed to study the changes in the resident microbes over the duration of probiotic consumption. We could find that chiefly *Shigella*, *Escherichia*, *Burkholderia*, *Camphylobacter*, *Lactobacillus*, *Clostridium*, *Eubacterium*, and *Blautia* define the response of the host to the probiotic strain. Moreover, we observed a shift in the gut profile of the non-respondents towards a respondent's signature gut profile after consuming the probiotic, which proves the importance of precise personalized selection of dosage for an effective tailored probiotic therapy.

Ethics

Approval for the study design was obtained from the Institutional Ethics Committee (IEC) of Shri Krishna Medical College Karamsad, Anand, Gujarat (HMPCMCE:HREC/FCT/41/01) and Anand Agricultural University (AAU), Anand (AAU/DR/RES/DM/IEC/659/2011). The trial was registered at ICMR Clinical Trial Registry (REF/2012/10/004135).

Author Contributions

Conceived and designed the experiments: JP, CJ, and HP; performed metagenomic analysis and manuscript writing: SS; product development: SV; clinical recruitment of participants: MG and US; clinical investigations and data interpretation: ST and RP; literature search and critical review of the manuscript: HAP; statistical data analysis: AP.

Acknowledgments

We are indebted to the technical staff of Ome Reserch Facility, Department of Animal Biotechnology, AAU, India whose names were not included in the author list, but who contributed to sequencing and data analysis of this work. The authors kindly acknowledge the funding support received from DBT, India, through Project BT/PR-14950/FNS/20/490/2010. Partial results of the study were presented in the Symposium on "Gut Microbiota, Probiotics and Their Impact throughout the Lifespan," conducted at Harvard Medical School, Boston, MA, USA. September 22–23, 2014.

References

Ahmed, S., Macfarlane, G. T., Fite, A., McBain, A. J., Gilbert, P., Macfarlane, S., et al. (2007). Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. *Appl. Environ. Microbiol.* 73, 7435–7442. doi:10.1128/AEM.01143-07

An, H. M., Baek, E. H., Jang, S., Lee, D. K., Kim, M. J., Kim, J. R., et al. (2010). Efficacy of lactic acid bacteria (LAB) supplement in management of constipation among nursing home residents. *Nutr. J.* 9, 5. doi:10.1186/1475-2891-9-5

Arboleya, S., Salazar, N., Solís, G., Fernández, N., Gueimonde, M., and de los Reyes-Gavilán, C. G. (2013). *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

Armougom, F., and Raoult, D. (2009). Exploring microbial diversity using 16S rRNA high-throughput methods. *J. Comput. Sci. Syst. Biol.* 2, 74–92. doi:10.4172/jcsb.1000019

Ashar, M. N., and Prajapati, J. B. (2001). Serum cholesterol level in humans fed acidophilus milk. *Indian J. Microbiol.* 41, 257–263.

Bartosch, S., Fite, A., Macfarlane, G. T., and McMurdo, M. E. (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

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

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

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M., et al. (2008). Decreased diversity of the fecal Microbiome in recurrent *Clostridium* difficile-associated diarrhea. *J. Infect. Dis.* 197, 435–438. doi:10.1086/525047

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

Coen, M., Want, E. J., Clayton, T. A., Rhode, C. M., Hong, Y. S., Keun, H. C., et al. (2009). Mechanistic aspects and novel biomarkers of responder and non-responder phenotypes in galactosamine-induced hepatitis. *J. Proteome Res.* 8, 5175–5187. doi:10.1021/pr9005266

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

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 Wouters, T., Doré, J., and Lepage, P. (2000). Does our food (environment) change our gut microbiome ('In-Vironment'): a potential role for inflammatory bowel disease. *Dig. Dis.* 30, 33–39. doi:10.1159/000342595

Ding, T., and Schloss, P. D. (2014). Dynamics and associations of microbial community types across the human body. *Nature* 509, 357–360. doi:10.1038/nature13178

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi:10.1126/science.1110591

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., and Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl Acad. Sci. U.S.A.* 104, 13780–13785. doi:10.1073/pnas.0706625104

Garrett, W. S., Gallini, C. A., Yatsunenko, T., Michaud, M., DuBois, A., Delaney, M. L., et al. (2010). Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. *Cell Host Microbe* 8, 292–300. doi:10.1016/j.chom.2010.08.004

Gerritsen, J., Smidt, H., Rijkers, G. T., and de Vos, W. M. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr.* 6, 209–240. doi:10.1007/s12263-011-0229-7

Ghosh, T. S., Sen Gupta, S., Bhattacharya, T., Yadav, D., Barik, A., Chowdhury, A., et al. (2014). Gut microbiomes of Indian children of varying nutritional status. *PLoS ONE* 9:e95547. doi:10.1371/journal.pone.0095547

Grzeskowiak, L., Collado, M. C., Mangani, C., Maleta, K., Laitinen, K., Ashorn, P., et al. (2012). Distinct gut microbiota in southeastern African and northern European infants. *J. Pediatr. Gastroenterol. Nutr.* 54, 812–816. doi:10.1097/MPG.0b013e318249039c

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

Gupta, S. S., Mohammed, M. H., Ghosh, T. S., Kanungo, S., Nair, G. B., and Mande, S. S. (2011). Metagenome of the gut of a malnourished child. *Gut Pathog.* 3, 7. doi:10.1186/1757-4749-3-7

Guslandi, M., Mezzi, G., Sorghi, M., and Testoni, P. A. (2004). *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Dig. Dis. Sci.* 45, 1462–1466. doi:10.1023/A:1005588911207

Hatakka, K., Mutanen, M., Holma, R., Saxelin, M., and Korpela, R. (2008). *Lactobacillus rhamnosus* LC705 together with *Propionibacterium freudenreichii* ssp shermanii JS administered in capsules is ineffective in lowering serum lipids. *J. Am. Coll. Nutr.* 27, 441–447. doi:10.1080/07315724.2008.10719723

Herron, K. L., Vega-Lopez, S., Conde, K., Ramjiganesh, T., Shachter, N. S., and Fernandez, M. L. (2003). Men classified as hypo- or hyper respondents to dietary cholesterol feeding exhibit differences in lipoprotein metabolism. *J. Nutr.* 133, 1036–1042.

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291, 881–884. doi:10.1126/science.291.5505.881

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

Hopkins, M. J., Sharp, R., and Macfarlane, G. T. (2001). Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance and community cellular fatty acid profiles. *Gut* 48, 198–205. doi:10.1136/gut.48.2.198

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

Kennedy, R. J., Hoper, M., Deodhar, K., Kirk, S. J., and Gardiner, K. R. (2000). Probiotic therapy fails to improve gut permeability in a hapten model of colitis. *Scand. J. Gastroenterol.* 35, 1266–1271. doi:10.1080/003655200453601

Korpela, K., Flint, H. J., Johnstone, A. M., Lappi, J., Poutanen, K., Dewulf, E., et al. (2014). Gut microbiota signatures predict host and microbiota responses to dietary interventions in obese Individuals. *PLoS ONE* 9:e90702. doi:10.1371/journal.pone.0090702

Kostic, A. D., Chun, E., Meyerson, M., and Garrett, W. S. (2013). Microbes and inflammation in colorectal cancer. *Cancer Immunol. Res.* 3, 150–157. doi:10.1158/2326-6066.CIR-13-0101

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

Lampe, J. W., Navarro, S. L., Hullar, M. A. J., and Shojaie, A. (2013). Inter-individual differences in response to dietary intervention: integrating omics platforms towards personalised dietary recommendations. *Proc. Nutr. Soc.* 72, 207–218. doi:10.1017/S0029665113000025

Lazarevic, V., Whiteson, K., Huse, S., Hernandez, D., Farinelli, L., Osterås, M., et al. (2009). Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. *J. Microbiol. Methods* 79, 266–271. doi:10.1016/j.mimet.2009.09.012

Lederberg, J. (2000). Infectious history. *Science* 288, 287–293. doi:10.1126/science.288.5464.287

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi:10.1038/4441022a

Louis, P. (2012). Dietary modulation of the human gut microbiota. *Agro Food Industry Hi-Tech* 23, 26–28.

Macfarlane, S. (2014). Antibiotic treatments and microbes in the gut. *Environ. Microbiol.* 16, 919–924. doi:10.1111/1462-2920.12399

Malchow, H. A. (1997). Crohn's disease and *Escherichia coli*. A new approach in therapy to maintain remission of colonic Crohn's disease. *J. Clin. Gastroenterol.* 25, 653–658. doi:10.1097/00004836-199712000-00021

Mariat, D., Firmesse, O., Levenez, F., Guimaraes, V., Sokol, H., Doré, J., et al. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* 9:123. doi:10.1186/1471-2180-9-123

Matsumoto, M., Sakamoto, M., and Benno, Y. (2009). Dynamics of fecal microbiota in hospitalized elderly fed probiotic LKM512 yogurt. *Microbiol. Immunol.* 53, 421–432. doi:10.1111/j.1348-0421.2009.00140.x

McLaughlin, S. D., Clark, S. K., Tekkis, P. P., Nicholls, R. J., and Ciclitira, P. J. (2010). The bacterial pathogenesis and treatment of pouchitis. *Therap. Adv. Gastroenterol.* 3, 335–348. doi:10.1177/1756283X10370611

McOrist, A. L., Miller, R. B., Bird, A. R., Keogh, J. B., Noakes, M., Topping, D. L., et al. (2011). Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. *J. Nutr.* 141, 883–889. doi:10.3945/jn.110.128504

Morgan, X. C., Tickle, T. L., Sokol, H., Gevers, D., Devaney, K. L., Ward, D. V., et al. (2004). Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* 13, R79. doi:10.1186/gb-2012-13-9-r79

Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., et al. (2006). Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl. Environ. Microbiol.* 72, 1027–1033. doi:10.1128/AEM.72.2.1027-1033.2006

Naruszewicz, M., Johansson, M. L., Zapolska-Downar, D., and Bukowska, H. (2002). Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. *Am. J. Clin. Nutr.* 76, 1249–1255.

Ott, S. J., Musfeldt, M., Wenderoth, D. F., Hampe, J., Brant, O., Fölsch, U. R., et al. (2004). Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* 53, 685–693. doi:10.1136/gut.2003.025403

Ouwehand, A. C., Tiihonen, K., Saarinen, M., Putaala, H., and Rautonen, N. (2009). Influence of a combination of *Lactobacillus acidophilus* NCFM and lactitol on healthy elderly: intestinal and immune parameters. *Br. J. Nutr.* 101, 367–375. doi:10.1017/S0007114508003097

Patil, D. P., Dhotre, D. P., Chavan, S. G., Sultan, A., Jain, D. S., Lanjekar, V. B., et al. (2012). Molecular analysis of gut microbiota in obesity among Indian individuals. *J. Biosci.* 37, 647–657. doi:10.1007/s12038-012-9244-0

Pitkala, K. H., Strandberg, T. E., FinneSoveri, U. H., Ouwehand, A. C., Poussa, T., and Salminen, S. (2007). Fermented cereal with specific bifidobacteria normalizes bowel movements in elderly nursing home residents. A randomized controlled trial. *J. Nutr. Health Aging* 2007, 305–311.

Prajapati, J. B., Khedkar, C. D., Chitra, J., Senan, S., Mishra, V., Sreeja, V., et al. (2011). Whole genome shotgun sequencing of an Indian-origin *Lactobacillus helveticus* strain MTCC 5463 with probiotic potential. *J. Bacteriol.* 193, 4282–4283. doi:10.1128/JB.05449-11

Prajapati, J. B., Nathani, N., Patel, A. K., Senan, S., and Joshi, C. G. (2013). Genomic analysis of dairy starter culture *Streptococcus thermophilus* MTCC 5461. *J. Microbiol. Biotechnol.* 23, 459–466. doi:10.4014/jmb.1210.10030

Prajapati, J. B., Senan, S., Momin, J. K., Damor, R., and Kamalia, K. B. (2012). A randomised double blind placebo controlled trial of potential probiotic strain *Lactobacillus helveticus* MTCC 5463: assessment of its safety, tolerance and influence on intestinal wellbeing and humoral immune response in healthy human volunteers. *Int. J. Health Pharm. Sci.* 1, 92–99.

Rajilić-Stojanović, M., Jonkers, D. M., Salonen, A., Hanevik, K., Raes, J., Jalanka, J., et al. (2015). Intestinal microbiota and diet in IBS: causes, consequences, or epiphenomena. *Am. J. Gastroenterol.* 110, 278–287. doi:10.1038/ajg.2014.427

Reid, G., Gaudier, E., Guarner, F., Huffnagle, G. B., Macklaim, J. M., Munoz, A. M., et al. (2010). Respondents and non-respondents to probiotic interventions: how can we improve the odds? *Gut Microbes* 1, 200–204. doi:10.4161/gmic.1.3.12013

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

Sadrzadeh-Yeganeh, H., Elmadfa, I., Djazayery, A., Jalali, M., Heshmat, R., and Chamary, M. (2010). The effects of probiotic and conventional yoghurt on lipid profile in women. *Br. J. Nutr.* 103, 1778–1783. doi:10.1017/S0007114509993801

Schloss, P. D., Iverson, K. D., Petrosino, J. F., and Schloss, S. J. (2014). The dynamics of a family's gut microbiota reveal variations on a theme. *Microbiome* 2, 25. doi:10.1186/2049-2618-2-25

Schmalenberger, A., Schwieger, F., and Tebbe, C. C. (2001). Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. *Appl. Environ. Microbiol.* 67, 3557–3563. doi:10.1128/AEM.67.8.3557-3563.2001

Schultz, M., Timmer, A., Herfarth, H. H., Sartor, R. B., Vanderhoof, J. A., and Rath, H. C. (2004). *Lactobacillus* GG in inducing and maintaining remission of Crohn's disease. *BMC Gastroenterol.* 4:5. doi:10.1186/1471-230X-4-5

Senan, S., Prajapati, J. B., and Joshi, C. G. (2015). Whole-genome based validation of the adaptive properties of Indian origin probiotic *Lactobacillus helveticus* MTCC 5463. *J. Sci. Food Agric.* 95, 321–328. doi:10.1002/jsfa.6721

Seto, C. T., Jeraldo, P., Orenstein, R., Chia, N., and DiBaise, J. K. (2014). Prolonged use of a proton pump inhibitor reduces microbial diversity: implications for *Clostridium* difficile susceptibility. *Microbiome* 2, 42. doi:10.1186/2049-2618-2-42

Sun, Y., Cai, Y., Liu, L., Yu, F., Farrell, M. L., McKendree, W., et al. (2009). ESPRIT: estimating species richness using large collections of 16S rRNA pyrosequences. *Nucleic Acids Res.* 37, e76. doi:10.1093/nar/gkp285

Tiihonen, J., Lonnqvist, J., Wahlbeck, K., Klaukka, T., Niskanen, L., Tanskanen, A., et al. (2009). 11-year follow-up of mortality in patients with schizophrenia: a population-based cohort study (FIN11 study). *Lancet* 374, 620–627. doi:10.1016/S0140-6736(09)60742-X

Turnbaugh, P. J., Ridaura, K., Faith, J. J., Rey, F. E., Knight, R., and Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* 1, 6ra14. doi:10.1126/scitranslmed.3000322

Tyakht, A. V., Kostryukova, E. S., Popenko, A. S., Belenikin, M. S., Pavlenko, A. V., Larin, A. K., et al. (2013). Human gut microbiota community structures in urban and rural populations in Russia. *Nat. Commun.* 4, 2469. doi:10.1038/ncomms3469

van Baarlen, P., Troost, F., van der Meer, C., Hooiveld, G., Boekschoten, M., Brummer, R. J., et al. (2011). Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. *Proc. Natl Acad. Sci. U.S.A.* 108, 4562–4569. doi:10.1073/pnas.1000079107

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., et al. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J.* 5, 220–230. doi:10.1038/ismej.2010.118

Woodmansey, E. J., McMurdo, M. E., 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

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., et al. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334, 105–108. doi:10.1126/science.1208344

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 Statement: 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 ARTICLE
published: 21 August 2014
doi: 10.3389/fmicb.2014.00437

Bifidobacterium bifidum as an example of a specialized human gut commensal

Francesca Turroni[1], Sabrina Duranti[2], Francesca Bottacini[1], Simone Guglielmetti[3], Douwe Van Sinderen[1] and Marco Ventura[2]*

[1] Alimentary Pharmabiotic Centre and Department of Microbiology, Bioscience Institute, National University of Ireland, Cork, Ireland
[2] Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy
[3] Department of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy

Edited by:
Maria De Angelis, University of Bari Aldo Moro, Italy

Reviewed by:
Stella Maris Reginensi Rivera, Universidad de la República Oriental del Uruguay, Uruguay
Diego Mora, Università degli Studi di Milano, Italy

***Correspondence:**
Marco Ventura, Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parco Area delle Scienze 11a, 43124 Parma, Italy
e-mail: marco.ventura@unipr.it

Bifidobacteria are considered dominant and for this reason key members of the human gut microbiota, particularly during the first one to two years following birth. A substantial proportion of the bifidobacterial population in the intestine of infants belong to the *Bifidobacterium bifidum* taxon, whose members have been shown to display remarkable physiological and genetic features involving adhesion to epithelia, as well as utilization of host-derived glycans. Here, we reviewed the current knowledge on the genetic features and associated adaptations of *B. bifidum* to the human gut.

Keywords: *Bifidobacterium bifidum*, bifidobacteria, probiotics, genomics, microbiota

GENERAL FEATURES OF THE GENUS *Bifidobacterium*

The genus *Bifidobacterium*, a member of the *Bifidobacteriaceae* family, belongs to the *Actinobacteria* phylum (Stackebrandt and Tindall, 2000). Bifidobacteria are Gram-positive microorganisms with a high G+C DNA content, which were first isolated from feces of a breast-fed infant by Tissier in 1899, and then named *Bacillus bifidus* (Tissier, 1900). However, because of their morphological and physiological features, which are similar to those of lactobacilli, they were classified as members of the genus *Lactobacillus* for most of the 20th century and only starting from 1974 have been recognized as a separate genus (Buchanan, 2009). Currently, the genus *Bifidobacterium* is comprised of 48 different taxa, 40 of which have been isolated from the gastro-intestinal tract (GIT) contents of mammals, birds, or insects, while the remaining eight from sewage and fermented milk (Ventura et al., 2007a, 2009b, 2014). In addition, two bifidobacterial taxa, i.e., *Bifidobacterium crudilactis* and *Bifidobacterium mongoliense*, were isolated from raw milk cheeses (St Marcellin, Vercors area, France) (Delcenserie et al., 2013). Taking their different ecological niches into account and combining this information with a comparative analysis of their 16S rRNA sequences, as well as with other housekeeping genes (*clp*C, *dna*J, *xfp*, *dna*B, *rpo*C, and *pur*F), the various *Bifidobacterium* taxa can be clustered into six different phylogenetic groups, designated as the *Bifidobacterium adolescentis*-, *Bifidobacterium asteroides*-, *Bifidobacterium boum*-, *Bifidobacterium longum*-, *Bifidobacterium pullorum*-, and *Bifidobacterium pseudolongum*-phylogenetic groups (Ventura et al., 2007a). In this context, the *B. bifidum* species was shown not to fit in any of the above mentioned phylogenetic groups, thus suggesting the existence of unique and specific genetic features.

BIFIDOBACTERIAL ECOLOGY

Bifidobacteria have been isolated from six different ecological niches. Bifidobacteria are widely distributed among animals whose offspring enjoy parental care, such as mammals, birds, social insects. Therefore, the reason for their ecological distribution may be due to direct transmission of bifidobacterial cells from mother/carer to offspring. Though bifidobacteria are commonly found in the animal gut, these microorganisms have also been found in three other ecological niches: (iv) human blood (*Bifidobacterium scardovii*), (v) sewage (e.g., *Bifidobacterium minimum* and *Bifidobacterium thermacidophilum*), and (vi) food products (e.g., *Bifidobacterium animalis* subsp. *lactis*). These atypical ecological niches are rather different from that of the GIT, and it is plausible that the identification of bifidobacteria in these environments is the consequence of contaminations from GIT (Ventura et al., 2007b).

Notably, bifidobacteria that belong to the species *Bifidobacterium animalis*, *Bifidobacterium adolescentis*, *Bifidobacterium dentium*, and *Bifidobacterium catenulatum* display a more cosmopolitan lifestyle (Lamendella et al., 2008).

Bifidobacterium bifidum, *Bifidobacterium breve*, and *Bifidobacterium longum* are specifically identified in the human gut and have been shown to represent part of the dominant bacterial members of the gut microbiota of breast-fed infants (Turroni et al., 2009a, 2012).

BIFIDOBACTERIAL POPULATION IN THE HUMAN GUT

Bifidobacteria quickly colonize the intestine of infants during the first weeks of life due to selection by breast or formula milk, as confirmed by metagenomic analyses (Roger et al., 2010; Koenig et al., 2011). In breast-fed infants, *B. breve* is the dominant species,

followed by *B. bifidum* and *B. longum* subsp. *infantis* (Turroni et al., 2012; Milani et al., 2013).

The fecal microbiota of infants is characterized by high levels of bifidobacteria (Harmsen et al., 2000; Turroni et al., 2012). The level of abundance of bifidobacteria within the human gut decreases with age, although ecological analyses based on FISH and metagenomic studies have estimated that their presence in the adult colon is around 4.3 ± 4.4% of fecal microbes (Eckburg et al., 2005; Mueller et al., 2006). In adult feces, *B. adolescentis* and *B. catenulatum* species are commonly detected, followed by *B. longum*. Recently, a study involving the isolation of bifidobacteria from human intestinal mucosal samples and fecal samples on selective media (i.e., by a culture-dependent method), followed by the sequencing of the 16S rRNA internal transcribed spacer (ITS) regions of individual isolates allowed a description of the biodiversity of the bifidobacterial population present in the human gut (Turroni et al., 2009a). This study identified the most abundant bifidobacterial species present in the human gut, being represented by *B. longum*, *B. pseudolongum*, *B. animalis* subsp. *lactis*, *B. adolescentis*, *B. bifidum*, *B. pseudocatenulatum*, and *B. breve*. Furthermore, this analysis highlighted that the distribution of bifidobacteria present in various human subjects underline both an inter-subject, and an intra-subject variability, as also confirmed through real-time quantitative polymerase chain reaction (qPCR) analyses of fecal samples from healthy adults (Guglielmetti et al., 2013).

Turroni et al. (2009b) evaluated the bifidobacterial composition of the human intestine by a microbiomic approach, through the analyses of five colonic mucosal samples from healthy adults. This work showed how each subject possesses a specific population of colonic bifidobacteria that is in agreement with the large inter-variability of the whole intestinal microbiota previously described (Eckburg et al., 2005; Palmer et al., 2007). These samples were dominated by 16S rRNA gene sequences closely related to the *B. pseudolongum* phylogenetic groups (74.5%), followed by members of the *B. longum* (17%) and *B. adolescentis* (8.5%) phylogenetic groups. Moreover, this culture-independent approach led to the identification of many novel bifidobacterial 16S rRNA gene sequences, which are presumed to represent as yet undefined novel bifidobacterial species.

As mentioned above, *B. bifidum* is among the first colonizers of the human gut, reaching high numbers in the infant gut, but also detected at low levels in adults (Turroni et al., 2012). Particularly, the analyses by qPCR of fecal specimens from healthy adults revealed the presence of *B. bifidum* in 76% of the analyzed samples ($n = 82$), with a mean log(10) number of cells per g of feces (±SD) of 6.5 ± 1.4, whereas the mean concentration of total bifidobacteria in the same samples was 8.6 ± 1.2 (Guglielmetti et al., 2013). Therefore, *B. bifidum* species is a frequent member of the intestinal bifidobacterial population in healthy adults.

GENOMICS INSIGHTS INTO THE *B. bifidum* TAXON

Due to the availability of novel whole-genome sequencing approaches, research in molecular microbiology, in particular that related to pathogens, has undergone dramatic changes during the last decade. In recent times, genome-decoding efforts have also been directed towards gut commensals and probiotic bacteria such as members of the genus *Bifidobacterium* (Schell et al., 2002; Lee et al., 2008; Sela et al., 2008; Barrangou et al., 2009; Turroni et al., 2010; Bottacini et al., 2011, 2012; O'Connell Motherway et al., 2011). In 2009, a genomics-based discipline, named probiogenomics, was established, which aims to provide insights into the diversity and evolution of beneficial gut commensals, and to reveal the molecular basis for their adaptation and interaction with the mammalian gut (Ventura et al., 2009a). Thanks to these probiogenomics efforts, we have significantly expanded our understanding of the biology of gut microorganisms, such as bifidobacteria, and we have generated a large amount of data on metabolic capabilities, genetics, and phylogeny of these bacteria. Within the genus *Bifidobacterium*, just eight members of the *B. bifidum* species have had their genome sequenced out of 23 currently publicly available complete bifidobacterial genome sequences (NCBI source). Notably, of these eight, only three genome sequences are complete, while the remaining five B. bifidum genome sequences are still fragmented in multiple contigs. The genome size of a *B. bifidum* taxon ranges from 2.14 to 2.28 Mb, whereas such a genome displays a GC content of about 62%, which is in line with the average values described for genomes of members of the genus *Bifidobacterium* (Ventura et al., 2007b, 2012; Turroni et al., 2013b). The currently NCBI-deposited reference genome of the *B. bifidum* species belongs to the infant stool isolate PRL2010 (Turroni et al., 2010), which was sequenced and published in 2010. The *B. bifidum* PRL2010 genome is also similar (89% identity at nucleotide level) to that of *B. longum* subsp. *infantis* ATCC15697 (Sela et al., 2008), even though they belong to two distinct bifidobacterial phylogenetic clusters, perhaps reflecting the fact that they share a common ecological niche (Ventura et al., 2007a). A functional classification of the genes present in the *B. bifidum* genomes according to the Cluster of Orthologous Genes (COG) families allowed the identification of a large proportion (>10%) of genes assigned to the COG family of carbohydrate metabolism and transport, including genes predicted to be involved in mucin metabolism (see below).

In silico analyses of the *B. bifidum* PRL2010 chromosome identified candidate genes displaying a deviant G+C content and for this reason possibly acquired through Horizontal Gene Transfer (HGT), collectively referred to as the mobilome. The predicted PRL2010 mobilome includes a prophage-like element Bbif-1 (Ventura et al., 2010) and two loci encompassing type I Restriction/Modification (R/M) systems as well as a type III R/M system (Turroni et al., 2010). Moreover, the predicted mobilome encompasses a 19 kb DNA region, which appears to represent an integrated plasmid.

The evaluation of genome variability within members of the *B. bifidum* species was assayed by Comparative Genomic Hybridization (CGH) experiments and the use of *B. bifidum* PRL2010-based microarrays (Turroni et al., 2010). In this way genes from the sequenced *B. bifidum* PRL2010 strain were assessed for their presence or absence in the genomes of a set of seven *B. bifidum* strains, which included isolates from various infant fecal samples as well as the neotype of *B. bifidum* species. Interestingly, among the variable regions of the CGH map there are those

predicted as the mobilome of PRL2010 as well as other genes predicted to be involved in bacterium–environment interaction, such as the genes specifying sortase-dependent pili (Turroni et al., 2010). In addition, analysis of the *B. bifidum* PRL2010 chromosome revealed novel insights into the metabolic strategies followed by this strain to degrade host-derived glycans, and in particular mucin-associated carbohydrates.

THE GENOMICS OF MUCIN BREAKDOWN IN *B. bifidum*

Mucin represents the main component of the mucus gel layer that is covering the epithelial surface of the GIT (Podolsky, 1985). The main carbohydrate monomers found in mucin include *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose, and galactose, which are sometimes linked to sialic acid and sulfate groups (Forstner et al., 1995). In addition, mucin contains salts, lipids, and many proteins, such as growth factors, lysozyme, defensins, immunoglobulins, trefoil factors, and several intestinal proteins (Johansson et al., 2008).

Among members of the genus *Bifidobacterium*, the ability to degrade mucin is a peculiar property of the *B. bifidum* species, which can hydrolyse the glycosydic bonds of mucin (Turroni et al., 2010, 2011) and utilize it as the sole carbon source (Guglielmetti et al., 2009).

In silico analyses of the genome sequences of PRL2010 revealed a relatively small set of genes dedicated to carbohydrate metabolism, which predominantly specified glycosyl hydrolases (GH), compared to other bifidobacteria (Turroni et al., 2011). Remarkably, about 60% of the identified GH-encoding enzymes from *B. bifidum* PRL2010 are predicted to be involved in the degradation of mucin-derived oligosaccharides, most of which are uniquely present in the *B. bifidum* chromosome relative to other currently available bifidobacterial genomes. Furthermore, according to the Carbohydrate Active Enzymes (CAZy) system (Cantarel et al., 2009), the *B. bifidum* PRL2010 genome is predicted to encode members of two carbohydrate-binding module (CBM) families, CBM32 and CBM51, that are suggested to bind to carbohydrate residues encountered in the mucin core structure. Notably, in bifidobacteria the genetic information corresponding to predicted CBM32 and CBM51 members were only detected in the genomes of *B. bifidum* (Turroni et al., 2011).

Additional insights into the behavior of *B. bifidum* PRL2010 to utilize mucin were obtained from functional genomics approaches, such as whole proteome profiling as well as transcriptomic investigations (Turroni et al., 2010). Several of the enzymes encoded by *B. bifidum* PRL2010 involved in mucin metabolism encompass extracellular enzymes, such as putative exo-α-sialidases, as well as a predicted 1,2-α-L-fucosidase and 1,3/4-α-L-fucosidase, and a putative cell wall-anchored endo-α-*N*-acetylgalactosaminidase (Ashida et al., 2008, 2009; Kiyohara et al., 2012). Additional PRL2010 encoded enzymes that are believed to be involved in mucin breakdown include four *N*-acetyl-β-hexosaminidases, and four β-galactosidases. The mucin-catabolic phenotype of PRL2010 is further facilitated by the presence of carbohydrate transporters belonging to various families, such as the ATP-binding cassette (ABC-type), phosphoenolpyruvate phosphotransferase system (PEP-PTS) and major facilitator superfamily (MFS).

The genome of *B. bifidum* PRL2010 encompasses a DNA region spanning eight genes, which encode enzymes for the breakdown of galacto-*N*-biose that forms one of the core structures of mucin-oligosaccharides. In this context it is believed that *B. bifidum* PRL2010 accesses mucin-derived galacto-*N*-biose by the action of extracellular enzymes like the exo-α-sialidases and 1,2-α-L-/α-1,3/4-fucosidases, which perform de-sialidation and de-fucosylation, respectively, of mucin-derived oligosaccharides to facilitate further breakdown by the action of other enzymes encoded by PRL2010 such as the lacto-*N*-biosidase and endo-α-*N*-acetylgalactosaminidase.

Comparative genomics analyses involving all other currently available *B. bifidum* genomes revealed a high conservation of the predicted genetic arsenal involved in mucin breakdown (Turroni et al., 2011). Thus, it is tempting to conclude that mucin metabolism in PRL2010 is a genetic feature of most if not all members of the *B. bifidum* species rather than of a unique strain. Mucin degradation, which is expected to reduce the mucin layer and consequently reduce the protective barrier covering the intestinal mucosa, is generally considered as an undesirable event. However, one may also consider breakdown as an evolved "host-settler mechanism." In fact, mucin production in the GIT normally initiates only several months after birth and reaches its mature level at about 12 months (Hooper et al., 1999). Interestingly, mucin breakdown activity as operated by *B. bifidum* could trigger the secretion of additional colonic mucin, thus increasing the thickness of the total amount of mucus layer covering the gut and so reinforcing the epithelial barrier function, which constitutes an important feature especially in those subjects affected by irritable bowel syndrome (Caballero-Franco et al., 2007). The capacity to efficiently use mucus is a typical feature also of *Akkermansia muciniphila*, a human intestinal species that has been associated with healthy intestines and disease prevention (Guglielmetti et al., 2008b).

PILI PRODUCTION BY *B. bifidum* AS KEY HOST–MICROBE EFFECTOR MOLECULES

Non-flagellar appendages decorating the microbial cell surface were identified in bacteria in the early 1950s and since then the molecular data about their assembly, composition, and function has greatly expanded, especially for pathogens (Telford et al., 2006). In this context, these extracellular structures are considered crucial in the initial establishment of pathogens inside the host and are consequently considered key effector molecules in pathogenesis. However, their identification in bifidobacteria was only very recently established (Foroni et al., 2011; O'Connell Motherway et al., 2011). It has been shown that bifidobacterial genomes belonging to *B. bifidum*, *B. longum* subsp. *longum*, *B. adolescentis*, *B. dentium*, *B. animalis* subsp. *Lactis*, and *B. breve* contain one to seven predicted sortase-dependent pilus gene loci, each of which are predicted to encode one major pilin subunit (represented by $FimA_{PRL2010}$ or $FimP_{PRL2010}$ for the $pil2_{PRL2010}$ and $pil3_{PRL2010}$ clusters, respectively) plus a minor pilin subunit (represented by $FimB_{PRL2010}$ and $FimQ_{PRL2010}$ for the $pil2_{PRL2010}$ and $pil3_{PRL2010}$ clusters, respectively), as well as a so-called sortase, a protein specifically dedicated to covalently assemble these pilin subunits (Foroni et al., 2011).

Very recently, the four *B. bifidum* genome sequences that are currently publicly available have been screened for sortase-dependent pili leading to the identification of three loci (Turroni et al., 2013a). Of these three identified loci, only two were shown to be genetically intact whereas the third appeared to be non-functional due to a frameshift within the coding region of the gene encoding major pilus subunit (Turroni et al., 2013a). When $FimA_{PRL2010}$ was compared to FimA homologs encoded by other *B. bifidum* strains, their amino acid sequences were shown to display much higher variability compared to the FimP homologs (Turroni et al., 2013a). Furthermore, $FimA_{PRL2010}$ includes a CnaB-type domain that is known to serve as a stalk in binding to components of the Extra Cellular Matrix proteins of the host, such as fibronectin, collagen types I to XV, and laminin (Deivanayagam et al., 2000).

Transcriptomic investigations performed on *B. bifidum* PRL2010 upon colonization of mice as well as upon contact with human cell lines, demonstrated a clear transcriptional up-regulation of those genes encompassing two sortase-dependent pili, named *pil2* and *pil3* (Turroni et al., 2013a). Heterologous expression of the pilus-encoding genes corresponding to Pil2 and Pil3 in the non-piliated, Gram-positive host *Lactococcus lactis* showed that both types of pili are modulating the adhesion to human enterocytes through extracellular matrix (ECM) proteins and bacterial aggregation. ECM deglycosylation provoked a dramatic reduction in PRL2010 pili-mediated binding ability compared to untreated ECM (Turroni et al., 2013a), pointing that N- and/or O-linked glycoproteins are involved in adhesion of PRL2010 pili to ECM. Furthermore, carbohydrate binding competition experiments demonstrated that mannose and fucose act as potential receptors for Pil2 of *B. bifidum* PRL2010 in a fashion that is reminiscent of that previously described for other enteric bacteria (Farfan et al., 2011), whereas the putative binding partners for Pil3 appear to encompass a larger set of carbohydrates (Turroni et al., 2013a).

Recombinant piliated *L. lactis* cells were also shown to evoke a higher tumor necrosis factor alpha (TNFα) response during murine colonization compared to their non-piliated parent, indicating that *B. bifidum* PRL2010 sortase-dependent pili not only contribute to adhesion but also display immunomodulatory activity (Turroni et al., 2013a). Triggering TNFα production by pili synthetized by *B. bifidum* PRL2010 may represent an intriguing feature of this species as one of the first colonizers of the human gut (Turroni et al., 2012). In this context, it is worth mentioning that cytokines belonging to the TNFα superfamily are not only linked to the occurrence of inflammatory diseases (Yasutake et al., 1999), but also exert a major role in the rejection of tumors and the response to infections (Wajant et al., 2003; Lebeer et al., 2010). Furthermore, the induction of TNFα may be crucial for the initiation of cross-talk among immune cells without provoking any inflammation or detrimental effects (Galdeano et al., 2007).

OTHER HOST RESPONSE EFFECTOR MOLECULES ENCODED BY *B. bifidum*

Recently, the murine lytic enzyme TgaA encoded by *B. bifidum* MIMBb75 has been molecularly characterized (Guglielmetti et al., 2014b). This peptidoglycan-degrading enzyme contains two active domains, i.e., a lytic murine transglycosylase and a cysteine histidine-dependent amidohydrolase/peptidase (CHAP) domain and was demonstrated to exert immunomodulatory effects (Guglielmetti et al., 2014a). The TgA-encoding gene does not appear to be widely distributed among the currently available *B. bifidum* genomes and thus represents an example of a strain-dependent gene (Guglielmetti et al., 2014b).

Other *B. bifidum* proteins involved in host interaction are represented by the surface lipoprotein BopA, which was originally described to be involved in adhesion to intestinal epithelium (Guglielmetti et al., 2008b; Gleinser et al., 2012). However, recently the role of BopA in the adhesion of *B. bifidum* was reassessed and, in contrast to what was published earlier, the strong adhesion of *B. bifidum* to epithelial cell lines is mainly BopA-independent (Kainulainen et al., 2013).

Within the surface proteins encoded by *B. bifidum* strains responsible of adhesion to the human intestine, the transaldolase Tal of *B. bifidum* A8 has been also proposed (Gonzalez-Rodriguez et al., 2012). Notably, such protein has been shown in modulating the adhesion to mucin as well as to promote bacterial aggregation, thus could act as a key colonization factor in driving the establishment of *B. bifidum* cells in the human gut.

IMMUNE RESPONSE OF *B. bifidum* STRAINS

Members of the *B. bifidum* species have been claimed to exert an important role in the evolution and maturation of the immune system of the host, which is still undeveloped at birth (Lopez et al., 2011). The interaction of *B. bifidum* with the host immune system has been assayed by investigating the impact of *B. bifidum* Z9 in combination with a second human gut commensal, *Lactobacillus acidophilus*, on the transcriptome of dendritic cells (DCs) (Weiss et al., 2010). This study highlighted that *B. bifidum* Z9 down-regulates the expression of genes involved to the adaptive immune system in murine DCs. Such findings corroborated other studies based on *in vitro* assays and involving various strains belonging to different *Bifidobacterium* species, which display a clear and distinct induction of cytokine profile by bifidobacteria. In particular, it was shown that *B. bifidum* strains, in contrast to representatives of other bifidobacterial species, provoked a significantly increased production of the IL-17 cytokine (Turroni et al., 2010; Lopez et al., 2011). The observation that *B. bifidum* strains induce an immune response affecting Treg/TH17 plasticity (Turroni et al., 2010; Lopez et al., 2011) leads to hypothesize that such commensal bacteria have a key role in mucosal tolerance, as also suggested by the demonstration that *B. bifidum*, differently from several other species of the genus, possesses the ability to induce IL-2 secretion by DCs (Guglielmetti et al., 2014a). In particular, it was demonstrated that the cell-surface-exposed molecule Tga of *B. bifidum* MIMBb75 is capable alone through its C-terminal CHAP domain of inducing DCs activation and IL-2 production (Guglielmetti et al., 2014b).

Recently, the host response triggered by the presence of *B. bifidum* PRL2010 cells was investigated thanks to a high-throughput gene expression technology and by utilizing both an *in vitro* cell line model as well as a murine model (Turroni et al., 2014a). Notably, the overall host-response scenario driven by *B.*

bifidum PRL2010 cells can be described as a pro-inflammatory response priming the immune system, yet at the same time attenuating the pro-inflammatory response by down-regulation of certain chemokines, heat shock proteins (HSP) as well as stimulating the up-regulation of defensin and tight junction genes. In addition, results from ELISA experiments displayed that exposure to *B. bifidum* PRL2010 triggers the synthesis of IL-6 and IL-8 cytokines, presumably through NF-κb activation (Turroni et al., 2014a).

Other *in vivo* observations involving *B. bifidum* strains have been performed under chemically-induced diseases such as colitis. In this context, administration of *B. bifidum* strain S17 to mice with colitis was shown to suppress intestinal inflammation with a significant reduction in histology scores and the levels of pro-inflammatory cytokines interleukin IL-1β, IL-6, keratinocyte-derived chemokine and the inflammatory markers cyclooxygenase and myeloperoxidase (Philippe et al., 2011).

B. bifidum STRAINS AS POTENTIAL HEALTH-PROMOTING CANDIDATES

Various strains of the *B. bifidum* species have been reported to exert health benefits to their human host, including antibacterial activities against pathogens such as *Helicobacter pylori* (Shirasawa et al., 2010; Chenoll et al., 2011), reduction of apoptosis in the intestinal epithelium of infants suffering from necrotizing enterocolitis (Khailova et al., 2010), modulation of the host-immune system (Fu et al., 2010; Philippe et al., 2011), and alleviation of anti-inflammatory activities associated with certain chronic large bowel dysfunctions (Mouni et al., 2009; Guglielmetti et al., 2011). In addition, *B. bifidum* together with other bifidobacterial species like *B. breve* and *B. longum* subsp. *infantis* are considered important for the establishment of a well-balanced, autochthonous intestinal microbiota in newborns (Tabbers et al., 2011). However, in order to exert a potential health-promoting activity in the human gut, bacteria need to reach this compartment in a viable form, while they should also be able to persist within the intestine. In this context, many *B. bifidum* strains have been shown to possess a strong adhesion phenotype to human epithelial intestinal cell monolayers (Caco-2 and HT29) (Guglielmetti et al., 2009; Serafini et al., 2013) and in a few cases, such as for *B. bifidum* PRL2010 and MIMBb75, were demonstrated to survive under gastrointestinal challenges (Serafini et al., 2013) and colonize the intestine impacting on the resident microbial communities at various intestinal loci (Singh et al., 2013). Another interesting phenotype displayed by probiotic bacteria is their displacement and competition against pathogens. Interestingly, *in vitro* trials based on HT29 monolayer involving *B. bifidum* PRL2010 cells displayed a clear inhibition of adhesion of pathogenic bacteria such as *Escherichia coli* and *Cronobacter sakazakii* (Serafini et al., 2013).

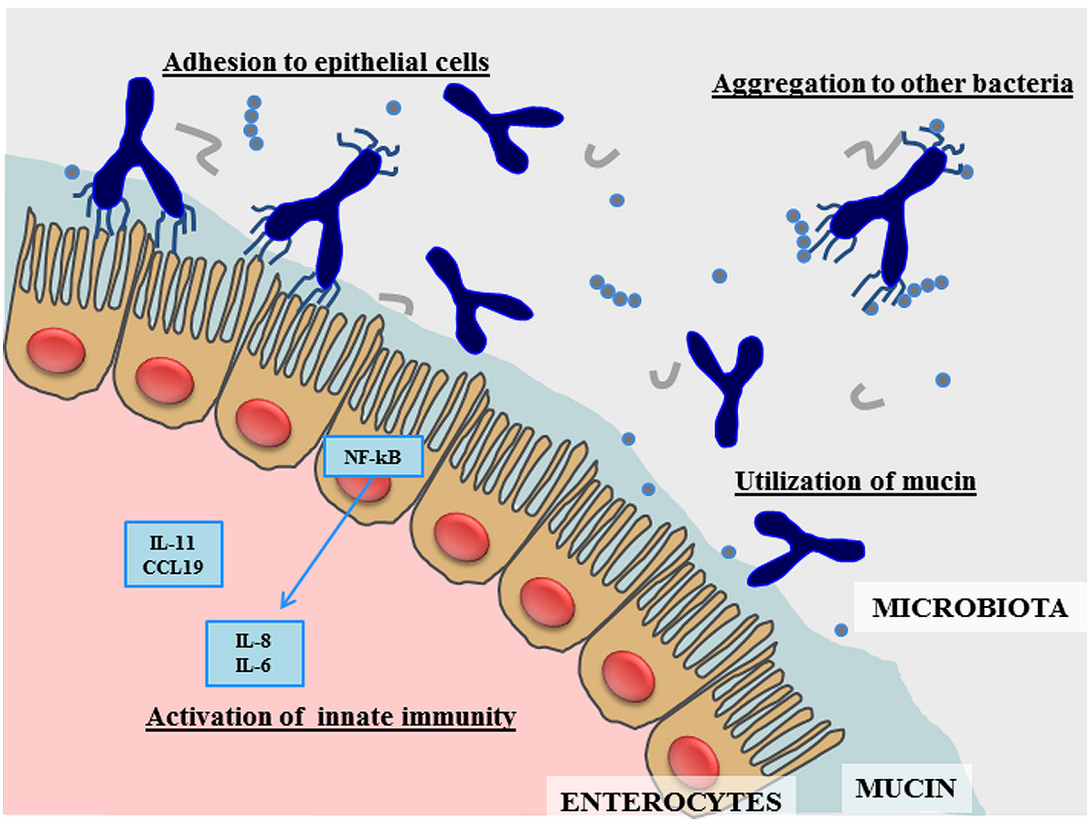


FIGURE 1 | Schematic representation of the main effects produced by *Bifidobacterium bifidum* in the human gut.

A health-promoting microorganism needs to be administered to its human host within a specific matrix in order to assure survival and biological functionality of its cells (e.g., producing probiotic molecules). Recently, kefir and kefiran were shown to affect the transcriptome of *B. bifidum* PRL2010 causing increased transcription of genes involved in the metabolism of dietary glycans as well as genes acting as host–microbe effector molecules such as pili (Serafini et al., 2014). Thus, the use of kefir and perhaps other (fermented) food products may be considered as a valuable means for the administration of *B. bifidum* cells to humans and may represent an effective food matrix to pre-adapt bifidobacterial cells to the host in order to enhance probiotic efficacy.

INDUSTRIAL USE OF *B. bifidum*

Due to their health-promoting activities, much effort has been invested in the incorporation of bifidobacteria into probiotic food, supplements, and pharmaceutical preparations. By regulatory definition, microbial cells must be alive in a sufficient number in order to define a product as probiotic. In this perspective, the commercial use of *B. bifidum* as a probiotic has been limited by the fact that members of this species are particularly sensitive to stresses such as acidity and, in particular, oxygen (Jayamanne and Adams, 2006). Strategies to preserve probiotic cell viability are available and involve, for instance, microencapsulation (Zhang et al., 2013), and the addition of prebiotic molecules to the formulation (Guglielmetti et al., 2008a). However, these strategies do not overcome the problem of the limited biomass yields generally obtained in industrial fermentations due to the intrinsic stress sensitivity of *B. bifidum*, resulting in a strong increase of production costs. In this context, an aspect of particular importance is represented by strain "domestication." In fact, once a bifidobacterial strain is isolated from its natural environment, its intrinsic sensitivity to oxygen decreases slowly and progressively during the numerous subcultivations under laboratory conditions. In this direction, an example is represented by strain *B. bifidum* MIMBb75, which was isolated about twelve years ago from a fecal sample of a healthy adult. Although originally very recalcitrant to laboratory cultivation, after hundreds of subculturings in aerobic atmosphere followed by incubation in a gas-pack with Anaerocult A, this strain drastically improved its ability to resist oxidative and other laboratory stresses (Guglielmetti S., personal communication). Thus, the selective pressure of a laboratory environment induced physiological changes that allowed the employment of strain *B. bifidum* MIMBb75 at industrial level; this strain, in fact, is now commercially available in a pharmaceutical probiotic product consisting of an encapsulated formulation, in which MIMBb75 cells can maintain a viability of at least 10^9 CFU per capsule during the entire shelf-life of the product (Guglielmetti et al., 2011; Guglielmetti S., personal communication).

CONCLUSION

During the last 5–10 years research in bifidobacteria has blossomed (Ventura et al., 2012, 2014; Turroni et al., 2014b). In this context, genomic investigations of bifidobacteria have revealed genetic repertoires that are considered crucial for conveying the typical saccharolytic phenotype of these bacteria, and which are worthy of detailed investigation for their potential roles in colonization of the human gut and their dependence on our diet. However, the discovery of the specific functional contribution to the host by each member of the bifidobacterial population in the human gut is still at its infancy. Furthermore, very little is known about the cross-talk that is believed to occur between individual members of the bifidobacterial microbiota (Egan et al., 2014) and with other members of the gut microbiota (Sonnenburg et al., 2006). The genetic data retrieved from the genome analysis of *B. bifidum* suggests the existence of various molecules that are responsible for specific health-promoting activities exerted by members of this bifidobacterial taxon (**Figure 1**). Future work needs to be carried out in order to better understand how these genetic features are exploited in the human gut through the use of high throughput metagenomic and post-genomic approaches. Nonetheless, currently available experimental data already supports the notion that *B. bifidum* represents a highly interesting bacterial species that is able to benefit human health in the prevention and treatment of gastrointestinal dysfunctions.

ACKNOWLEDGMENTS

We thank GenProbio srl for the financial support of the Laboratory of Probiogenomics. This publication has emanated from research conducted with the financial support of Science Foundation Ireland (SFI) under Grant Numbers 07/CE/B1368 and SFI/12/RC/2273.

REFERENCES

Ashida, H., Maki, R., Ozawa, H., Tani, Y., Kiyohara, M., Fujita, M., et al. (2008). Characterization of two different endo-alpha-N-acetylgalactosaminidases from probiotic and pathogenic enterobacteria, *Bifidobacterium longum* and *Clostridium perfringens*. *Glycobiology* 18, 727–734. doi: 10.1093/glycob/cwn053

Ashida, H., Miyake, A., Kiyohara, M., Wada, J., Yoshida, E., Kumagai, H., et al. (2009). Two distinct alpha-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. *Glycobiology* 19, 1010–1017. doi: 10.1093/glycob/cwp082

Barrangou, R., Briczinski, E. P., Traeger, L. L., Loquasto, J. R., Richards, M., Horvath, P., et al. (2009). Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. lactis DSM 10140 and Bl-04. *J. Bacteriol.* 191, 4144–4151. doi: 10.1128/JB.00155-09

Bottacini, F., Dal Bello, F., Turroni, F., Milani, C., Duranti, S., Foroni, E., et al. (2011). Complete genome sequence of *Bifidobacterium animalis* subsp. lactis BLC1. *J. Bacteriol.* 193, 6387–6388. doi: 10.1128/JB.06079-11

Bottacini, F., Milani, C., Turroni, F., Sanchez, B., Foroni, E., Duranti, S., et al. (2012). *Bifidobacterium asteroides* PRL2011 genome analysis reveals clues for colonization of the insect gut. *PLoS ONE* 7:e44229. doi: 10.1371/journal.pone.0044229

Buchanan, R. E. (2009). *Bergey's Manual of Systematic Bacteriology*, ed. N. E. Gibbons. New York: Springer.

Caballero-Franco, C., Keller, K., De Simone, C., and Chadee, K. (2007). The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292, G315–G322. doi: 10.1152/ajpgi.00265.2006

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238. doi: 10.1093/nar/gkn663

Chenoll, E., Casinos, B., Bataller, E., Astals, P., Echevarria, J., Iglesias, J. R., et al. (2011). Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. *Appl. Environ. Microbiol.* 77, 1335–1343. doi: 10.1128/AEM.01820-10

Deivanayagam, C. C., Rich, R. L., Carson, M., Owens, R. T., Danthuluri, S., Bice, T., et al. (2000). Novel fold and assembly of the repetitive B region of the *Staphylococcus aureus* collagen-binding surface protein. *Structure* 8, 67–78. doi: 10.1016/S0969-2126(00)00081-2

Delcenserie, V., Taminiau, B., Gavini, F., De Schaetzen, M. A., Cleenwerck, I., Theves, M., et al. (2013). Detection and characterization of *Bifidobacterium crudilactis* and *B. mongoliense* able to grow during the manufacturing process of French raw milk cheeses. *BMC Microbiol.* 13:239. doi: 10.1186/1471-2180-13-239

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi: 10.1126/science.1110591

Egan, M., O'connell Motherway, M., Ventura, M., and Van Sinderen, D. (2014). Metabolism of sialic acid by *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 80, 4414–4426. doi: 10.1128/AEM.01114-14

Farfan, M. J., Cantero, L., Vidal, R., Botkin, D. J., and Torres, A. G. (2011). Long polar fimbriae of enterohemorrhagic *Escherichia coli* O157:H7 bind to extracellular matrix proteins. *Infect. Immun.* 79, 3744–3750. doi: 10.1128/IAI.05317-11

Foroni, E., Serafini, F., Amidani, D., Turroni, F., He, F., Bottacini, F., et al. (2011). Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb. Cell Fact.* 10(Suppl. 1), S16. doi: 10.1186/1475-2859-10-S1-S16

Forstner, J. F., Oliver., M. G., and Sylvester, F. A. (1995). *Production, Structure and Biologic Relevance of Gastrointestinal Mucins.* New York: Raven Press.

Fu, Y. R., Yi, Z. J., Pei, J. L., and Guan, S. (2010). Effects of *Bifidobacterium bifidum* on adaptive immune senescence in aging mice. *Microbiol. Immunol.* 54, 578–583. doi: 10.1111/j.1348-0421.2010.00255.x

Galdeano, C. M., De Moreno De Leblanc, A., Vinderola, G., Bonet, M. E., and Perdigon, G. (2007). Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin. Vaccine Immunol.* 14, 485–492. doi: 10.1128/CVI.00406-06

Gleinser, M., Grimm, V., Zhurina, D., Yuan, J., and Riedel, C. U. (2012). Improved adhesive properties of recombinant bifidobacteria expressing the *Bifidobacterium bifidum*-specific lipoprotein BopA. *Microb. Cell Fact.* 11, 80. doi: 10.1186/1475-2859-11-80s

Gonzalez-Rodriguez, I., Sanchez, B., Ruiz, L., Turroni, F., Ventura, M., Ruas-Madiedo, P., et al. (2012). Role of extracellular transaldolase from *Bifidobacterium bifidum* in mucin adhesion and aggregation. *Appl. Environ. Microbiol.* 78, 3992–3998. doi: 10.1128/AEM.08024-11

Guglielmetti, S., Balzaretti, S., Taverniti, V., Miriani, M., Milani, C., Scarafoni, A., et al. (2014a). Characterization of TgaA, a VirB1-like component belonging to a putative type IV secretion system of *Bifidobacterium bifidum* MIMBb75. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.01413-14 [Epub ahead of print].

Guglielmetti, S., Zanoni, I., Balzaretti, S., Miriani, M., Taverniti, V., De Noni, I., et al. (2014b). The murein lytic enzyme TgaA of *Bifidobacterium bifidum* MIMBb75 modulates dendritic cell maturation through its CHAP amidase domain. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.00761-14 [Epub ahead of print].

Guglielmetti, S., Ciranna, A., Mora, D., Parini, C., and Karp, M. (2008a). Construction, characterization and exemplificative application of bioluminescent *Bifidobacterium longum* biovar longum. *Int. J. Food Microbiol.* 124, 285–290. doi: 10.1016/j.ijfoodmicro.2008.03.033

Guglielmetti, S., Tamagnini, I., Mora, D., Minuzzo, M., Scarafoni, A., Arioli, S., et al. (2008b). Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. *Appl. Environ. Microbiol.* 74, 4695–4702. doi: 10.1128/AEM.00124-08

Guglielmetti, S., Fracassetti, D., Taverniti, V., Del Bo, C., Vendrame, S., Klimis-Zacas, D., et al. (2013). Differential modulation of human intestinal *Bifidobacterium* populations after consumption of a wild blueberry (*Vaccinium angustifolium*) drink. *J. Agric. Food Chem.* 61, 8134–8140. doi: 10.1021/jf402495k

Guglielmetti, S., Mora, D., Gschwender, M., and Popp, K. (2011). Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life – a double-blind, placebo-controlled study. *Aliment. Pharmacol. Ther.* 33, 1123–1132. doi: 10.1111/j.1365-2036.2011.04633.x

Guglielmetti, S., Tamagnini, I., Minuzzo, M., Arioli, S., Parini, C., Comelli, E., et al. (2009). Study of the adhesion of *Bifidobacterium bifidum* MIMBb75 to human intestinal cell lines. *Curr. Microbiol.* 59, 167–172. doi: 10.1007/s00284-009-9415-x

Harmsen, H. J., Wildeboer-Veloo, A. C., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., et al. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J. Pediatr. Gastroenterol. Nutr.* 30, 61–67. doi: 10.1097/00005176-200001000-00019

Hooper, L. V., Xu, J., Falk, P. G., Midtvedt, T., and Gordon, J. I. (1999). A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9833–9838. doi: 10.1073/pnas.96.17.9833

Jayamanne, V. S., and Adams, M. R. (2006). Determination of survival, identity and stress resistance of probiotic bifidobacteria in bio-yoghurts. *Lett. Appl. Microbiol.* 42, 189–194. doi: 10.1111/j.1472-765X.2006.01843.x

Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L., and Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15064–15069. doi: 10.1073/pnas.0803124105

Kainulainen, V., Reunanen, J., Hiippala, K., Guglielmetti, S., Vesterlund, S., Palva, A., et al. (2013). BopA does not have a major role in the adhesion of *Bifidobacterium bifidum* to intestinal epithelial cells, extracellular matrix proteins, and mucus. *Appl. Environ. Microbiol.* 79, 6989–6997. doi: 10.1128/AEM.01993-13

Khailova, L., Mount Patrick, S. K., Arganbright, K. M., Halpern, M. D., Kinouchi, T., and Dvorak, B. (2010). *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 299, G1118–G1127. doi: 10.1152/ajpgi.00131.2010

Kiyohara, M., Nakatomi, T., Kurihara, S., Fushinobu, S., Suzuki, H., Tanaka, T., et al. (2012). Alpha-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

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., et al. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proc. Natl. Acad. Sci. U.S.A.* 108(Suppl. 1), 4578–4585. doi: 10.1073/pnas.1000081107

Lamendella, R., Santo Domingo, J. W., Kelty, C., and Oerther, D. B. (2008). Bifidobacteria in feces and environmental waters. *Appl. Environ. Microbiol.* 74, 575–584. doi: 10.1128/AEM.01221-07

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat. Rev. Microbiol.* 8, 171–184. doi: 10.1038/nrmicro2297

Lee, J. H., Karamychev, V. N., Kozyavkin, S. A., Mills, D., Pavlov, A. R., Pavlova, N. V., et al. (2008). Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. *BMC Genomics* 9:247. doi: 10.1186/1471-2164-9-247

Lopez, P., Gonzalez-Rodriguez, I., Gueimonde, M., Margolles, A., and Suarez, A. (2011). Immune response to *Bifidobacterium bifidum* strains support Treg/Th17 plasticity. *PLoS ONE* 6:e24776. doi: 10.1371/journal.pone.0024776

Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G. A., 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

Mouni, F., Aissi, E., Hernandez, J., Gorocica, P., Bouquelet, S., Zenteno, E., et al. (2009). Effect of *Bifidobacterium bifidum* DSM 20082 cytoplasmic fraction on human immune cells. *Immunol. Invest.* 38, 104–115. doi: 10.1080/08820130802608303

Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., et al. (2006). Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl. Environ. Microbiol.* 72, 1027–1033. doi: 10.1128/AEM.72.2.1027-1033.2006

O'Connell Motherway, M., Zomer, A., Leahy, S. C., Reunanen, J., Bottacini, F., Claesson, M. J., et al. (2011). Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11217–11222. doi: 10.1073/pnas.1105380108

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biol.* 5:e177. doi: 10.1371/journal.pbio.0050177

Philippe, D., Heupel, E., Blum-Sperisen, S., and Riedel, C. U. (2011). Treatment with *Bifidobacterium bifidum* 17 partially protects mice from Th1-driven inflammation in a chemically induced model of colitis. *Int. J. Food Microbiol.* 149, 45–49. doi: 10.1016/j.ijfoodmicro.2010.12.020

Podolsky, D. K. (1985). Oligosaccharide structures of isolated human colonic mucin species. *J. Biol. Chem.* 260, 15510–15515.

Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L., and Mccartney, A. L. (2010). Examination of faecal *Bifidobacterium populations* in breast- and formula-fed infants during the first 18 months of life. *Microbiology* 156, 3329–3341. doi: 10.1099/mic.0.043224-0

Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., et al. (2002). The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14422–14427. doi: 10.1073/pnas.212527599212527599

Sela, D. A., Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., et al. (2008). The genome sequence of *Bifidobacterium longum* subsp. infantis reveals adaptations for milk utilization within the infant microbiome. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18964–18969. doi: 10.1073/pnas.0809584105

Serafini, F., Strati, F., Ruas-Madiedo, P., Turroni, F., Foroni, E., Duranti, S., et al. (2013). Evaluation of adhesion properties and antibacterial activities of the infant gut commensal *Bifidobacterium bifidum* PRL2010. *Anaerobe* 21, 9–17. doi: 10.1016/j.anaerobe.2013.03.003

Serafini, F., Turroni, F., Ruas-Madiedo, P., Lugli, G. A., Milani, C., Duranti, S., et al. (2014). Kefir fermented milk and kefiran promote growth of *Bifidobacterium bifidum* PRL2010 and modulate its gene expression. *Int. J. Food Microbiol.* 178, 50–59. doi: 10.1016/j.ijfoodmicro.2014.02.024

Shirasawa, Y., Shibahara-Sone, H., Iino, T., and Ishikawa, F. (2010). *Bifidobacterium bifidum* BF-1 suppresses *Helicobacter pylori*-induced genes in human epithelial cells. *J. Dairy Sci.* 93, 4526–4534. doi: 10.3168/jds.2010-3274

Singh, N., Arioli, S., Wang, A., Villa, C. R., Jahani, R., Song, Y. S., et al. (2013). Impact of *Bifidobacterium bifidum* MIMBb75 on mouse intestinal microorganisms. *FEMS Microbiol. Ecol.* 85, 369–375. doi: 10.1111/1574-6941.12124

Sonnenburg, J. L., Chen, C. T., and Gordon, J. I. (2006). Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *PLoS Biol.* 4:e413. doi: 10.1371/journal.pbio.0040413

Stackebrandt, E., and Tindall, B. J. (2000). Appreciating microbial diversity: rediscovering the importance of isolation and characterization of microorganisms. *Environ. Microbiol.* 2, 9–10.

Tabbers, M. M., De Milliano, I., Roseboom, M. G., and Benninga, M. A. (2011). Is *Bifidobacterium breve* effective in the treatment of childhood constipation? Results from a pilot study. *Nutr. J.* 10:19. doi: 10.1186/1475-2891-10-19

Telford, J. L., Barocchi, M. A., Margarit, I., Rappuoli, R., and Grandi, G. (2006). Pili in Gram-positive pathogens. *Nat. Rev. Microbiol.* 4, 509–519. doi: 10.1038/nrmicro1443

Tissier. (1900). *Recherchers Sur La Flora Intestinale Normale et Pathologique Du Nourisson*. Ph.D. thesis, University of Paris, Paris.

Turroni, F., Bottacini, F., Foroni, E., Mulder, I., Kim, J. H., 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., Foroni, E., Pizzetti, P., Giubellini, V., Ribbera, A., Merusi, P., et al. (2009a). Exploring the diversity of the bifidobacterial population in the human intestinal tract. *Appl. Environ. Microbiol.* 75, 1534–1545. doi: 10.1128/AEM.02216-08

Turroni, F., Marchesi, J. R., Foroni, E., Gueimonde, M., Shanahan, F., Margolles, A., et al. (2009b). Microbiomic analysis of the bifidobacterial population in the human distal gut. *ISME J.* 3, 745–751. doi: 10.1038/ismej.2009.19

Turroni, F., Milani, C., Van Sinderen, D., and Ventura, M. (2011). Genetic strategies for mucin metabolism in *Bifidobacterium bifidum* PRL2010: an example of possible human–microbe co-evolution. *Gut Microbes* 2, 183–189. doi: 10.4161/gmic.2.3.16105

Turroni, F., Peano, C., Pass, D. A., Foroni, E., Severgnini, M., Claesson, M. J., et al. (2012). Diversity of bifidobacteria within the infant gut microbiota. *PLoS ONE* 7:e36957. doi: 10.1371/journal.pone.0036957

Turroni, F., Serafini, F., Foroni, E., Duranti, S., O'connell Motherway, M., Taverniti, V., et al. (2013a). Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium–host interactions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11151–11156. doi: 10.1073/pnas.1303897110

Turroni, F., Ventura, M., Butto, L. F., Duranti, S., O'toole, P. W., Motherway, M. O., et al. (2013b). Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. *Cell. Mol. Life Sci.* 71, 183–203. doi: 10.1007/s00018-013-1318-0

Turroni, F., Taverniti, V., Ruas-Madiedo, P., Duranti, S., Guglielmetti, S., Lugli, G. A., et al. (2014a). *Bifidobacterium bifidum* PRL2010 modulates the host innate immune response. *Appl. Environ. Microbiol.* 80, 730–740. doi: 10.1128/AEM.03313-13

Turroni, F., Ventura, M., Butto, L. F., Duranti, S., O'Toole, P. W., Motherway, M. O., et al. (2014b). Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. *Cell. Mol. Life. Sci.* 71, 183–203. doi: 10.1007/s00018-013-1318-0

Ventura, M., Canchaya, C., Fitzgerald, G. F., Gupta, R. S., and Van Sinderen, D. (2007a). Genomics as a means to understand bacterial phylogeny and ecological adaptation: the case of bifidobacteria. *Antonie Van Leeuwenhoek* 91, 351–372. doi: 10.1007/s10482-006-9122-6

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., et al. (2007b). Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbiol. Mol. Biol. Rev.* 71, 495–548. doi: 10.1128/MMBR.00005-07

Ventura, M., O'flaherty, S., Claesson, M. J., Turroni, F., Klaenhammer, T. R., Van Sinderen, D., et al. (2009a). Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat. Rev. Microbiol.* 7, 61–71. doi: 10.1038/nrmicro2047

Ventura, M., Turroni, F., Canchaya, C., Vaughan, E. E., O'toole, P. W., and Van Sinderen, D. (2009b). Microbial diversity in the human intestine and novel insights from metagenomics. *Front. Biosci. (Landmark Ed.)* 14:3214–3221. doi: 10.2741/3445

Ventura, M., Turroni, F., Foroni, E., Duranti, S., Giubellini, V., Bottacini, F., et al. (2010). Analyses of bifidobacterial prophage-like sequences. *Antonie Van Leeuwenhoek* 98, 39–50. doi: 10.1007/s10482-010-9426-4

Ventura, M., Turroni, F., Lugli, G. A., and Van Sinderen, D. (2014). Bifidobacteria and humans: our special friends, from ecological to genomics perspectives. *J. Sci. Food Agric.* 94, 163–168. doi: 10.1002/jsfa.6356

Ventura, M., Turroni, F., Motherway, M. O., Macsharry, J., and Van Sinderen, D. (2012). Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends Microbiol.* 20, 467–476. doi: 10.1016/j.tim.2012.07.002

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death Differ.* 10, 45–65. doi: 10.1038/sj.cdd.4401189

Weiss, G., Rasmussen, S., Nielsen Fink, L., Jarmer, H., Nohr Nielsen, B., and Frokiaer, H. (2010). Bifidobacterium bifidum actively changes the gene expression profile induced by *Lactobacillus acidophilus* in murine dendritic cells. *PLoS ONE* 5:e11065. doi: 10.1371/journal.pone.0011065

Yasutake, N., Matsuzaki, T., Kimura, K., Hashimoto, S., Yokokura, T., and Yoshikai, Y. (1999). The role of tumor necrosis factor (TNF)-alpha in the antitumor effect of intrapleural injection of *Lactobacillus casei* strain Shirota in mice. *Med. Microbiol. Immunol.* 188, 9–14. doi: 10.1007/s004300050099

Zhang, F., Li, X. Y., Park, H. J., and Zhao, M. (2013). Effect of microencapsulation methods on the survival of freeze-dried *Bifidobacterium bifidum*. *J. Microencapsul.* 30, 511–518. doi: 10.3109/02652048.2012.758178

Conflict of Interest Statement: 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.

Received: 12 June 2014; accepted: 01 August 2014; published online: 21 August 2014.
*Citation: Turroni F, Duranti S, Bottacini F, Guglielmetti S, Van Sinderen D and Ventura M (2014) Bifidobacterium bifidum as an example of a specialized human gut commensal. Front. Microbiol. **5**:437. doi: 10.3389/fmicb.2014.00437*
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 17 February 2015
doi: 10.3389/fmicb.2015.00058

Discovering probiotic microorganisms: *in vitro*, *in vivo*, genetic and omics approaches

***Konstantinos Papadimitriou*[1]*†, *Georgia Zoumpopoulou*[1†], *Benoit Foligné*[2†], *Voula Alexandraki*[1], *Maria Kazou*[1], *Bruno Pot*[2‡] *and Effie Tsakalidou*[1‡]**

[1] *Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece*
[2] *Bactéries Lactiques et Immunité des Muqueuses, Institut Pasteur de Lille, Centre d'Infection et d'Immunité de Lille, Université Lille Nord de France, CNRS UMR8204, Lille, France*

Edited by:
Maria De Angelis, University of Bari Aldo Moro, Italy

Reviewed by:
Marcela Carina Audisio, Instituto de Investigaciones Para la Industria Química – Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina
Giorgio Giraffa, Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie, Italy

***Correspondence:**
Konstantinos Papadimitriou, Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens, Iera Odos 75, Athens 118 55, Greece
e-mail: kpapadimitriou@aua.gr

†These authors have contributed equally to this work.
‡These authors have supervised equally this work.

Over the past decades the food industry has been revolutionized toward the production of functional foods due to an increasing awareness of the consumers on the positive role of food in wellbeing and health. By definition probiotic foods must contain live microorganisms in adequate amounts so as to be beneficial for the consumer's health. There are numerous probiotic foods marketed today and many probiotic strains are commercially available. However, the question that arises is how to determine the real probiotic potential of microorganisms. This is becoming increasingly important, as even a superficial search of the relevant literature reveals that the number of proclaimed probiotics is growing fast. While the vast majority of probiotic microorganisms are food-related or commensal bacteria that are often regarded as safe, probiotics from other sources are increasingly being reported raising possible regulatory and safety issues. Potential probiotics are selected after *in vitro* or *in vivo* assays by evaluating simple traits such as resistance to the acidic conditions of the stomach or bile resistance, or by assessing their impact on complicated host functions such as immune development, metabolic function or gut–brain interaction. While final human clinical trials are considered mandatory for communicating health benefits, rather few strains with positive studies have been able to convince legal authorities with these health claims. Consequently, concern has been raised about the validity of the workflows currently used to characterize probiotics. In this review we will present an overview of the most common assays employed in screening for probiotics, highlighting the potential strengths and limitations of these approaches. Furthermore, we will focus on how the advent of omics technologies has reshaped our understanding of the biology of probiotics, allowing the exploration of novel routes for screening and studying such microorganisms.

Keywords: probiotics, screening, mechanism, *in vitro* model, *in vivo* model, omics, molecular marker, health claim

INTRODUCTION

Probiotic research faces new challenges. Currently, there is an increased legislative pressure, in both the EU and the USA, to strictly limit the health communications of probiotics. While a more strict regulation is of course not a major problem as such, it may considerably hamper the release of new probiotic strains and applications. This is especially worrisome, as the boost of the metagenomics research efforts starts to pay off by creating numerous new and interesting working hypothesis for microbiota manipulation in maintaining and restoring health. Results of this research, for the first time, allow to avoid the tedious screening of large numbers of strains and to identify potential new health promoting bacteria from the comparison of population with different health status (lean versus obese, allergic versus non-allergic, etc.). Several new applications have been suggested already either via a supplemented diet or via a pharmaceutical approach. Improving *Faecalibacterium prausnitzii* levels has been suggested as beneficial for inflammatory bowel disease (IBD) patients (Miquel et al., 2013), the use of *Akkermansia muciniphila* has recently been patented for treating metabolic disorders (Cani et al., 2014) and the role of dietary bioactive proteins and peptides in autism spectrum disorders may also result in new probiotic strains in the market (Siniscalco and Antonucci, 2013). The legislative framework today is not ready to cope with these new applications. Approval will require a full analysis of the mechanism of action. A full inventory of the risks will have to be determined in different populations, at different doses and using different delivery modes and matrices. The research approaches presented in this review aim to assist in this process. While many of the *in vitro* models described here may seem outdated, they are still used for cost and ethical reasons. The use of new molecular omics-based technologies is increasing fast and it will most probably replace traditional screening methods. Omics technologies may also turn out to be very effective in the follow-up analysis of probiotic candidate strains resulting from *in vitro* and/or *in vivo* screening with current methodologies. Genome sequencing, as an example, will allow to quickly detect and eliminate strains that pose a potential risk, through the presence of antibiotic resistance or virulence genes. The new research approaches will also facilitate the analysis and description of functional mechanisms,

facilitating the construction of health claim or pharmaceutical dossiers. Another consequence of this focus on mechanisms might be that live microorganisms will no longer be necessary, but they will be replaced by the active ingredients or metabolites identified as the active compound. This might cause a shift for certain applications from the food to the pharma area. However, it remains to be shown if this shift will also result in active ingredients that have no side effects, as it is currently expected from probiotics. The use of the models and research strategies described in this review, alone or in combination, may help to answer that type of questions.

DOCUMENTING PROBIOTICS WITH *IN VITRO* ASSAYS

SURVIVING STRESS WITHIN THE HOST

Since the early days of probiotics research, *in vitro* screening of probiotics was a preferable choice due to the simplicity and the low cost of such approaches (**Table 1**). Even though some of these tests may seem outdated they are still in use and they can be found in recent reports. Perhaps the most important advantage of *in vitro* assays is their ability to screen multiple strains simultaneously.

According to current definitions, probiotics should be viable, even though sometimes dead microbial cells can also exert health benefits (Salminen et al., 1999). It is also recommended that probiotics must be able to reach the desired body niches alive. An initial screening of strains based on various stress tolerance assays is of utmost importance (Upadrasta et al., 2011), especially for non-encapsulated strains directly used in food. Hence, appropriate *in vitro* tests have been adopted to select strains based on their ability to survive transit through the different compartments of the gastrointestinal tract (GIT; Joint FAO/WHO Working Group, 2002).

Survival of potential probiotic bacteria under simulated GIT conditions has been extensively studied over the last decades and strain-specific differences are marked throughout the literature. Following ingestion, probiotics first encounter the harsh conditions of the stomach and they must be able to survive under the extreme acidic conditions and the activities of the digestive enzymes. The pH of the stomach is known to fluctuate from 1–2 up to 4–5 after food consumption but most *in vitro* assays have been developed to select strains that withstand extreme low pH values. Most conventional methodologies include experiments studying the survival of strains in buffers with no nutrients like PBS or modified growth media, all adjusted to low pH. Similar experiments have not been reported for high values of pH, mimicking the slightly alkaline conditions of the small intestine, perhaps reflecting the notion that most probiotic strains are resistant to alkaline conditions. Acid tolerance tests are among the simplest tests that can be performed, allowing the routine screening of large numbers of strains. However, given the unrealistic harsh pH conditions employed during these tests, they may result in the loss of relatively acid sensitive probiotic candidates. For example, acid sensitive strains could be protected from the acid challenge of the stomach due to the buffering properties of food vehicles or specific food ingredients. Furthermore, the strains are most often challenged as pure cultures in either log or stationary phase, while in reality, probiotics are delivered to the host already stressed due to extended fermentation periods, food processing conditions, and storage. This pre-stressed state of probiotics may lead either to enhanced or diminished stress resistance during passage through the host, a property that may be species or even strain dependent.

Except for these simplified survival tests, artificial gastric as well as pancreatic juices have been developed to better represent the *in vivo* conditions (Charteris et al., 1998). The survival in true gastric juice obtained from human individuals has been reported (Conway et al., 1987). Generally, synthetic gastric and pancreatic juices include the enzymes pepsin and pancreatin, respectively, and controlled incubation of strains in these juices have been investigated to mimic the time spent by probiotics in the upper and the lower GIT (Lavermicocca et al., 2008). Bile secreted in the small intestine reduces the survival of bacteria by disrupting the cell membrane, by inducing protein misfolding and denaturation and by damaging DNA. Bile salt hydrolase (BSH) is an enzyme that hydrolyses the amino acids of conjugated bile salts (glycine or taurine), reducing their toxicity. Tolerance to bile salt concentrations between 0.15 and 0.5% has been recommended for probiotics, which is in the range of the physiological concentrations met in the GIT (Gorbach and Goldin, 1992). Again, bile tolerance assays may be easy to perform, but they may not particularly facilitate the reliable selection of probiotics, for several reasons. For example, in most cases strains are separately studied for acid or bile tolerance, despite the fact that these two stresses are actually sequential during passage through the GIT, increasing the stress pressure. The use of non-human bile may also raise some questions, as bovine or porcine bile do not have the same impact on microorganisms as human bile (Begley et al., 2005).

The need for more elaborate *in vitro* assays for testing the fate of probiotic strains in the GIT led to the development of several GIT simulators. More precisely, a multi-compartmental dynamic computer-controlled model simulating the stomach and the small intestine (Minekus et al., 1999) has been used to quantify the survival of lactic acid bacteria (LAB) and the data obtained correlated well with those obtained from human subjects (Marteau et al., 1997). In other cases, *in vitro* systems reproduce not only the conditions of the stomach and the small intestine but also those occurring in the oral cavity using an oro-gastric-intestinal (OGI) system (Bove et al., 2012). The simulator of the human intestinal microbial ecosystem (SHIME) was developed by inoculating human fecal material in a fermenter-based simulator to establish a GIT-like microbial population (Molly et al., 1994). Experiments with SHIME revealed similar survival rates of microorganisms to those obtained with *in vivo* tests (Cook et al., 2012). A modification of the SHIME system involved the incorporation of a mucosal environment in the SHIME model, resulting in a more representative colonization ability for the test strains (Van den Abbeele et al., 2012). Another system that relied on two separate fermenters was designed to better simulate the physiological events of ingestion and digestion in the upper GIT. Using this system, it was possible to investigate the survival of probiotics through more realistic pH values, i.e., those that prevail prior, during and after a meal (Mainville et al., 2005). Obviously, GIT simulators offer many advantages over independent *in vitro* tests and the

Table 1 | *In vitro* assays employed during screening for novel probiotic strains.

Probiotic property	Assays	Representative references
Surviving stress within the host	Low pH and bile (e.g., artificial gastric and pancreatic juices and GIT simulators)	Conway et al. (1987), Molly et al. (1994), Marteau et al. (1997), Charteris et al. (1998), Minekus et al. (1999), Mainville et al. (2005), Lavermicocca et al. (2008), Bove et al. (2012), Cook et al. (2012), Van den Abbeele et al. (2012)
Safety assays	Antibiotic resistance	Delgado et al. (2007), Argyri et al. (2013), Pisano et al. (2014)
	Hemolytic activity	Pisano et al. (2014)
	Adhesion to mammalian cells	Harty et al. (1994)
	Production of enzymes (e.g., glycosidases)	Oakey et al. (1995), Bernardeau et al. (2006)
	Production of toxins (e.g., cytolysins)	Tan et al. (2013)
	Production of biogenic amines	Halász et al. (1994), Bover-Cid and Holzapfel (1999)
Colonization of the host	Cell surface hydrophobicity	Ouwehand et al. (1999), Vinderola et al. (2004), Kaushik et al. (2009), Jena et al. (2013), García-Cayuela et al. (2014)
	Adhesion to mucus (e.g., adhesion to mucin, enzymatic activity of GAPDH)	Kirjavainen et al. (1998), Laparra and Sanz (2009), Ferreira et al. (2011), Kinoshita et al. (2013)
	Auto-aggregation screening	Collado et al. (2008), Bao et al. (2010), Malik et al. (2013), Botta et al. (2014)
	Adhesion to intestinal epithelium (e.g., cell-lines, tissue fragments and whole tissue models)	Vesterlund et al. (2005), Muller et al. (2009), Tassell and Miller (2011)
Antimicrobial assays	Production of antimicrobial metabolites such as organic acids and bacteriocins (e.g., simple inhibition tests, turbidometric assays, bioluminescence assay, streak methods)	Tagg and McGiven (1971), Jacobsen et al. (1999), Strus et al. (2005), Lahtinen et al. (2007), Parkes et al. (2009), Guo et al. (2010), Haukioja (2010), Lahteinen et al. (2010), Chenoll et al. (2011), Cresci et al. (2013), Kechaou et al. (2013), Al Kassaa et al. (2014), Coman et al. (2014)
	Co-aggregation with pathogens	Collado et al. (2007), Bao et al. (2010)
	Enhancement of intestinal barrier function (e.g., TER measurement, immunofluorescence of tight junction protein antibodies, tight junctional protein phosphorylation)	Resta-Lenert and Barrett (2003), Zyrek et al. (2007), Ewaschuk et al. (2008)
Immunomodulation	Bacterial translocation in the GIT	Corthesy et al. (2007)
	Co-culture models mimicking *in vivo* situation (e.g., co-culture models or three component models with epithelium cells, immune cells and bacteria)	Borchers et al. (2009), Cencic and Langerholc (2010)
	Interaction of host immune system with bacterial compounds (e.g., lipoteichoic acids and peptidoglycan)	Miettinen et al. (1998), Corthesy et al. (2007), Steinberg et al. (2014)
	Regulation of epithelial tight junctions	McKay et al. (1997)
	Anti-inflammatory immune-stimulating properties (e.g., alleviation of IBD and allergic symptoms)	Fujiwara et al. (2004), Smits et al. (2005), Foligne et al. (2007)
	β-Hexosaminidase release assay (alleviation of allergic reactions)	Kim et al. (2013)

(Continued)

Table 1 | Continued

Probiotic property	Assays	Representative references
Cardiovasular diseases	Deconjugation of bile salts (e.g., BSH activity)	Dashkevicz and Feighner (1989), Smet et al. (1994), Zheng et al. (2013)
	Conversion of cholesterol to coprostanol	Lye et al. (2010)
	Peptides from bacterial metabolism with ACE inhibitory activity	Papadimitriou et al. (2007)
Anticancer	Ames test	Burns and Rowland (2004)
	Comet assay	Pool-Zobel et al. (1996)
	Nitrosamine degrading assay	Duangjitcharoen et al. (2014)
	Preventing colon cancer cell invasion	Choi et al. (2006)
	Induction of apoptosis of cancer cells	Castro et al. (2010)
	Binding to mutagenic compounds (HCAs)	Faridnia et al. (2010)
	Removal of toxins and toxic metals	Halttunen et al. (2007), Nybom et al. (2008)
	Bacterial fermentation and production of SCFAs	Boffa et al. (1992), Mariadason et al. (1999), Sakata et al. (2003), Cousin et al. (2012)
Additional health benefits	β-Galactosidase activity	Hughes and Hoover (1995)
	Production of vitamins	Pompei et al. (2007)
	Linolenic acid test	Kullisaar et al. (2002)
	Oxalate-degradation	Campieri et al. (2001)

selection of probiotic strains using these systems may be more reliable. However, such simulators do not allow rapid screening of multiple strains and they may be relatively expensive to maintain and operate. Today advancements in encapsulation technology allow the targeted delivery of probiotic strains to different compartments of the GIT in an active state irrespectively of their stress robustness.

SAFETY ASSAYS

Another important aspect in selecting probiotic strains is their safety status. While in Europe QPS regulation has identified the microorganisms that can be safely used in foods, there might be some safety aspects that may need to be evaluated before commercial probiotic cultures are put on the market (Joint FAO/WHO Working Group, 2002). Laboratory tests applied for the safety evaluation of probiotic cultures include *in vitro* assays examining different intrinsic properties of the strains. Initially, the minimum inhibitory concentrations (MICs) for the most relevant antibiotics is usually determined and evaluated using protocols given by EFSA (2008). The microdilution-broth test performed on 96-well microplates (Argyri et al., 2013), the disk-diffusing method (Pisano et al., 2014) and ready-to-use commercial kits (Delgado et al., 2007) have been applied to specify MIC values of known antibiotics for potential probiotic strains in many cases. Hemolytic activity is also examined (Joint FAO/WHO Working Group, 2002). Clear zones of hydrolysis, partial hydrolysis or no reaction around the streaking of strains on blood agar plates indicate the hemolytic ability of probiotics (Pisano et al., 2014). *In vitro* tests of pathogenic traits concern the ability of bacteria to bind to mammalian cells such as platelets, which is coupled with their binding to fibronectin, fibrinogen and collagen (Harty et al., 1994). The production of certain enzymes (e.g., glycosidases, proteases and gelatinases) is also perceived as a potential pathogenicity trait (Oakey et al., 1995; Bernardeau et al., 2006). Strains should be tested with appropriate *in vitro* assays for the production of known human toxins (e.g., cytolysins; Tan et al., 2013). Biogenic amines are usually generated by decarboxylation of the corresponding amino acids through substrate-specific decarboxylases of bacteria. Assays performed on solid media are based on the pH change of the medium after bacterial growth, corresponding to positive decarboxylase activity (Bover-Cid and Holzapfel, 1999). Quantitative analysis of biogenic amines is generally accomplished by chromatography using amino acid analyzers (Halász et al., 1994). *In vitro* safety tests are generally very useful to identify and exclude clean-cut cases of pathogenic strains from being used as probiotics. For example, a hemolytic or a toxin producing strain can be easily identified and excluded from further analysis. The problem with the *in vitro* safety assays concerns the identification of false negative strains. A virulence trait may be simply non-active under the specific conditions of the assay and thus remain undetectable (e.g., a toxin that may be down-regulated *in vitro*). Virulence is a complex phenomenon that sometimes needs an active interaction with the host to be triggered and for this reason *in vivo* models may be more appropriate. The screening of the bacterial genome for the presence of virulence and resistance genes (see below) is also a way to predict the possibility of non-expressed safety risk factors.

COLONIZATION OF THE HOST AS A PREREQUISITE TO EXERT CERTAIN HEALTH BENEFITS

Although research in the probiotic area has considerably progressed the last decades, the correlation of specific cultures with specific health claims is still ambiguous. In a relatively limited number of cases specific *in vitro* assays have been devised to investigate the protective or therapeutic role of probiotic candidates against certain diseases. The simplest application is the competition of the probiotics with potential pathogens for resources and space in the GIT. Adherence to mucus and epithelial cells is still considered a controversial topic in probiotics research. On the one hand it is a desirable probiotic trait, as it facilitates colonization of the host and antagonism against pathogens, but on the other hand it is considered as a risk for translocation. The latter might be especially important in highly sensitive populations of immune depressed patients where probiotic applications are often considered (Sanders et al., 2010).

The hydrophobicity phenotype of bacterial cell surface is related to their adhesive capacity and colonization of the gut (Ouwehand et al., 1999). Generally, cell surface hydrophobicity is determined according to the capacity of the bacteria to partition into hydrocarbons (i.e., hexadecane, xylene, toluene; Kaushik et al., 2009; Jena et al., 2013), thus, reflecting the non-specific adhesion capability related to cell surface characteristics (García-Cayuela et al., 2014). Controversial results of hydrophobicity studies show that this feature might be questionable (Vinderola et al., 2004). In general, assessing the adhesive capacity of probiotic strains based on surface hydrophobicity is rather outdated.

Adhesion tests of probiotics to human intestinal mucus obtained from infants or healthy human feces have been performed (Kirjavainen et al., 1998; Ferreira et al., 2011). Moreover, high-throughput screening methods based on immobilized commercially available mucin have also been reported (Laparra and Sanz, 2009). Mucins are large glycoproteins that fortify intestinal mucosal surfaces forming a protective shield for the epithelial cells against harmful environmental conditions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressed on the bacterial cell surface aid binding to human colonic mucin and the evaluation of this enzymatic activity has been reported as a simple screening method (Kinoshita et al., 2013). Alternatively, other studies have focused on the ability of probiotic bacteria to form cellular aggregates via self-aggregation (auto-aggregation; Del Re et al., 2000) by measuring absorbance of bacterial suspensions that are left standing for certain time intervals (Collado et al., 2008). Auto-aggregation capacity of LAB is correlated to their adhesion to different kind of host cells (Malik et al., 2013), and it is considered as a desirable characteristic for preliminary probiotic screening (Bao et al., 2010; Botta et al., 2014). Intestinal epithelial cell (IEC) lines are often presumed to better represent conditions in the tissues of the GIT (i.e., adhesion ability and colonization of probiotic strains). Several studies have been conducted using human epithelial cell lines (like HT-29, HT-29MTX, and Caco-2) to screen the adhesion of probiotic strains (Muller et al., 2009). In general, the *in vitro* testing of the adhesion potential is considered experimentally difficult. Reproducibility issues have been observed among laboratories due to the use of different variants of a given cell line

and of high background levels (Kirjavainen et al., 1998). These tests can only yield rough indications of a strain's potential to adhere *in vivo*. Additionally, resected intestinal tissue fragments have been used unprocessed or immobilized on microtitre plates for adhesion assays (Vesterlund et al., 2005). Finally, the whole tissue models consisting of the epithelial tissue with the mucus layer in the presence of commensal microbiota may allow the assessment of more complex adhesive interactions between probiotics and the host (Tassell and Miller, 2011).

Several molecules that are actively aiding the binding to host's cells have been identified. The problem with the *in vitro* assays is their inability to recapture the actual conditions prevailing in the GIT. In most cases adhesion is studied for single strains thus in the absence of any additional microbiota that would mimic the gut microbiome. This is a very important drawback for most assays, since there is fierce competition for adhesion sites among the different microbes *in vivo*. The use of cancer cells is also a bit controversial since their extracellular matrix and surface properties may differ significantly from that of healthy IECs. Nevertheless, strains shown to adhere with high efficiency to human cells *in vitro* usually behaves in a similar manner *in vivo*.

ANTIMICROBIAL ASSAYS

Another desired attribute is the production of antimicrobial compounds by probiotics. Perturbation of the GIT microbiome plays an important role in the pathophysiology of common gastrointestinal infectious diseases. Researchers have proposed that probiotics may prevent gastrointestinal disorders by maintaining homeostasis of the gut microbiome or by competitively inhibiting the growth of pathogens (Hickson, 2011). Selection of probiotic bacteria active against infectious diarrhea attributed to viruses (e.g., rotavirus and norovirus; Al Kassaa et al., 2014) or bacteria (e.g., *Escherichia coli, Salmonella* and *Campylobacter* sp.) or to *Clostridium difficile* infection (Parkes et al., 2009), is usually based on antimicrobial properties of probiotic strains. This is also the case for antibiotic-associated diarrhea (Cresci et al., 2013) and *Helicobacter pylori* infection (Chenoll et al., 2011), as well as for infections related to sites of the human body other than the GIT, such as the oral cavity (Haukioja, 2010), the upper respiratory tract (Kechaou et al., 2013), and the urogenital system (Strus et al., 2005). In addition to the production of known antimicrobial metabolites such as organic acids, probiotic bacteria may also produce specialized inhibitory agents, like bacteriocins. Target strains commonly include both Gram positive and Gram negative bacteria, as well as fungal strains, comprising of not only pathogenic bacteria but also strains representative of the predominant human GIT microbiota (Gagnon et al., 2011). In general, antagonistic activity is evaluated *in vitro* using simple inhibition tests performed on solid media. More precisely, the agar spot test (Jacobsen et al., 1999), the paper-disk diffusion assay (Guo et al., 2010), and the well diffusion assay (Tagg and McGiven, 1971) have been extensively used as methods for evaluating antimicrobial activity. Moreover, inhibitory effects of probiotic culture filtrates assessed by an automated turbidometric assay that monitors the ability of the indicator bacteria to grow has also been reported (Lahteinen et al., 2010). In some cases, bioluminescent indicator strains were also used to investigate the possible production of antimicrobial compounds from probiotic bacteria (Lahtinen et al., 2007). Assays as the cross-streak and the radial streak methods are comparatively more efficient in terms of examining the inhibitory properties of intact probiotic cells and not only properties attributed to their producing metabolites (Coman et al., 2014).

Antimicrobial ability of probiotics refers not only to the production of antimicrobial compounds or acids that affect luminal pH (Suardi et al., 2013), but also the competitive exclusion of pathogens. Probiotics may compete with pathogens for binding sites on the surface of the GIT. The *in vitro* adhesion assays mentioned earlier can be used to assess the binding competition between probiotic and pathogenic strains. In this context, bacterial aggregation between genetically distinct cells (co-aggregation) is of considerable importance. Thus, protective properties of probiotics against pathogen infections can also be evaluated through co-aggregation assays based not only on simple absorbance measurements but also on radiolabelling and fluorescence detection (Collado et al., 2007).

Antimicrobial properties of probiotics are also correlated with the enhancement of the intestinal barrier function (Mennigen and Bruewer, 2009). Barrier properties can be investigated by measuring trans-epithelial electrical resistance (TEER) before and after apical exposure of IEC lines to bacteria (Zyrek et al., 2007). Promotion of tight junction integrity is known to block paracellular transport of pathogenic bacteria. Alterations of tight junction proteins are examined *in vitro* either by immunofluorescence using specific antibodies (Ewaschuk et al., 2008), or by evaluating the capacity of probiotics to alter tight junctional protein phosphorylation (Resta-Lenert and Barrett, 2003).

Once more, the *in vitro* production of antimicrobial substances alone cannot provide us with important information about the probiotics application *in vivo*. We cannot know if the selected strain will be able to be incorporated in the microbiome and whether the conditions prevailing in the GIT will allow it to produce its antimicrobial(s) compounds at sufficient amounts to have an effect. Usually, probiotic strains produce more than one antimicrobial substance that may act synergistically, increasing the spectrum of targeted microorganisms. This property may be desirable as long as this antimicrobial spectrum is restricted to pathogenic microorganisms but it cannot be excluded that it will not affect the normal microbiota of the gut as well. Similarly to other tests, antimicrobial assays may lead to false negatives, i.e., strains that are capable of biosynthesizing antimicrobial(s) but they do not produce it under the *in vitro* conditions employed. In addition, antimicrobials of probiotics are generally perceived as safe and in most cases the toxicity to host's cells is rarely investigated. There is a clear need for more elaborate assays that would better represent the complex interactions between the probiotics and the host microbiome to understand the consequences of the *in situ* production of antimicrobials by the former.

IMMUNOMODULATION

Modulation of host immunity is one of the most commonly proposed health benefits attributed to the consumption of probiotics. Probiotic selection that is correlated to the protection against

microbial pathogens has been associated with the stimulation of antibody secretion, as well as cell-mediated immune responses (Cross, 2002). Evaluation of the bacterial translocation in the GIT can be used to screen potential probiotic strains, considering that some strains may be capable of triggering dendritic cells (DCs) or M cells from the Peyer's patches and thereby manage to cross the epithelium (Corthesy et al., 2007). In most of the *in vitro* experiments, researchers have attempted to reconcile the mechanisms underlying the complex and dynamic immune interactions of the gut by using co-culture models (Cencic and Langerholc, 2010) or 3D models (Borchers et al., 2009). The use of three component models (epithelia, immune cells and microbiota) closely mimics the *in vivo* situation (Fontana et al., 2013). In addition, several reports highlight the complex interaction between the host immune system and different bacterial compounds, including chromosomal DNA, cell wall components such as lipoteichoic acids and peptidoglycan, as well as soluble metabolites (Corthesy et al., 2007). In these assays, cytokines like IL-5, IL-10, IL-12b, IL-17a, IFN-γ, TNF-α, and TGF-β, as well as the levels of the secretory immunoglobulin type A (sIgA) are used to assess stimulation of the immune response and the inflammation status (Miettinen et al., 1998; Steinberg et al., 2014).

IBD covers a family of chronic diseases affecting the GIT, having as most common forms Crohn's disease (CD) and ulcerative colitis (UC). Propitious functions of the probiotics for alleviation of IBD symptoms involve the ability to restore biodiversity within the microbiota, the antagonism against pathogens, the improvement of mucus production, the stimulation of epithelial proliferation, the modulation of intestinal permeability and the mediation of pro-inflammatory effects (Scaldaferri et al., 2013). *In vitro* models for probiotic selection in IBD research are alike to those mentioned previously. Specifically, assays that investigate the antimicrobial activity against microbes that help alter microbial biodiversity within the gut, the regulation of epithelial tight junctions and the induction of an anti-inflammatory cytokine profile (high IL-10/IL-12 ratio) from immune cells, are commonly used (McKay et al., 1997; Foligne et al., 2007).

Allergy is a complex immune response to environmental or food antigenic stimuli and it is mostly correlated with the hypersensitivity reaction mediated by the interaction of immune cells coated with allergen-specific IgE that requires the involvement of T-cells with a Th2-skewed cytokine profile (Furrie, 2005). Th2-skewed immune cells have extensively been studied to select probiotic strains that exert certain immune-stimulating properties for further *in vivo* use (Fujiwara et al., 2004). Alleviation of allergic symptoms has also been correlated to the induction of the immunosuppressive cytokines IL-10 and TGF-β or the reduction of T-cell proliferation (Smits et al., 2005). The β-hexosaminidase release assay helps to identify potential anti-allergic probiotics, since the secretion of this molecule corresponds to a hallmark of allergic reactions resulting from exposure of mast cells to antigens (Kim et al., 2013).

Disturbance in the balance of the normal microbiome in different body niches can lead to inflammation. Probiotics known for their anti-inflammatory cytokine profile from immune cells can be efficacious in moderating this inflammation as in the case of caries and periodontal disease or of functional digestive disorders such as the irritable bowel syndrome (IBS; Krasse et al., 2006; Gill et al., 2009).

While we found the measurement of cytokine levels produced by peripheral blood mononuclear cells (PBMCs) upon stimulation with probiotics to be a reliable and reproducible technique to identify strains with potential pro- or anti-inflammatory properties, the *in vivo* relevance can be questioned as the test involves only one type of immune cells and ignores the complexity of the *in vivo* communication between different cell types. The model is also blind for the differences between the innate and the adaptive immune system. Moreover, the model does not reflect differences of the immune system in relation to certain pathologies. Despite these limitations, experiments with PBMCs are of major interest, as they will allow to quantitatively classify strains and select strains with opposite profiles, e.g., for further mechanistic investigation.

CARDIOVASCULAR DISEASES (CVDs)

Several reports have demonstrated that manipulating the gut microbiome with probiotics may affect host metabolism and ultimately reduce the risk for CVDs. Increased bacterial BSH activity in probiotic strains can result in decreased body weight gain, lower levels of plasma cholesterol, and liver triglycerides (Cani and Van Hul, 2015). Bile salt hydrolyzing activity can be evaluated qualitatively by the plate assay method using taurodeoxycholic acid (TDCA) sodium salt (Dashkevicz and Feighner, 1989), and quantitatively by high-performance liquid chromatography (Smet et al., 1994). The deconjugation of bile salts can lead to secretion of cholesterol and lipids via the fecal route (Zheng et al., 2013). An *in vitro* assay for the conversion of cholesterol to coprostanol by the action of bacterial cholesterol reductase has also been described (Lye et al., 2010). In relation to cholesterol, coprostanol is less absorbed in the intestine and it can be easier removed with feces. Furthermore, the assimilation of cholesterol present in the growth media by probiotic strains has been tested *in vitro* (Tomaro-Duchesneau et al., 2014).

Probiotic strains and metabolic by-products potentially confer benefits to the heart by the prevention and therapy of heart syndromes, as well as by lowering serum cholesterol (Ebel et al., 2014). The ACE enzyme has a key role in the rennin–angiotensin system which controls the arterial blood pressure and the equilibrium of water and salt in the body. The hydrolysis of angiotensin I to angiotensin II, which is a strong vasoconstrictor agent from the ACE enzyme, can lead to an increase in blood pressure. During proteolysis of extracellular proteins like casein, peptides are being released that may inhibit ACE-I activity which is used as a screening tool (Papadimitriou et al., 2007).

There are several lines of evidence that support the positive implication of probiotics to the prevention of cardiovascular diseases (Ebel et al., 2014). However, the actual mechanisms involved are not well understood and thus the *in vitro* assays available for this type of health claims are relatively restricted. Improving the barrier is generally considered an effective way to decrease basic physiological inflammation of e.g., adipose tissue, contributing to a reduced risk for the development of overweight and metabolic syndrome and therefore positively affecting CVD risks. A general comment can be made regarding the currently available assays for

cholesterol absorption or its conversion to coprostanol. The length of these *in vitro* test protocols (often more than 20 h) may not match the *in vivo* time window for the absorption of cholesterol in the intestine after emulsification.

ANTICANCER

The gut microbiota is considered to be related to the risk of cancer and it has been suggested that consumption of probiotics may decrease this risk. The important role of probiotics in retarding carcinogenesis is attributed to their ability to influence metabolic, immunologic, and protective functions in the body (Wollowski et al., 2001). Antimutagenic activities of probiotics have been evaluated by the Ames test (Burns and Rowland, 2004). Probiotics also exerted an antigenotoxic activity related to decreased DNA damage of colon cells treated with carcinogens as evaluated by the "comet assay" (Pool-Zobel et al., 1996). The nitrosamine degrading assay (Duangjitcharoen et al., 2014) and the evaluation of the antioxidant properties of bacterial samples, i.e., intact cells and cell-free culture supernatants, are used for the detection of potential probiotics against cancer (Amaretti et al., 2013). Bacterial cell-free culture supernatants of probiotic strains have also been tested for preventing colon cancer cell invasion *in vitro* (Choi et al., 2006). Strains showing *in vitro* inhibitory activity on tumor cell proliferation, induction of apoptotic cell death, and ability of cellular sphingolipidic metabolism, have been recognized as promising candidates for cancer prevention (Castro et al., 2010). Moreover, a possible mechanism of anti-carcinogenic properties of probiotic strains involves the physical binding by the bacterial cell of the mutagenic compounds, such as heterocyclic amines (HCAs). Specifically, bacterial strains that are able to sequester HCAs could decrease their absorption by the human intestine via their elimination through feces carryover (Faridnia et al., 2010). Furthermore, *in vitro* removal of toxins and toxic metals present in aqueous solutions or in drinking water has been studied for selected probiotics (Halttunen et al., 2007; Nybom et al., 2008). Also, luminal short chain fatty acids (SCFAs), produced in the colonic lumen during bacterial fermentation, are known anti-carcinogenic agents within the gut (Commane et al., 2005). In the past, the influence of probiotic bacteria on the production of SCFAs by fecal bacteria was studied *in vitro* using batch-culture and continuous-culture techniques (Sakata et al., 2003). *In vitro* GIT models as described above can also be used.

The aforementioned *in vitro* assays for anticancer properties of probiotic bacteria provide minimal information about the actual *in vivo* efficacy. On one hand, probiotic bacteria showing antimutagenic and/or antigenotoxic activities may exert generalized prophylaxis against gut related cancers. On the other hand, probiotics producing SCFAs may have a more direct effect by selectively killing cancer cells as it has been demonstrated for propionic acid bacteria (Cousin et al., 2012) or by assisting in the renewal of the colonic epithelia by butyric acid (Mariadason et al., 1999) or its effect on histone deacytelase (Boffa et al., 1992).

Generally, the existing *in vitro* assays are not sufficient to truly screen probiotics for anticancer properties and thus *in vivo* assays will be necessary. In any case, the idea of using bacteria (some of which are known probiotics, like bifidobacteria) in the treatment of cancer is gaining momentum (Chong, 2014). As the mechanisms of cancer prevention and therapy become clearer, new and more elaborate *in vitro* assays may be developed in the future.

ADDITIONAL HEALTH BENEFITS

There are several additional health benefits that have been attributed to probiotics. Lactose intolerance, attributed to an insufficient amount of lactase in the small intestine to early hydrolyze lactose, is an important problem when consuming milk or lactose containing foods (Kim and Gilliland, 1983; Céspedes et al., 2013). Dairy products containing probiotic bacteria could aid the digestion of lactose by their β-galactosidase enzyme when crossing or colonizing the gut. Screening for β-galactosidase activity in potential probiotics is assessed through the hydrolysis of the *o*-nitrophenol-β-galactopyranoside (Hughes and Hoover, 1995).

Other nutritional effects of probiotics relate to the production of vitamins. They play a major role in helping humans to meet their needs for these essential nutrients (Eck and Friel, 2013). *In vitro* studies have documented the capacity of some probiotic strains to synthesize vitamin K, folic acid, vitamin B2, and B12 (Pompei et al., 2007).

Probiotics may also have a protective role against oxidative stress in the host (Songisepp et al., 2005). Oxidative stress has many physiological consequences to the host including aging, carcinogenesis, etc. Evaluation of the antioxidative activity of probiotics is usually assessed by the linolenic acid test (LA-test; Kullisaar et al., 2002).

Furthermore, the role of oxalate-degrading bacteria in the treatment of kidney stone disease is of particular interest (Abratt and Reid, 2010). Identification of potential probiotic strains through the evaluation of oxalate degradation by pure cultures has been reported (Campieri et al., 2001).

Lastly, volatile sulfur compounds (VSCs) produced by oral bacteria can cause halitosis. An *in vitro* test was developed by Kang et al. (2006) in which the influence of LAB on VSC production by *Fusobacterium nucleatum* could be assessed. Interestingly, inhibition of VSC production was related to co-aggregation of the LAB strains with *F. nucleatum* as well as the production of H_2O_2 by the former.

FUTURE PERSPECTIVES FOR THE USE OF *IN VITRO* TESTS IN PROBIOTIC RESEARCH

There are several *in vitro* assays that are being employed in an attempt to predetermine or document probiotic properties in relation to health claims. Even though such assays are useful to screen probiotic candidates they exhibit variable effectiveness. Regulatory authorities have attempted the standardization of the *in vitro* assays by publishing detailed protocols and directives. Unfortunately, even a superficial examination of the literature reveals that *in vitro* tests are being performed in a rather arbitrary manner. This makes it difficult to compare findings between different studies. Reproducibility issues have also been reported, making it difficult to rely solely on the outcomes of *in vitro* tests for the selection of probiotic strains. Apparently, *in vivo* assays may be more appropriate but in most cases they cannot be used for high throughput screening due to the increased cost and for ethical reasons. Therefore, *in vitro* screening is and will be an indispensable part of the

discovery of new probiotics. More research is needed to improve and standardize the available experimental protocols aiming at improving reproducibility and decreasing the percentages of false positively or negatively identified strains with probiotic potential. Additionally, novel methods might need to be developed that will extend the health-promoting properties currently assessed with *in vitro* tests.

DISCOVERING PROBIOTIC MICROORGANISMS: *IN VIVO* APPROACHES

THE RIGHT MODEL FOR THE RIGHT PURPOSE

The use of *in vivo* approaches to explore probiotic potential resulted in the description of a huge diversity of biological models of various complexity, ranging from simple multicellular organisms such as worms and invertebrates over sophisticated knock-out (KO) models in rodents to human clinical trials in different types of the population (**Figure 1**; **Table 2**). While all these models can teach us something, they also represent certain disadvantages and integrating results from different models remains difficult. Therefore, while final assessment of probiotic functionality should ideally be performed directly in the target population (general population or a subgroup with a given condition; Rijkers et al., 2010), the pre-selection of strains to be included in these expensive clinical trials might need to be made using appropriate *in vivo* models.

Indeed, while rudimentary models such as *Caenorhabditis elegans*, or *Drosophila* exhibit obvious benefits for (large) screening purposes, they are also not devoid of relevance in deciphering more universal signaling pathways, even related to mammalian innate immunity, as shown by the work of the Nobel prize winner Hoffmann and his team (Vogel, 2012). *C. elegans* was successfully applied to establish the anti-infective, antioxidative and lifespan extending impact of lactobacilli (Wang et al., 2011; Grompone et al., 2012). The prototype worm is currently proposed to screen probiotics (Park et al., 2014), or to establish antitumor activity (Fasseas et al., 2013). In a similar way, the fly is useful to explore metabolic, immune and antioxidant effects of the *Lactobacillus*-host mutualism (Jones et al., 2013; Matos and Leulier, 2014).

Quite recently, the zebrafish (*Danio rerio*) has garnered intense interest as a human disease model (Lieschke and Currie, 2007) due to its many advantages as an experimental vertebrate. It now appears that the zebrafish can be used for high-throughput screening (e.g., of drug libraries) in the discovery process of promising new therapeutics (Lessman, 2011). The latter was successfully developed for probiotics, showing that probiotic administration may enhance the zebrafish welfare by modulating the innate immune response and improving hepatic stress tolerance, involving stress and apoptosis genes, and genes from the innate immune system (Gioacchini et al., 2014; Rieu et al., 2014). Of note, zebrafish can also partly mimic characteristics of IBDs when larvae are subjected to chemicals such as trinitrobenzene sulfonic acid (TNBS; Fleming et al., 2010) or when encountering unfavorable conditions, including dysbiosis of the intestinal microbiota (He et al., 2013). Modifications include colitis susceptibility genes like *NOD1* and *NOD2* (Oehlers et al., 2011), enabling the routine evaluation of anti-inflammatory compounds.

RODENTS AS THE NECESSARY COMPROMISE

The small animal models mentioned above clearly meet the needs for cost effective and public-acceptable screening but are still far away from an integrated mammalian physiology. Therefore more pertinent experimental models are required for the evaluation of probiotic functionality, allowing to study various dynamic states and when addressing specific diseases with multifactorial origins.

Accuracy of the results of animal models is not always in perfect accordance with human outcomes and may sometimes appear

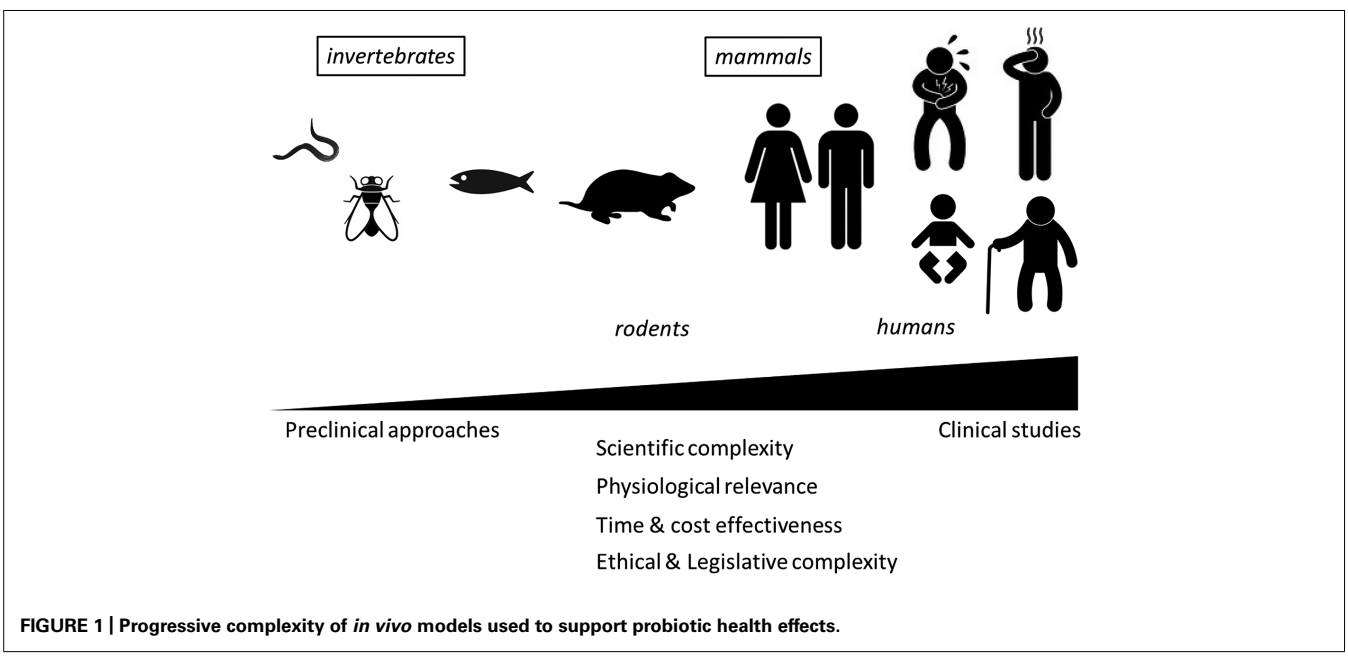


FIGURE 1 | Progressive complexity of *in vivo* models used to support probiotic health effects.

Table 2 | Advantages of using small animals/rodent models for probiotic research support.

Functionality	Possible intervention/improvement	Representative references
Physiological relevance for humans (Immune system, neuroendocrinological system)	Transgenic mice (knock-out/ knock-in)	Helm and Burks (2002)
Closely related innate and adaptive immunity (PRRs and signaling cascades, secretory Ig, T and B cells, DCs, etc.)	Tissue-specific knock-out	Sodhi et al. (2012)
Sharing of similar immune response types (Th1, Th2, Th17, Treg cells and cytokine responses)	Conditional knock-out	Sodhi et al. (2012)
Hosting complex microbiota (gut, vagina, lung, skin)	Humanized mice	Martin et al. (2008)
	Axenic mice	Verdu and Collins (2004)
	Monocolonized mice	Eaton et al. (2011)
	Microbiota transplantation	Le Roy et al. (2013)
	Co-housing	Henao-Mejia et al. (2012)
	Selective antibiotic treatment	Ichinohe et al. (2011), Viaud et al. (2013)
Responsiveness to many infectious, immune and other disorders	Allergy, inflammation (asthma, COPD, IBD, etc.)	Kim et al. (2014)
	Bacteria, virus, fungi and parasites pathogens	Alak et al. (1997), Villena et al. (2011), Collins et al. (2014), Kikuchi et al. (2014)
	Neurologic disorders (EAE, visceral pain)	Eutamene et al. (2007), Rousseaux et al. (2007), Kwon et al. (2013)
	Stress, cognitive functions	Gilbert et al. (2013), Hsiao et al. (2013)

problematic as it was for example recently stated: "Inflammatory findings on species extrapolations: humans are definitely no 70-kg mice" (Leist and Hartung, 2013; Seok et al., 2013). Whether animals can be used to predict human response to drugs, chemicals or foods (including probiotics) is apparently a contentious issue. While some will promote a ban on animal experimentation which lacks scientific evidence for human predictivity (Knight, 2007), the relevance of e.g., mice disease models for humans has been judged rather positively during a dedicated European Commission workshop held in the UK[1]. However, a certain level of criticism on the relevance of animal models does apply. Shanks et al. (2009) illustrate with numerous examples their importance, or lack thereof, in the different steps of the complete research process. They discuss, as an example, the bioavailability differences between primates, rodents, and dogs. When specifically considering probiotic interactions with the host microbiota, a deeper comprehension of the symbiosis between animals and bacteria is key to understanding the complex biology of environmental, genetic and microbiome interactions in relation to human health and disease evolution. Kostic et al. (2013) reviewed how model systems are influencing the current understanding of host–microbiota interactions by exploring recent human microbiome studies. They conclude that experimental model systems, including mice, fish, insects, and the Hawaiian bobtail squid, continue to provide critical insight into how host–microbiota homeostasis is constructed and maintained. Taking also into account the dynamics of the human microbiome through human life stages, predictive value of simple models may indeed have its limits, but their use may be crucial in understanding mechanisms of interactions and regulation of metabolic, physiological, and immunological activities.

Effective use of rodent models will depend on extreme standardization, including the microbiota composition. Relevance for the human situation needs to be considered at any time, as e.g., many bacterial species which are commensal for humans can be pathogenic in mice and *vice versa* (Baker, 1998; Pan et al., 2014). Despite all these possible drawbacks, small animals such as rats and mice will inevitably be continued to be used as models to address numerous research questions related to probiotics, including the evaluation of immune and metabolic responsiveness, regulatory processes or neuro-endocrinological and nutritional aspects, which all play important roles in the complex microbiota–host relationships. Moreover, small animals permit to mimic specific diseases, e.g., by using genetically modified specimens (conditional and tissues-specific knock-in/knock-out mutants, **Table 2**) or specific chemicals (e.g., TNBS to induce intestinal inflammation) and infectious challenges, while manipulation of the microbiota allows to question the role of (a) specific microorganism(s) (**Table 2**).

FITTING ETHICS AND LEGISLATION

Controversy on animal experimentation has always been high. Researchers in need of animal models have to cope with ethical and legal considerations, as well as with a public opinion that often is not fully aware of the economic and societal importance of the research envisaged, nor of the regulatory context which also limits the unethical use of animal suffering. Researchers are also actively required to ensure that animal models (i) are scientifically

[1]http://ec.europa.eu/research/health/pdf/summary-report-25082010_en.pdf

(and statistically) validated (ii) cannot be replaced by *in vitro* alternatives and (iii) minimize animal suffering by limiting the number of animals and the length of the experimentation to what is statistically required. Research strategies and methods should be challenged continuously and reviewed objectively with respect to the 3Rs rule established more than 50 years ago, i.e., use opportunities to replace, reduce and refine (Russel and Burch, 1959). In addition to the proper management of pain by analgesia and anesthesia, welfare accommodations improved tremendously following the most recent US and EU animal housing guidelines. Mice and rats should be allowed sufficient space of adequate complexity, allowing to express a wide range of normal behaviors, and providing enrichment possibilities to promote physical exercise, foraging, manipulative and cognitive activities, whenever possible (Richmond, 2000).

In this review we do not focus on the use of large mammals (pigs, dogs, or monkeys) as these are not widely available and ethical considerations are considerable. Non-invasive dietary interventions with pigs, however, may have an interest as the GIT of pigs resembles very well the human GIT.

MODELING DISEASES TO SUBSTANTIATE HEALTH EFFECTS

Probiotic activity is situated on three main levels (Rijkers et al., 2010): (i) influence on the growth or survival of pathogenic microorganisms in the gut lumen (level 1), (ii) improvement of the mucosal barrier function and the mucosal immune system (level 2) and, (iii) beyond the gut, effects on the systemic immune system, remote organs and systems such as the liver and the brain (level 3). Correspondingly, *in vivo* approaches intended to substantiate probiotic effects might consider these three levels through gastrointestinal infection models, mucosal immune disorder models and models dealing with dedicated experimental neuro-metabolic pathologies respectively.

Maintaining or improving overall "health" is difficult to demonstrate in humans and *a fortiori* in animals. Health claim evidence for probiotics obviously starts by proving innocuousness of the probiotic strain and its matrix and by clearly establishing its safety, using adapted procedures (Vankerckhoven et al., 2008; Sanders et al., 2010). In a second step, the model must fit the purpose of showing convincingly the projected functionality of the probiotic strain, either in a prophylactic or therapeutic way, or demonstrating a reduction of risk activity. This functionality must be illustrated either using read-outs that are relevant for the human situation, in appropriate terms of expected changes in metabolism, physiology, immunology, etc. or by a measurable limitation of the severity or frequency of disease. Discriminating, relevant physiopathological markers are crucial and should reflect the targeted probiotic functionality. Clearly, the anti-infectious impact of probiotics, depicted for example by the survival rate of a defined pathogen, can only be considered for that particular pathogen and should not be extended to other infections. Consequently, optimal read-outs have to be selected as clear markers of pathology. In the case of inflammation, various pathological as well as beneficial changes may take place. For instance, we routinely determine IL-10 production in inflamed tissues and observed increased amounts of this anti-inflammatory cytokine in the colon of mice following TNBS or dextran sodium sulfate (DSS) treatment, as compared to healthy mice. While IL-10 is a "regenerating" mediator, rapidly induced by injured tissues in response to damage (Barada et al., 2007), it is not an appropriate marker for efficacy in inflamed situations, where IL-10 is to be considered a marker of inflammation. However, in the context of a healthy mucosa, probiotics and other prophylactic anti-inflammatory drugs may be able to substantially increase baseline levels of IL-10 and TGF-α, and thus prevent further injuries.

INTESTINAL MODELS FOR INFLAMMATION AND INFECTION

For the evaluation of the anti-inflammatory activity of bacteria, several colitis models can currently be used. In many cases the reduction of chemically induced inflammation, e.g., induced by TNBS or DSS, is measured (see Claes et al., 2011 for a complete review). These models are mostly acute models, while chronic ones are less common. They obviously only mimic symptoms of IBD such as UC and CD but do not cause the real disease. Therefore, each model has its own specificity and some interventions may have opposite effects in different models. TNBS evokes an inflammatory process involving T cells while the acute DSS model is more likely to induce epithelial barrier disturbances (Foligne et al., 2010a).

The IL-10 KO mouse model may also be used to study probiotics, but the spontaneous colitis that progressively will occur is not homogenous, difficult to control in time and highly dependent on animal facility conditions. Sometimes additional treatments such as *Helicobacter* infections or small amounts of DSS are required to trigger the onset of inflammation. Clearly, the choice of a model will depend on the putative mode of action of the probiotic used. The IL-10 KO mouse model abolishes a key regulatory cytokine and may thus exclude a virtually protective probiotic if the main mechanism is IL-10 dependent. Similarly, while *nod1*/*nod2* KO mice are good models for CD, the fact that they lack the cellular receptor for peptidoglycan and derived anti-inflammatory products (i.e., certain muramidyl di- or tri-peptides), will at the same time exclude the efficient study of bacteria that execute their anti-inflammatory effect in this way (Macho Fernandez et al., 2011). In general, KO mice models, especially those deficient for key receptors such as TLRs, are appropriate for case-by-case studies, often aiming at confirming specific mechanisms of action, but are less suitable for broader screening purposes.

Finally, some other models of colitis involve adoptive transfer of specific T cells ($CD4^+CD45rb$) in immune-deficient animals (SCID, $Rag^{-/-}$) allowing to explore the impact of probiotics on adaptive immunity. *Citrobacter rodentium* is a murine pathogen that induces, depending on the genetic background of the mice, variable degrees of infectious and inflammatory responses, ranging from infectious colitis to severe and fast lethal inflammation. It shares several pathogenic mechanisms with enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), two clinically important human gastrointestinal pathogens, showing severe adhesion-based virulence in the intestinal mucosa. The models also display crypt hyperplasia, demonstrating similarities with pre-carcinogenic states. Some probiotics have been shown to positively alleviate *C. rodentium*-induced colitis as demonstrated for lactobacilli, bifidobacteria, propionibacteria, yeasts and spores of *Bacillus subtillis* (Chen et al., 2005; Johnson-Henry et al., 2005; Wu

et al., 2008; Foligne et al., 2010b; Collins et al., 2014). This model, when appropriately used, can be considered a model of choice to investigate overall probiotic functionality (Borenshtein et al., 2008) as it reveals information on the anti-infectious as well as the anti-inflammatory potential of the strains tested.

Clostridium difficile can be the causative agent of primary and recurrent antibiotic-associated diarrhea and colitis in hospitalized patients. Mice models to mimic this type of infection exist but require the administration of a broad range of antibiotics that are not always compatible with the planned probiotic interventions (Chen et al., 2008; Sun et al., 2011). Therefore, the preferential use of yeasts such *Saccharomyces boulardii* has been suggested (Barc et al., 2008), or the window of intervention is to be kept quite short to demonstrate substantial effects (Kaur et al., 2010). Partial efficacy has been shown on inflammation or stool consistency parameters, although the infection was not completely cured (Fitzpatrick et al., 2011). Further efforts are needed to optimize the model for more detailed study of promising probiotic strains.

Similarly to *Clostridium difficile*, a *Salmonella typhimurium* infection in mice requires the administration of antibiotics during the colonization (Hapfelmeier and Hardt, 2005), limiting also the readouts of the model. However, infectious challenges with *S. enterica* can be useful to study mortality and translocation (Zoumpopoulou et al., 2008; de LeBlanc Ade et al., 2010; Zacarias et al., 2014), focusing on indirect anti-inflammatory effects and on the impact of the probiotics on the intestinal barrier.

Finally, listeriosis is not spontaneously modeled in mice and infectivity would require genetically modified humanized animals having the necessary receptor to allow the pathogen to attach and disseminate within the host. When established, it might allow to explore anti-infective probiotic potentials against *Listeria* (Archambaud et al., 2012).

CANCER MODELS

While animal models exist for chemopreventive and chemotherapeutical drugs for e.g., mammary and ovary cancer, bladder, and prostate cancer, esophagus and colon cancer, lung and pancreas cancer, skin head and neck cancer, most studies involving probiotics focused on colorectal cancer (CRC), as it represents the most common malignancy of the GIT and has been linked to dietary habits and a Western lifestyle. Many indications have indeed pointed toward the importance of the microbiota in the increased risk for CRC development. Several possible mechanisms for reducing the risk for CRC development have been suggested, each supported by *in vitro* and *in vivo* models (Uccello et al., 2012). Probiotic intervention intends to alter the metabolism of the microbiota by reducing intestinal enzymes that promote the production of potential carcinogenic substances: β-glucuronidase that produces aglycons, or nitroreductase and azoreductase, which produce free amines from aromatic nitroso compounds and azo dyes respectively (Goldin and Gorbach, 1984).

A second mechanism includes the direct inactivation of potential carcinogenic compounds, such as mutagenic derivatives of heterocyclic aromatic amines. Commensal bacteria have been found to bind or metabolize these compounds, resulting in reduced mutagenicity in HCA exposure models (Kumar et al., 2010), reduced bioavailability of other mutagens in the GIT and tissues in mice (Orrhage et al., 2002) or increased detoxification in rats (Challa et al., 1997). Another way to study probiotic efficiency in reducing the prevalence of CRC is the IL-10 KO mice model (O'Mahony et al., 2001), leading to spontaneous colitis and colon cancer development.

Improving the host's immune response by activation of antigen-presenting cells, natural killer cells or subsets of T and B cells is another way to promote antitumor activity in mice (Sekine et al., 1985) and may explain some of the observations in syngeneic mice and guinea pigs on Lewis lung carcinoma and line-10 hepatoma (Matsuzaki et al., 1985), as well as in bladder cancer in man (Aso et al., 1995). The same probiotic strain was also shown to have a positive effect on transplantable tumor cells and to suppress chemically-induced carcinogenesis in rodents (Matsuzaki et al., 1988). A component of the polysaccharide peptidoglycan complex was shown to improve colitis-associated cancer in mice (Matsumoto et al., 2009). Measuring survival rates of mice injected with tumor cells is another model that can be used to test or compare the potential of different probiotic strains to increase cellular immunity (Lee et al., 2004).

Anti-proliferative effects by regulation of apoptosis and cell differentiation have been described in response to the carcinogen azoxymethane (AOM; Le Leu et al., 2005), which may be linked to reduced levels of ornithine decarboxylase involved in polyamine biosynthesis (Moorehead et al., 1987). Butyrate also enhances cellular differentiation and reduces proliferation (Topping and Clifton, 2001). Butyrate may be used as an energy source by the colonocytes and together with other SCFAs they may reduce the colonic pH and the concentration of secondary bile salts. In addition, conjugated linoleic acids (CLAs) were also shown to have anti-inflammatory and anti-carcinogenic effects (Kim and Park, 2003; Ewaschuk et al., 2006; Evans et al., 2010), possibly through the activation of PPARγ.

Besides for CRC, the Apc^{Min} mouse model can also be used in the case of mammary tumorigenesis (Moser et al., 1995). Chen et al. (2009) used this model to examine the quantitative and mechanistic effects of probiotic yeast on the induction of intestinal tumors. Clearly a large number of models exist for the investigation of probiotic activities on cancer, reflecting the many possible mechanisms of probiotic activity, as well as the intense cancer research activity.

MODELS LOOKING INTO METABOLIC DISORDERS

Metabolic syndrome (MS) is a complex multifactorial disorder involving genetic predisposition, life style, diet, and environmental factors and is almost always accompanied by an increased risk of cardiovascular diseases and type 2 diabetes, and possibly also non-alcoholic fatty liver disease (NAFLD) and hypertension. Obesity is the most important precursor for MS, especially on a longer term. Pro- and prebiotics can play a role in weight management, as obesity was found to be linked to a dysbalance of the microbiota, both in mice and man (Ley et al., 2005). Turnbaugh et al. (2008) investigated the inter-relationship between diet, microbial ecology of the gut and energy balance using a Western diet-induced obesity model in mice. This diet induced a dominance of the Firmicutes in the distal gut microbiota which could be reversed by subsequent dietary manipulations to limit weight gain. Interestingly

the transplantation to germ-free lean mice of the microbiota from mice with diet-induced obesity, increased significantly more the adiposity in the recipient mice than transplants from lean donor mice. Possible mechanisms include a change in intestinal permeability, leading to decreased translocation of lipopolysaccharides in the microbiota of lean mice and therefore to decreased inflammation and abdominal adiposity (Cani et al., 2008). Also the importance of *A. muciniphila* in this process has recently been illustrated (Everard et al., 2013). Overall these observations have fuelled the idea of using probiotics and prebiotics in dietary strategies for weight management (Nicholson et al., 2005; Cani et al., 2009). Again, several mechanisms, and thus several models, can be considered to improve gut microbial balance: CLA production (Lee et al., 2006), polyphenol supplementation, low amounts of probiotics (Rastmanesh, 2011), prebiotic intake, decreased food intake, increased satiety, increased barrier function or ways to decreased abdominal adiposity or total cholesterol levels (Yadav et al., 2008). Since it is impossible to describe all MS related animal models here, it is important to note that the cause/consequence relationship is not clear when only considering the composition change of the microbiota related to obesity, as many of the models involve high-fat diets, also directly affecting the microbial composition (Hildebrandt et al., 2009). The use of germ-free mice with standardized nutrition might help to study the direct impact of a particular microbiota composition on MS. The observed shifts in the microbiota composition may have an impact on the barrier function, but they have also been linked to functional shifts (e.g., production of SCFAs) in the microbiota which can contribute to an obese phenotype (Turnbaugh et al., 2006). Important to notice as well, is the observation that the situation in humans seems to be different from mice, since bacteroidetes-related taxa were either reported to increase, to remain unchanged, or to decrease after weight loss (Duncan et al., 2008; Nadal et al., 2009). The detection of the *ob* and *db* genes in mice led to the development of several animal models to study obesity effects (Ingalls et al., 1950; Hummel et al., 1966; Comuzzie and Allison, 1998). The *ob* gene, located on chromosome 6, encodes the hormone leptin and renders *ob/ob* mice massively obese, with marked hyperphagia and mild transient diabetes, while the *db* gene, located on chromosome 4, codes for the leptin receptor and renders the *db/db* mice markedly obese with hyperphagia, but with severe, life-shortening diabetes.

One can conclude that the complex interplay between genetics, environment, diet, the microbiota and its metabolism or the barrier and immune function of the host, make it difficult to develop all-round animal models. Partial mechanisms may need to be put forward for independent evaluation, with the total picture being obtained through the combination of different *in vitro* and *in vivo* models. Translation from animal to man may also prove to be difficult, because of this complexity.

MODELS FOR AUTO-IMMUNE DISEASES

Similarly to MS, auto-immune disease (AID) covers a broad range of possible diseases (type 1 diabetes, multiple sclerosis, rheumatoid arthritis, pemphigus vulgaris, scleroderma, Sjögren's syndrome, etc.) involving, besides genetic factors, also an aberrant intestinal microbiota, a disturbed mucosal barrier and altered intestinal immune responsiveness. All these factors share functional links and will therefore determine the models to be considered for more in depth study of probiotic mechanisms or for comparing different strains. Since probiotics have the potential to interfere with all three factors, one will need to try and find strains that have the capacity to change in a positive direction any, or any combination, of these factors. As for the intestinal immune responsiveness, interest will be in anti-inflammatory probiotics, as chronic inflammation underlies many AIDs and is often at the start of its development, as in rheumatoid arthritis. It is also not clear if the AID is caused by inflammation, or the other way around.

Animal models such as the non-obese diabetic mice will spontaneously develop diabetes resembling human insulin-dependent diabetes mellitus (Gepts and Lecompte, 1981; Kataoka et al., 1983). The fact that in these mice a progressive lymphocytic infiltration of autoreactive $CD8^+$ T cells into the islets of Langerhans will cause insulitis positioned the model as a model of AID. In another model, considered a good model of rheumatoid arthritis in humans, a probiotic strain prevented the onset of type II collagen-induced arthritis in DBA/1 mice (Kato et al., 1998). Results suggest that the probiotic was able to modify the systemic humoral and cellular immunity and could elicit alterations of the immune state in this model. In general there are many conflicting data on the effect of probiotics in AID. In part this uncertainty comes from the diversity of the strains evaluated, while the different genetic backgrounds of the host might also be an important reason.

FUTURE PERSPECTIVES FOR THE USE OF *IN VIVO* TESTS IN PROBIOTIC RESEARCH

To increase the accuracy of animal models, multi-humanized mice can be considered, carrying functional human genes, cells, tissues, or organs. Immune-deficient mice are often used as recipients for human cells or tissues, because they can relatively easily accept heterologous cells due to lack of host immunity. Traditionally, nude mice and severe combined immunodeficiency (SCID) mice have been used for this purpose, but many other models have been shown to engraft human cells and tissues even more efficiently (Ito et al., 2008). These humanized mouse models may assist to model the human immune system in various scenarios of health and disease, and may enable the evaluation of therapeutic candidates in an *in vivo* setting more close to human physiology. While those specific humanized mice are commonly used in biological and medical research for human therapeutics, they do not frequently appear in probiotic research yet.

Given the importance of the microbiota for many immune and metabolic functionalities of the host, the development and use of mice models with an artificially composed microbiota, e.g., a human microbiota, might help to better mimic the human condition. The use of axenic or monoxenic mice may help to unravel the "egg or the chicken" question mentioned above. The impact of a dietary intervention with or without a microbiota can learn interesting things about the direct influence of the administered probiotic versus e.g., an indirect metabolic or microbiological effect or can show the direct impact on the immune system of any planned intervention.

As for the ethical problem of using animal models, interesting developments such as seen at the Wageningen University in The Netherlands, may bring some solution in the future. *In silico* solutions may try to represent the interactions of the pig gut, the nutrients and other feed/food components with the residing microbes and with epithelial cells. All elements are considered as nodes in a mathematical model, together with their mutual, quantitative dependencies[2]. Using these model interactions, a number of higher level processes related to intestinal immunity, tolerance and barrier functions can possibly be simulated, and conditions as gut homeostasis could possibly be better understood. On a longer term, the model may even evolve into a dynamic and predictive one, allowing to test hypotheses.

DISCOVERING PROBIOTICS WITH GENETICS AND OMICS

STRESS RESPONSES

Over the past two decades several attempts have been made to identify molecular markers that would facilitate the rapid selection of probiotic strains (**Table 3**). To elucidate the complex adaptation mechanisms of probiotic microorganisms under stress conditions, several genetic and omics studies have been conducted in an attempt to identify gene expression and/or protein production patterns related to stress. The exposure of cells to gastric acidity causes reduction of intracellular pH, which adversely affects numerous cell wall and transmembrane-based processes and damages proteins, nucleic acids and other cellular macromolecules. To resist acid stress, microorganisms employ mechanisms aiming at the maintenance of the intracellular pH homeostasis, the repair of macromolecular structures like the cell envelope or ribosomes and other damaged molecules (Lebeer et al., 2008). Heat shock proteins are molecular chaperones involved in the repair of acid-damaged proteins. Many studies have demonstrated that several heat shock proteins, e.g., DnaK, GroES, GroEL, GrpE are induced by acid stress, but their induction varies among different species/strains (Hamon et al., 2014). In parallel, the Clp proteases (e.g., ClpP, ClpE, ClpL) are also induced under acid stress targeting denatured proteins to re-fold them to the appropriate structure or to degrade them if they are beyond repair (Ferreira et al., 2013; Hamon et al., 2014). Genes implicated in DNA repair were also found to be up-regulated under acidic stress (e.g., *uvrB*, *uvrD1*, *vsr;* Jin et al., 2012). Furthermore, an essential component in the response against low pH is the up-regulation of the F_1F_0-ATPase (Sanchez et al., 2008; Jin et al., 2012; Koponen et al., 2012). The various subunits of this multimeric enzyme are being encoded by eight genes found on the *atp* acid inducible operon. The F_1F_0-ATPase lowers the cytoplasmic concentration of protons by virtually extruding them to the extracellular environment at the expense of ATP which can ultimately lead to energy depletion and growth arrest. The composition of the cell envelope is also altered upon exposure to acidic conditions to decrease its permeability to protons. Genes and proteins involved in peptidoglycan biosynthesis (e.g., *manB*, *glmU*, *dapA*, glycosyltransferases), in D-alanylation of lipoteichoic acids (e.g., *dlt* operon), in fatty acid (e.g., *fab* genes) and exopolysaccharide (EPS; e.g., Etk-like tyrosine kinase) biosynthesis were induced to ameliorate the cells' resistance to acid stress (Perea Velez et al., 2007; Jin et al., 2012; Koponen et al., 2012). In addition proteins involved in cell envelope biogenesis (e.g., FabF, RfbB, RfbC) were shown to have a strain-specific role in acid tolerance (Hamon et al., 2014). The gene *luxS* exhibited enhanced expression under acidic conditions in several probiotics, implicating quorum sensing (QS) in acid stress resistance (Moslehi-Jenabian et al., 2009; Koponen et al., 2012).

As discussed above, having survived the hostile environment of the stomach, probiotics next have to face bile in the duodenum. Many transcriptomics and proteomics studies have been performed to determine bile resistance factors in probiotic strains. Interestingly, several of the pathways that are activated during acid stress in the stomach seem to be involved in the ability of probiotics to adapt to bile, as well. Responses against bile include the increased expression of molecular chaperones (e.g., GroEL, GroES, HSP20, DnaK), proteases (e.g., Clps, DegQ), DNA repair proteins (e.g., helicase) and of the F_1F_0-ATPase (Wu et al., 2010; Hamon et al., 2011; Ruiz et al., 2012; Ferreira et al., 2013; An et al., 2014). Genes involved in EPS (e.g., *eps*, *welG*) or fatty acid biosynthesis (e.g., *acc*, *fab*) have been found down-regulated (Koskenniemi et al., 2011; An et al., 2014). In contrast the *dlt* operon was up-regulated (Koskenniemi et al., 2011). It is generally accepted that cells attempt to protect the integrity of the cell envelope by appropriately regulating cell wall and cell membrane processes (Lebeer et al., 2008). Apart from these generic mechanisms that may also be induced by other stresses, there are some that are obviously specific for bile stress. The BSHs encoding genes were found to be up-regulated under bile stress in many studies (Hamon et al., 2011; Koskenniemi et al., 2011; An et al., 2014). However, this is not always the case indicating differences in the regulation of BSH among probiotic strains (Sanchez et al., 2007). Bile export from the cell is another mechanism to counterbalance bile toxicity. Permeases of the major facilitator superfamily (MFS) have been found to be up-regulated and could play a similar role with the bile-inducible efflux transporter BetA. Also ABC (ATP-binding cassette) transporters could play a role in bile expulsion (Koskenniemi et al., 2011; Ruiz et al., 2012; An et al., 2014). Probiotic microorganisms also use multidrug resistance (MDR) bile efflux transporters to actively pump bile salts out of the cytoplasm. Several MDR genes (e.g., *betA*, *ctr*) have been reported to be induced under bile stress (Price et al., 2006; Gueimonde et al., 2009).

The molecular basis of the stress physiology of LAB and other probiotics has advanced rapidly over the past years (Bron et al., 2011; Upadrasta et al., 2011). As our understanding of the stress response mechanisms have increased, a plethora of genes could have been selected as molecular markers for identifying robust probiotic strains. However, this approach has not been followed yet for a number of reasons. A closer look at the lists of genes involved in stress resistance reveals that many of them are well conserved and thus their presence does not reveal anything for the strain under investigation (e.g., heat shock proteins, F_1F_0-ATPase, etc.). In fact, several of them are housekeeping genes involved in central cellular processes and thus it is unlikely that they will be missing from the bacterial genome. In such cases it is the enzymatic activity of the relevant protein or protein complex that is

[2]http://www.wageningenur.nl/en/project/Models-of-dietary-effects-on-immune-responses-in-pigs-6.htm

Table 3 | Potential gene/protein markers related to different probiotic properties identified during genetic and omics studies.

Gene/protein	Function	Representative references
Stress responses (acid and bile)		
Heat shock proteins (e.g., DnaK, GroES, GroEL, GrpE)	Repair of damaged proteins	Wu et al. (2010), Ruiz et al. (2012), Hamon et al. (2014)
Clp proteases (e.g., ClpP, ClpE, ClpL)	Refolding or degrading denatured proteins	Ferreira et al. (2013), Hamon et al. (2014)
uvrB, uvrD1, vsr, helicases	DNA repair	Jin et al. (2012), An et al. (2014)
F_1F_0-ATPase	Decrease of intracellular pH	Sanchez et al. (2008), Hamon et al. (2011), Jin et al. (2012), Koponen et al. (2012)
manB, glmU, dapA, glycosyltransferases	Peptidoglycan biosynthesis	Jin et al. (2012)
dlt operon	D-alanylation of lipoteichoic acids	Perea Velez et al. (2007), Koskenniemi et al. (2011)
fab genes	Fatty acid biosynthesis	Koponen et al. (2012), An et al. (2014)
Etk-like tyrosine kinase, *welG*	Exopolysaccharide biosynthesis	Koskenniemi et al. (2011), Jin et al. (2012)
FabF, RfbB, RfbC	Cell envelope biogenesis	Hamon et al. (2014)
luxS	Cell-to-cell communication	Moslehi-Jenabian et al. (2009), Wu et al. (2010), Koponen et al. (2012)
bsh	Deconjugation of bile salts	Hamon et al. (2011), Koskenniemi et al. (2011), An et al. (2014)
Transporters	Bile efflux	Price et al. (2006), Gueimonde et al. (2009), Koskenniemi et al. (2011), Ruiz et al. (2012), An et al. (2014)
Adhesion		
Mub	Cell-surface proteins with cell wall anchoring motif (LPXTG)	Azcarate-Peril et al. (2008), Mackenzie et al. (2010), Weiss and Jespersen (2010), Turpin et al. (2012)
slpA	S-layer protein	Falentin et al. (2010), Ashida et al. (2011), Beganovic et al. (2011), Turpin et al. (2012)
Apf	Aggregation promoting factor	Ventura et al. (2002), Turpin et al. (2012)
srtA	Sortase-dependent surface protein	Munoz-Provencio et al. (2012)
spaCBA, spaFED, pil2, pil3, fim1, fim2, fim3	Sortase-dependent biosynthesis of pili	Kankainen et al. (2009), Foroni et al. (2011), Gilad et al. (2011), Westermann et al. (2012), Douillard et al. (2013), Turroni et al. (2013)
FbpA, E1 β-subunit of the pyruvate dehydrogenase complex	Fibronectin binding protein	Azcarate-Peril et al. (2008), Weiss and Jespersen (2010), Vastano et al. (2014)
tad	Assembly of tide adherence pilus	Westermann et al. (2012)
Degradation of HMOs and Mucus		
43 kbp gene cluster	Catabolism of HMOs	Sela et al. (2008)
F1SBPs	Import of oligosaccharides	Sela et al. (2008)
β-Galactosidases	Degradations of type-1 and type-2 HMOs	Yoshida et al. (2012)
Glycosylases	Degradation of HMOs	Kim et al. (2009)
Glycosyl hydrolases, exo-α-sialidases, fucosidases, PTS systems, ABC-type carriers, specific permeases, *engBF, afcA,* NagBb, AgnB	Mucin degradation	Yang et al. (2007), Ruas-Madiedo et al. (2008), Turroni et al. (2010a, 2011), Kiyohara et al. (2012), Shimada et al. (2014)

(Continued)

Table 3 | Continued

Gene/protein	Function	Representative references
adh	Adhesion and stimulation of mucin secretion	Mack et al. (2003)
Soluble protein p40	Stimulation of mucin production	Wang et al. (2014)
Modulation of the immune system		
p40 and p75 proteins and homologues	Activation Akt, promotion of cell growth, inhibition of TNF-α	Yan et al. (2007), Bauerl et al. (2010)
ClpB, Rpf	Potential immunogenic proteins	Gilad et al. (2011)
SLPs (SlpA, InlA, LspA, SlpE, SlpB), additional disperse genetic loci	Regulation of anti- or pro-inflammatory immune responses (e.g., induction of the IL-10 and IL-6 regulatory cytokines)	Konstantinov et al. (2008), Meijerink et al. (2010), van Hemert et al. (2010), Beganovic et al. (2011), Le Marechal et al. (2014)
ser	Inhibition of elastases	Turroni et al. (2010b)
Cell surface-associated EPS	Adaptive immune response and protection against the gut pathogen *Citrobacter rodentium*	Fanning et al. (2012)
Flagellin	Induction of human β-defensin 2 (hBD-2)	Schlee et al. (2007)
Production of antimicrobial compounds		
Bacteriocins	Protection against enteropathogens	Corr et al. (2007)
Genes involved in plantaricin biosynthesis and secretion	Regulation of pro- and anti-inflammatory cytokines of DCs	Meijerink et al. (2010)
Quorum sensing		
luxS	Induction of anti-inflammatory cytokines	Jacobi et al. (2012)
	Adhesion and competitive exclusion of pathogens	Buck et al. (2009), Moslehi-Jenabian et al. (2011)
lamBDCA operon, *lamKR* operon	*agr*-like quorum sensing systems	Sturme et al. (2005), Fujii et al. (2008)
Quorum sensing system related peptide (CHWPR)	Induction of *c-myc* and *IL-6* genes in somatic cells	Mitsuma et al. (2008)
Production of nutrients and other beneficial processes		
Vitamins, essential amino acids, SCFAs	*In situ* production of important nutrients	Falentin et al. (2010), Saulnier et al. (2011)
fos	Processing of health-promoting fructooligosaccharides	Ryan et al. (2005), Kim et al. (2009)
ABC carbohydrate transporters	High production of acetate and protection from enteropathogenic infection	Fukuda et al. (2012)
ccpA	Influencing blood cholesterol	Lee et al. (2010)

decisive and it needs to be experimentally confirmed. For example a bile-resistant mutant of *Bifidobacterium lactis* subsp. *animalis* overexpessing the F_1F_0-ATPase resisted acid stress better than the parental strain (Sanchez et al., 2006). Finally, at this stage, very few genes could be directly related to the robustness to a specific stress. The presence of BSHs genes as an example, is indicative for resistance to bile stress. However, this information on its own is not sufficient to provide us with the overall behavior of a strain under the multitude of probiotic stresses. The identification of more sequences, linked to their respective specific phenotypes, may lead to the construction of databases with a more predictive value.

ADHESION TO THE HOST

As discussed above, adhesion to the host's cells could be a significant characteristic of probiotics. Temporary colonization may be necessary for the probiotic to exert its properties e.g., the competitive exclusion of pathogens and the modulation of the immune system. A potential mechanism for adhesion to the host implicates the binding of molecules exposed on the surface of microbial cells to the mucus layer of the host's intestine. Mucus-binding proteins (Mubs) hold an important role in the process of probiotics' adherence to the host. Mubs are cell-surface proteins, characterized by the presence of a *C*-terminal cell wall anchoring motif (LPXTG) and multiple Mub repeats, homologous to the MucBP domains (Boekhorst et al., 2006) which bind to mucins and glycans. Several *mub* genes and Mub proteins have been determined in probiotic strains (Azcarate-Peril et al., 2008; Mackenzie et al., 2010; Weiss and Jespersen, 2010; Turpin et al., 2012). Surface (S-) layer proteins also play a pivotal role in the adhesion of probiotics. The *slpA* gene encoding the surface-layer protein A (SlpA) has been shown to be involved strongly in adhesion capacity (Ashida et al., 2011; Beganovic et al., 2011; Turpin et al., 2012) and has been also identified in the genome of probiotic propionibacteria (Falentin et al., 2010). Some probiotic strains which are devoid of S-layer proteins, encode the aggregation promoting factor (Apf) which shares several features with the S-layer proteins (Ventura et al., 2002; Turpin et al., 2012). Additionally, sortase-dependent surface proteins may also play an important role concerning the adhesion to the host. It has been demonstrated that disruption of the housekeeping sortase gene *srtA* led to reduced bacterial adhesion to epithelial cells (Munoz-Provencio et al., 2012). A major target for bacterial adhesins is fibronectin, an extracellular matrix glycoprotein. A transcriptomics study revealed that the gene encoding a fibronectin-binding protein was significantly up-regulated during incubation in duodenal juice and bile (Weiss and Jespersen, 2010). Genome analysis of a probiotic strain showed that the presence of FbpA protein may be responsible for adhesion to the extracellular matrix of epithelial cells (Azcarate-Peril et al., 2008). In a recent study, Vastano et al. (2014) demonstrated that the moonlighting protein E1 β-subunit of the pyruvate dehydrogenase complex, which is encoded by the *pdhB* gene, is an element related to fibronectin-binding. Some probiotic strains are equipped with proteinaceous surface appendages, such as pili or fimbriae, which facilitate their adhesion to human gut cells. In Gram positive bacteria the assembly of pili relies mostly upon a sortase-dependent mechanism (Mandlik et al., 2008). Gene clusters responsible for the biosynthesis of pili have been identified in the genomes of probiotic lactobacilli (e.g., *spaCBA*, *spaFED*; Kankainen et al., 2009; Douillard et al., 2013) and bifidobacteria (e.g., *pil2*, *pil3*, *fim1*, *fim2*, *fim3*; Gilad et al., 2011; Westermann et al., 2012; Turroni et al., 2013). Moreover, genetic analysis of several probiotic bifidobacteria strains revealed the existence of pilus gene clusters in their genome. Each cluster was organized in an operon and contained the major pilin subunit-encoding gene (*fimA* or *fimP*) along with one or two minor pilin subunit-encoding genes (*fimB* and/or *fimQ*) and a gene encoding a sortase enzyme (*strA*; Foroni et al., 2011). Genes involved in the assembly of the tide adherence (Tad) pilus found in bifidobacteria has also been reported (Westermann et al., 2012). Proteomic and genomic analyses have shown that moonlighting proteins (e.g., ENO, GAPDH, EF-Tu) and proteins related to stress response (e.g., DnaK, GrpE, GroEL, GroES) promote the adhesion of probiotics (Sanchez et al., 2005; Candela et al., 2009; Izquierdo et al., 2009; Gilad et al., 2011; Turpin et al., 2012; Le Marechal et al., 2014).

In contrast to stress related genes, genes involved in adhesion may be more informative about the properties of a strain. The presence of genes encoding adhesive molecules is still considered beneficial for selecting a probiotic strain as it might increase its interaction with the host. The adhesion biology of bacteria (both probiotics and pathogens) is a field evolving fast and detailed molecular mechanisms are being elucidated. These developments are expected to significantly improve the selection of probiotic strains. For example *in silico* analysis of these molecules may help to determine the nature of the adhesion sites (e.g., binding to fibronectin, mucin, etc.). Validation of the predicted adhesive potential of a strain is relatively straightforward with *in vitro* and *in vivo* assays as described earlier.

HUMAN MILK OLIGOSACCHARIDES AND MUCUS DEGRADATION

Mucins can modulate bacterial colonization as a direct energy source (Derrien et al., 2010). The gene pool of bifidobacteria contains several genes involved in the metabolism of human milk oligosaccharides (HMOs) and host-derived carbohydrates, like mucins. These genes allow the adaptation of these bacteria to the human GIT (Pokusaeva et al., 2011; De Bruyn et al., 2013). Genomic analysis of several bifidobacterial strains has given useful insights about the molecular mechanisms supporting these processes.

The genome sequence of *B. longum* subsp. *infantis* ATCC 15697 was found to contain a novel 43 kbp gene cluster encoding genes predicted to be involved in the catabolism of HMOs (Sela et al., 2008) as well as a great number of solute binding proteins (F1SBPs). The latter are part of ABC transporters and they are associated with the import of oligosaccharides. Furthermore, the expression of specific binding proteins related to HMO isomers import was induced under growth on HMO (Garrido et al., 2011). It was also shown that this probiotic bacterium uses two different β-galactosidases for the degradations of type-1 and type-2 HMOs (Yoshida et al., 2012; De Bruyn et al., 2013). Furthermore, in the genome sequence of *B. animalis* subsp. *lactis* AD011, several glycosylases were identified which are associated with the degradation of HMOs (Kim et al., 2009). Proteomic analysis of *B. bifidum* PRL2010 showed that among the mucin induced proteins were

a variety of glycosyl hydrolases, while transcriptional profiling led to the identification of several mucin-induced genes encoding for different components (e.g., exo-α-sialidases, fucosidases, PTS systems, ABC-type carriers, specific permeases; Turroni et al., 2010a, 2011). Two *B. bifidum* strains containing *engBF* and *afcA* genes, encoding for endo-α-*N*-acetylgalactosaminidase and 1,2-α-L-fucosidase respectively, were able to degrade high-molecular weight porcine mucin *in vitro*. The expression of both genes was highly induced in the presence of mucin (Ruas-Madiedo et al., 2008). Moreover, two novel α-*N*-acetylgalactosaminidases from *B. bifidum* JCM 1254 have been identified, NagBb (Kiyohara et al., 2012) and AgnB (Shimada et al., 2014). These enzymes exhibit activity against the core structures in mucin O-glycans. Concerning the probiotic lactobacilli, proteomic analysis of *Lactobacillus fermentum* I5007 after exposure to jejunal environment *in vivo*, disclosed the induction of a glycoside hydrolase, a mucin degrading enzyme (Yang et al., 2007).

In addition, mucins play a crucial role in the protection of the intestinal barrier function. Mucin degradation by intestinal bacteria and its use as a carbon source stimulate goblet cells to increase mucus production. Probiotics may also influence the production of mucin directly during adhesion or through other mechanisms. A mutant of the probiotic *L. plantarum* 299v strain lacking the adhesion gene (*adh*), lost the potential to induce mucin secretion (Mack et al., 2003). A recent study demonstrated that the soluble protein p40 from the probiotic strain *L. rhamnosus* GG, stimulates the activation of epidermal growth factor receptor (EGFR), which promotes the up-regulation of mucin production in goblet cells. Therefore p40 may contribute to the protective mechanism of the intestinal epithelium from injury and inflammation (Wang et al., 2014).

The ability of probiotics to degrade hosts glycans and use host oligosaccharides as an energy source is a very important property. The presence of genes whose products are involved in these processes in the genome of a strain is a clear indication about the adaptation of this particular strain to the GIT. Such strains may have a competitive advantage over other strains in prevailing and colonizing the GIT. The stimulation of mucin production by probiotics is also very interesting as it can facilitate increased binding sites for probiotics and improved gut barrier functions.

MODULATION OF THE IMMUNE SYSTEM

Probiotic bacteria can modulate the response of the host's immune system, interacting with IECs and DCs. It was demonstrated that the p40 and p75 proteins, purified from *L. rhamnosus* GG, stimulate activation of protein kinase B (Akt), promote cell growth, and inhibit TNF-α (Yan et al., 2007). Homologues of these proteins have been also found in several *L. casei* strains (Yan et al., 2007; Bauerl et al., 2010). The study on the extracellular proteome of *B. animalis* subsp. *lactis* BB-12 revealed six proteins with potential immunogenic effect (e.g., ClpB and Rpf; Gilad et al., 2011). In *L. plantarum* strains, six genetic loci were determined with potential impact on the production of the cytokines IL-10 and IL-12 by PBMCs. These loci included genes which might induce anti- or pro-inflammatory immune responses in the intestine (van Hemert et al., 2010). Genetic loci that might regulate the immune response of DCs were also identified in *L. plantarum* WCFS1 (Meijerink et al., 2010). SlpA has been shown to induce IL-10 production in DCs (Konstantinov et al., 2008) and to intensify immune protection by conferring resistance to infection by *Salmonella enterica* serovar Typhimurium FP1 (Beganovic et al., 2011). Moreover, proteomic analysis of the surface proteins of *Propionibacterium freudenreichii* ITG P20 strain revealed that several SLPs (e.g., InlA, LspA, SlpE, SlpA, SlpB) are contributing factors in the induction of the IL-10 and IL-6 regulatory cytokines (Le Marechal et al., 2014).

Serine protease inhibitor (serpin)-encoding genes (*ser*) are found in several bifidobacteria and they are involved in the inhibition of elastases, components related to intestinal inflammation (Turroni et al., 2010b). It has also been shown that *B. breve* UCC2003 produces a cell surface-associated EPS, encoded by each half of a bidirectional gene cluster, which evokes a weak adaptive immune response and it provides protection against the gut pathogen *Citrobacter rodentium* (Fanning et al., 2012).

Defensins are inducible antimicrobial peptides of the innate immune system, which play an important role in host defenses (Ganz, 2003). It has been demonstrated that the protein flagellin, produced by the probiotic strain *E. coli* Nissle 1917, induces the expression of human β-defensin 2 (hBD-2) by the intestinal epithelium (Schlee et al., 2007). Mutants devoid of the gene responsible for the production of flagellin, presented decreased ability to induce hBD-2.

Our understanding of the mechanisms involved in the modulation of the host's immune responses by probiotics is far from complete. Even though several genetic and omics studies have shed some light on the relevant mechanisms there is much ground to be covered. Novel developments in meta-transcriptomics and meta-proteomics are expected to speed up research in this field since they will allow the direct study of the interactions between microorganisms and the cell of the host. Currently, it is very difficult to determine *a priori* whether a strain could modulate immune responses (beneficial or not), based solely on sequence data. This is the main reason making it necessary to assess immunomodulatory properties during screening for probiotics with *in vitro* and/or *in vivo* tests.

PRODUCTION OF ANTIMICROBIAL COMPOUNDS

Probiotic strains often produce an array of antimicrobial compounds. Several of these, like organic acids (e.g., lactic acid) are primary metabolites. In most of these cases, the molecular players of these metabolic pathways for those molecules have been well studied. Other antimicrobial compounds are secondary metabolites, like bacteriocins. The biosynthetic regulons of many bacteriocins have been described and their mode of action has been elucidated. The beneficial effect of bacteriocin-producing probiotic strains against invasive enteropathogens has been well established *in vivo*. It has been demonstrated that the bacteriocin Abp118, produced by *L. salivarius* UCC118, was active against *Listeria monocytogenes* in mice (Corr et al., 2007). High throughput sequencing of bacterial genomes allows the rapid identification of genetic loci related to bacteriocin production and/or immunity. However, bacteriocins should not strictly be considered as antimicrobials. For example it has been demonstrated that six genes of *L. plantarum* WCFS1 associated with the plantaricin biosynthesis and

secretion, regulated the production of pro- and anti-inflammatory cytokines of DCs (Meijerink et al., 2010).

QUORUM SENSING

QS is a communication mechanism among bacterial cells which allows the orchestrated expression of genes within bacterial populations. Its function relies on signaling molecules known as autoinducers. In Gram negative bacteria QS signaling relies on *N*-acylhomoserine lactones (AHL), while in Gram positive bacteria this procedure is dependent upon small cyclic and linear peptides (Waters and Bassler, 2005).

In *L. plantarum* WCFS1 genome an *agr*-like two component regulatory system, encoded by the *lamBDCA* operon, was identified. This operon contained four genes encoding an autoinducing signaling peptide (AIP) modification protein (*lamB*), an AIP (*lamD*), a membrane-located histidine protein kinase (*lamC*) and a cytoplasmic response regulator (*lamA*). This system encodes for a cyclic thiolactone autoinducing peptide (CVGIW) which regulates the adhesion capability of the strain (Sturme et al., 2005). The same strain was shown to carry a second *agr*-like QS system encoded by the *lamKR* operon, which was similar to the *lamBDCA* operon, suggesting analogous function (Fujii et al., 2008). The probiotic strain *E. coli* Nissle 1917 was shown to produce AI-2 molecules (e.g., furanosyl borate diester), involving the *luxS* (autoinducer) gene expression. These molecules were found to influence the induction of anti-inflammatory cytokines in a mouse model of acute colitis (Jacobi et al., 2012). In *L. acidophilus* NCFM, the transcription of the *luxS* gene was notably increased after co-cultivation with live *Listeria monocytogenes* cells, indicating the important role of signaling molecules to the adhesion and the competitive exclusion of pathogens in the GIT (Buck et al., 2009; Moslehi-Jenabian et al., 2011). *B. animalis* subsp. *lactis* BB-12 produces a QS system related peptide (CHWPR). It has been demonstrated that this peptide enhances the expression of two genes in somatic cells, the gene *c-myc*, the deregulation of which has been associated with several forms of cancer, and IL-6, an anti- and pro-inflammatory cytokine (Mitsuma et al., 2008). The role of AI-2 signaling molecule to the adhesive potential of probiotic lactobacilli has also been addressed. It has been established that the disruption of the *luxS* gene reduced significantly the adherence to IECs (Buck et al., 2009).

QS is a very important aspect that may dramatically affect the efficacy of probiotics *in vivo*. Probiotics are entering an already established microbiome, including established biofilms, in which they need to be incorporated. In such an ecological system inter- and intra-species communication is vital to secure temporary colonization of the host, release of antimicrobial compounds and competitive exclusion of pathogens. Evidently, there is an ongoing communication between the microbiome (including any probiotic strain present) and the cells of the host, perhaps beyond immunomodulation. Detailed knowledge about these phenomena may allow us to optimize the probiotic effect.

PRODUCTION OF NUTRIENTS AND OTHER BENEFICIAL PROCESSES

Production of nutrients in the GIT by probiotic bacteria is an essential process for both the host and the microbiome. *In silico* genome analysis of two *L. reuteri* strains revealed genes responsible for the production of vitamins, essential amino acids, lactate and SCFA (Saulnier et al., 2011). It was shown that both strains hold complete biosynthetic pathways for folate and vitamin B12 and that one of the strains carried also a pathway for the production of vitamin B1. Analysis of the *P. freudenreichii* CIRM-BIA1^T genome revealed genes associated with the production of SCFAs and the precursor of menaquinone (vitamin K2), a bifidogenic compound (Falentin et al., 2010). The *fos* gene cluster of *B. animalis* subsp. *lactis* AD011 is another bifidogenic agent, since it is implicated in the processing of health-promoting fructooligosaccharides (Kim et al., 2009). This cluster was shown to have high similarity to the relevant operon described for *B. breve* UCC2003 (Ryan et al., 2005). Furthermore, probiotic bacteria appear to hold a significant role in the modulation of nutrient absorption and in the regulation of the host's energy balance. Several bifidobacteria possess genes encoding for ABC carbohydrate transporters. The latter contribute to the high consumption rate of specific carbohydrates resulting in high production of acetate, a metabolite which confers protection from enteropathogenic infection (Fukuda et al., 2012). Furthermore, genetic and proteomic analysis of *L. acidophilus* A4 revealed that the catabolite control protein A (*ccpA*) is probably involved in the reduction of total serum cholesterol by influencing the expression of several membrane associated proteins. These proteins may play a role in the adhesion of the cholesterol to the bacterial cells and consequently, in the process of lowering blood cholesterol (Lee et al., 2010).

FUTURE PERSPECTIVES FOR THE USE OF OMICS IN PROBIOTIC RESEARCH

Several aspects of the molecular mechanisms that underpin probiotic properties have been elucidated. Original studies relied on molecular analysis of single genes and proteins. Over the last decade the advent of omics technologies have allowed the study of probiotic organisms at the whole genome level (Gueimonde and Collado, 2012). Today, metagenomics methodologies are revealing the composition of complex ecosystems and their biology (Qin et al., 2010; Upadrasta et al., 2011). All niches of the human body carry microbiomes that diverge according to the specific compartment, the age and the dietary habits of the individual and many other factors (Ravel et al., 2014). Meta-omics offer for the first time the proper tools for understanding the *in vivo* behavior of probiotics in contrast to simulated conditions involving pure or only a handful of microbial cultures. As omics technologies become cheaper the genome sequencing of microorganisms will become a routine practice. Incorporation of novel functional data through high throughput transcriptomics and proteomics into databases will ultimately facilitate the *in silico* assessment of the probiotic potential of candidate microorganisms. It is too early to speculate whether *in silico* analysis will totally abolish experimental approaches. It is certain though that as our understanding of the molecular biology of probiotic properties improves, we will be able to design more efficient and more sophisticated *in vitro* and *in vivo* tests. Finally the cataloging of the human microbiome has already opened up the door for considering new categories of microorganisms

as potential probiotics beyond the usual suspects (i.e., LAB and bifidobacteria).

CONCLUSION

In this review we presented a detailed overview of the different methodologies employed for the discovery of new probiotic strains. The diversity of screening assays is considerable and their efficacy variable. Some assays are more applicable for screening high numbers of strains while others are more appropriate for validating the probiotic properties of a handful of strains. There is no bulletproof procedure or workflow for selecting probiotics than perhaps the actual testing of candidate strains on the target population (Rijkers et al., 2010). However, considering the limitations of human trials, traditional *in vitro* and *in vivo* assays along with novel omics approaches will remain important. To take full advantage of probiotics for the health of humans a methodological evolution is needed. For example, new "humanized" animal models may be necessary to study host–microbe interactions. Such developments need to go hand in hand with improvements in legislation, ethics, if we want to meet the scientific and technological challenges of probiotic research.

Today, in most parts of the Western world, the acceptance of health claim dossiers is very difficult. In Europe the use of the term "probiotic" has been banned in communications toward the consumer. In the food area, except for a health claim on lactose tolerance for yogurt, none of the 300 bundled health claim dossiers have been approved. It can be hoped that a more profound study of the mechanisms of action and a better understanding of the microbiome functioning and its dynamics with the host, will provide the health claim evaluation panels with the necessary evidence to consider a wider legal acceptance of health claims. While some of the technological evolutions mentioned in this review have the potential to contribute significantly to this, the willingness to consider new types of probiotics, such as *F. prausnitzii* or *A. muciniphila*, currently not yet available on the market, will be crucial.

Not treated in this review is the importance of the production process. With these new, highly anaerobic organisms such as *F. prausnitzii* or *A. muciniphila* in mind, it will be important for probiotic producers to come up with new production processes and modified preservation and administration strategies to guarantee the delivery of active strains to the consumer or patient. As several papers have shown (van Baarlen et al., 2009; Lebeer et al., 2011, 2012; Bron et al., 2012; van Bokhorst-van de Veen et al., 2012) the (industrial) processing of a probiotic preparation has a fundamental impact on the functionality in the host. Viability, the presence or absence of pili, the cell wall condition, the matrix or the growth stage of the probiotic, they all seem to have an important influence on its performance and its interaction with the host. Defining the mechanism of action of a probiotic might therefore also include some critical parameters of the production process.

Without any doubt, the continued development of omics technologies will assist in alleviating the shortages currently faced with the traditional *in vitro* and *in vivo* models. Although it may take a while before we can predict probiotic functionality directly from genomic and metagenomic information, the use of omics approaches to follow up on interesting *in vitro* or *in vivo* observations is very likely going to speed up research progress in the field of probiotics in the near future.

ACKNOWLEDGMENTS

The present work was cofinanced by the European Social Fund and the National resources EPEAEK and YPEPTH through the Thales project. In addition, this work was cofinanced by the European Regional Development Fund (ERDF) of the EU and by the Greek National Resources under the Operational Program Competitiveness and Entrepreneurship (OPCE II), Action "COOPERATION 2011", Project "ProbioDairyMeat". BP is indebted to the "*Fondation pour la Recherche Médicale*" (FRM), the "*Institut Pasteur de Lille*" (IPL), and the "*Centre National de la Recherche Scientifique*" (CNRS) for research funding.

REFERENCES

Abratt, V. R., and Reid, S. J. (2010). "Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease," in *Advances in Applied Microbiology*, eds S. S. Allen, I. Laskin, and M. G. Geoffrey (San Diego, CA: Academic Press), 63–87.

Alak, J. I., Wolf, B. W., Mdurvwa, E. G., Pimentel-Smith, G. E., and Adeyemo, O. (1997). Effect of *Lactobacillus reuteri* on intestinal resistance to *Cryptosporidium parvum* infection in a murine model of acquired immunodeficiency syndrome. *J. Infect. Dis.* 175, 218–221. doi: 10.1093/infdis/175.1.218

Al Kassaa, I., Hober, D., Hamze, M., Chihib, N. E., and Drider, D. (2014). Antiviral potential of lactic acid bacteria and their bacteriocins. *Probiotics Antimicrob. Proteins* 6, 177–185. doi: 10.1007/s12602-014-9162-6

Amaretti, A., Di Nunzio, M., Pompei, A., Raimondi, S., Rossi, M., and Bordoni, A. (2013). Antioxidant properties of potentially probiotic bacteria: *in vitro* and *in vivo* activities. *Appl. Microbiol. Biotechnol.* 97, 809–817. doi: 10.1007/s00253-012-4241-7

An, H., Douillard, F. P., Wang, G., Zhai, Z., Yang, J., Song, S., et al. (2014). Integrated transcriptomic and proteomic analysis of the bile stress response in a centenarian-originated probiotic *Bifidobacterium longum* BBMN68. *Mol. Cell Proteomics* 13, 2558–2572. doi: 10.1074/mcp.M114.039156

Archambaud, C., Nahori, M. A., Soubigou, G., Becavin, C., Laval, L., Lechat, P., et al. (2012). Impact of lactobacilli on orally acquired listeriosis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 16684–16689. doi: 10.1073/pnas.1212809109

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A., Tsakalidou, E., Nychas, G. J., Panagou, E. Z., et al. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* 33, 282–291. doi: 10.1016/j.fm.2012.10.005

Ashida, N., Yanagihara, S., Shinoda, T., and Yamamoto, N. (2011). Characterization of adhesive molecule with affinity to Caco-2 cells in *Lactobacillus acidophilus* by proteome analysis. *J. Biosci. Bioeng.* 112, 333–337. doi: 10.1016/j.jbiosc.2011.06.001

Aso, Y., Akaza, H., Kotake, T., Tsukamoto, T., Imai, K., and Naito, S. (1995). Preventive effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double-blind trial. The BLP Study Group. *Eur. Urol.* 27, 104–109.

Azcarate-Peril, M. A., Altermann, E., Goh, Y. J., Tallon, R., Sanozky-Dawes, R. B., Pfeiler, E. A., et al. (2008). Analysis of the genome sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. *Appl. Environ. Microbiol.* 74, 4610–4625. doi: 10.1128/AEM.00054-08

Baker, D. G. (1998). Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. *Clin. Microbiol. Rev.* 11, 231–266.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., et al. (2010). Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control* 21, 695–701. doi: 10.1016/j.foodcont.2009.10.010

Barada, K. A., Mourad, F. H., Sawah, S. I., Khoury, C., Safieh-Garabedian, B., Nassar, C. F., et al. (2007). Up-regulation of nerve growth factor and interleukin-10 in inflamed and non-inflamed intestinal segments in rats with experimental colitis. *Cytokine* 37, 236–245. doi: 10.1016/j.cyto.2007.04.005

Barc, M. C., Charrin-Sarnel, C., Rochet, V., Bourlioux, F., Sandre, C., Boureau, H., et al. (2008). Molecular analysis of the digestive microbiota in a gnotobiotic mouse model during antibiotic treatment: influence of *Saccharomyces boulardii*. *Anaerobe* 14, 229–233. doi: 10.1016/j.anaerobe.2008.04.003

Bauerl, C., Perez-Martinez, G., Yan, F., Polk, D. B., and Monedero, V. (2010). Functional analysis of the p40 and p75 proteins from *Lactobacillus casei* BL23. *J. Mol. Microbiol. Biotechnol.* 19, 231–241. doi: 10.1159/000322233

Beganovic, J., Frece, J., Kos, B., Lebos Pavunc, A., Habjanic, K., and Suskovic, J. (2011). Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. *Antonie Van Leeuwenhoek* 100, 43–53. doi: 10.1007/s10482-011-9563-4

Begley, M., Gahan, C. G., and Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiol. Rev.* 29, 625–651. doi: 10.1016/j.femsre.2004.09.003

Bernardeau, M., Guguen, M., and Vernoux, J. P. (2006). Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. *FEMS Microbiol. Rev.* 30, 487–513. doi: 10.1111/j.1574-6976.2006.00020.x

Boekhorst, J., Helmer, Q., Kleerebezem, M., and Siezen, R. J. (2006). Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiology* 152, 273–280. doi: 10.1099/mic.0.28415-0

Boffa, L. C., Lupton, J. R., Mariani, M. R., Ceppi, M., Newmark, H. L., Scalmati, A., et al. (1992). Modulation of colonic epithelial cell proliferation, histone acetylation, and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats. *Cancer Res.* 52, 5906–5912.

Borchers, A. T., Selmi, C., Meyers, F. J., Keen, C. L., and Gershwin, M. E. (2009). Probiotics and immunity. *J. Gastroenterol.* 44, 26–46. doi: 10.1007/s00535-008-2296-0

Borenshtein, D., Mcbee, M. E., and Schauer, D. B. (2008). Utility of the *Citrobacter rodentium* infection model in laboratory mice. *Curr. Opin. Gastroenterol.* 24, 32–37. doi: 10.1097/MOG.0b013e3282f2b0fb

Botta, C., Langerholc, T., Cencic, A., and Cocolin, L. (2014). *In vitro* selection and characterization of new probiotic candidates from table olive microbiota. *PLoS ONE* 9:e94457. doi: 10.1371/journal.pone.0094457

Bove, P., Gallone, A., Russo, P., Capozzi, V., Albenzio, M., Spano, G., et al. (2012). Probiotic features of *Lactobacillus plantarum* mutant strains. *Appl. Microbiol. Biotechnol.* 96, 431–441. doi: 10.1007/s00253-012-4031-2

Bover-Cid, S., and Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food. Microbiol.* 53, 33–41. doi: 10.1016/S0168-1605(99)00152-X

Bron, P., Van Bokhorst-Van De Veen, H., Wels, M., and Kleerebezem, M. (2011). "Engineering robust lactic acid bacteria," in *Stress Responses of Lactic Acid Bacteria*, eds E. Tsakalidou and K. Papadimitriou (New York: Springer), 369–394. doi: 10.1007/978-0-387-92771-8_16

Bron, P. A., Wels, M., Bongers, R. S., Van Bokhorst-Van De Veen, H., Wiersma, A., Overmars, L., et al. (2012). Transcriptomes reveal genetic signatures underlying physiological variations imposed by different fermentation conditions in *Lactobacillus plantarum*. *PLoS ONE* 7:e38720. doi: 10.1371/journal.pone.0038720

Buck, B. L., Azcarate-Peril, M. A., and Klaenhammer, T. R. (2009). Role of autoinducer-2 on the adhesion ability of *Lactobacillus acidophilus*. *J. Appl. Microbiol.* 107, 269–279. doi: 10.1111/j.1365-2672.2009.04204.x

Burns, A. J., and Rowland, I. R. (2004). Antigenotoxicity of probiotics and prebiotics on faecal water-induced DNA damage in human colon adenocarcinoma cells. *Mutat. Res.* 551, 233–243. doi: 10.1016/j.mrfmmm.2004.03.010

Campieri, C., Campieri, M., Bertuzzi, V., Swennen, E., Matteuzzi, D., Stefoni, S., et al. (2001). Reduction of oxaluria after an oral course of lactic acid bacteria at high concentration. *Kidney Int.* 60, 1097–1105. doi: 10.1046/j.1523-1755.2001.0600031097.x

Candela, M., Biagi, E., Centanni, M., Turroni, S., Vici, M., Musiani, F., et al. (2009). Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. *Microbiology* 155, 3294–3303. doi: 10.1099/mic.0.028795-0

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

Cani, P., Everard, A., Belzer, C., and De, V. W. (2014). *Use of Akkermansia for Treating Metabolic Disorders*. Patent no. WO2014075745A1.

Cani, P. D., Lecourt, E., Dewulf, E. M., Sohet, F. M., Pachikian, B. D., Naslain, D., et al. (2009). Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *Am. J. Clin. Nutr.* 90, 1236–1243. doi: 10.3945/ajcn.2009.28095

Cani, P. D., and Van Hul, M. (2015). Novel opportunities for next-generation probiotics targeting metabolic syndrome. *Curr. Opin. Biotechnol.* 32, 21–27. doi: 10.1016/j.copbio.2014.10.006

Castro, M. S., Molina, M. A., Di Sciullo, P., Azpiroz, M. B., Leocata Nieto, F., Sterin-Speziale, N. B., et al. (2010). Beneficial activity of *Enterococcus faecalis* CECT7121 in the anti-lymphoma protective response. *J. Appl. Microbiol.* 109, 1234–1243. doi: 10.1111/j.1365-2672.2010.04747.x

Cencic, A., and Langerholc, T. (2010). Functional cell models of the gut and their applications in food microbiology-a review. *Int. J. Food Microbiol.* 141(Suppl. 1), S4–S14. doi: 10.1016/j.ijfoodmicro.2010.03.026

Céspedes, M., Cárdenas, P., Staffolani, M., Ciappini, M. C., and Vinderola, G. (2013). Performance in nondairy drinks of probiotic *L. casei* strains usually employed in dairy products. *J. Food Sci.* 78, M756–M762. doi: 10.1111/1750-3841.12092

Challa, A., Rao, D. R., Chawan, C. B., and Shackelford, L. (1997). *Bifidobacterium longum* and lactulose suppress azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis* 18, 517–521. doi: 10.1093/carcin/18.3.517

Charteris, W. P., Kelly, P. M., Morelli, L., and Collins, J. K. (1998). Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J. Appl. Microbiol.* 84, 759–768. doi: 10.1046/j.1365-2672.1998.00407.x

Chen, C. C., Louie, S., Shi, H. N., and Walker, W. A. (2005). Preinoculation with the probiotic *Lactobacillus acidophilus* early in life effectively inhibits murine *Citrobacter rodentium* colitis. *Pediatr. Res.* 58, 1185–1191. doi: 10.1203/01.pdr.0000183660.39116.83

Chen, X., Fruehauf, J., Goldsmith, J. D., Xu, H., Katchar, K. K., Koon, H. W., et al. (2009). *Saccharomyces boulardii* inhibits EGF receptor signaling and intestinal tumor growth in *Apc^{min}* mice. *Gastroenterology* 137, 914–923. doi: 10.1053/j.gastro.2009.05.050

Chen, X., Katchar, K., Goldsmith, J. D., Nanthakumar, N., Cheknis, A., Gerding, D. N., et al. (2008). A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology* 135, 1984–1992. doi: 10.1053/j.gastro.2008.09.002

Chenoll, E., Casinos, B., Bataller, E., Astals, P., Echevarría, J., Iglesias, J. R., et al. (2011). Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. *Appl. Environ. Microbiol.* 77, 1335–1343. doi: 10.1128/AEM.01820-10

Choi, S. S., Kim, Y., Han, K. S., You, S., Oh, S., and Kim, S. H. (2006). Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress *in vitro*. *Lett. Appl. Microbiol.* 42, 452–458. doi: 10.1111/j.1472-765X.2006.01913.x

Chong, E. (2014). A potential role of probiotics in colorectal cancer prevention: review of possible mechanisms of action. *World J. Microbiol. Biotechnol.* 30, 351–374. doi: 10.1007/s11274-013-1499-6

Claes, I. J., De Keersmaecker, S. C., Vanderleyden, J., and Lebeer, S. (2011). Lessons from probiotic-host interaction studies in murine models of experimental colitis. *Mol. Nutr. Food Res.* 55, 1441–1453. doi: 10.1002/mnfr.201100139

Collado, M., 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

Collado, M. C., Meriluoto, J., and Salminen, S. (2007). Measurement of aggregation properties between probiotics and pathogens: *in vitro* evaluation of different methods. *J. Microbiol. Methods* 71, 71–74. doi: 10.1016/j.mimet.2007.07.005

Collins, J. W., Chervaux, C., Raymond, B., Derrien, M., Brazeilles, R., Kosta, A., et al. (2014). Fermented dairy products modulate *Citrobacter rodentium*-induced colonic hyperplasia. *J. Infect. Dis.* 210, 1029–1041. doi: 10.1093/infdis/jiu205

Coman, M. M., Verdenelli, M. C., Cecchini, C., Silvi, S., Orpianesi, C., Boyko, N., et al. (2014). *In vitro* evaluation of antimicrobial activity of *Lactobacillus rhamnosus* IMC 501®, *Lactobacillus paracasei* IMC 502® and SYNBIO® against pathogens. *J. Appl. Microbiol.* 117, 518–527. doi: 10.1111/jam.12544

Commane, D. M., Shortt, C. T., Silvi, S., Cresci, A., Hughes, R. M., and Rowland, I. R. (2005). Effects of fermentation products of pro- and prebiotics on trans-epithelial electrical resistance in an *in vitro* model of the colon. *Nutr. Cancer* 51, 102–109. doi: 10.1207/s15327914nc5101-14

Comuzzie, A. G., and Allison, D. B. (1998). The search for human obesity genes. *Science* 280, 1374–1377. doi: 10.1126/science.280.5368.1374

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. doi: 10.3168/jds.S0022-0302(87)79974-3

Cook, M. T., Tzortzis, G., Charalampopoulos, D., and Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *J. Control Release* 162, 56–67. doi: 10.1016/j.jconrel.2012.06.003

Corr, S. C., Li, Y., Riedel, C. U., O'toole, P. W., Hill, C., and Gahan, C. G. (2007). Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7617–7621. doi: 10.1073/pnas.0700440104

Corthesy, B., Gaskins, H. R., and Mercenier, A. (2007). Cross-talk between probiotic bacteria and the host immune system. *J. Nutr.* 137, 781S–790S.

Cousin, F. J., Jouan-Lanhouet, S., Dimanche-Boitrel, M.-T., Corcos, L., and Jan, G. (2012). Milk fermented by *Propionibacterium freudenreichii* induces apoptosis of HGT-1 human gastric cancer cells. *PLoS ONE* 7:e31892. doi: 10.1371/journal.pone.0031892

Cresci, G., Nagy, L. E., and Ganapathy, V. (2013). *Lactobacillus* GG and tributyrin supplementation reduce antibiotic-induced intestinal injury. *J. Parenter. Enteral Nutr.* 37, 763–774. doi: 10.1177/0148607113486809

Cross, M. L. (2002). Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunol. Med. Microbiol.* 34, 245–253. doi: 10.1111/j.1574-695X.2002.tb00632.x

Dashkevicz, M. P., and Feighner, S. D. (1989). Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. *Appl. Environ. Microbiol.* 55, 11–16.

De Bruyn, F., Beauprez, J., Maertens, J., Soetaert, W., and De Mey, M. (2013). Unraveling the Leloir pathway of *Bifidobacterium bifidum*: significance of the uridylyltransferases. *Appl. Environ. Microbiol.* 79, 7028–7035. doi: 10.1128/AEM.02460-13

de LeBlanc Ade, M., Castillo, N. A., and Perdigon, G. (2010). Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar Typhimurium infection. *Int. J. Food Microbiol.* 138, 223–231. doi: 10.1016/j.ijfoodmicro.2010.01.020

Delgado, S., O'sullivan, E., Fitzgerald, G., and Mayo, B. (2007). Subtractive screening for probiotic properties of *Lactobacillus* species from the human gastrointestinal tract in the search for new probiotics. *J. Food Sci.* 72, M310–M315. doi: 10.1111/j.1750-3841.2007.00479.x

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett. Appl. Microbiol.* 31, 438–442. doi: 10.1046/j.1365-2672.2000.00845.x

Derrien, M., Van Passel, M. W., Van De Bovenkamp, J. H., Schipper, R. G., De Vos, W. M., and Dekker, J. (2010). Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes* 1, 254–268. doi: 10.4161/gmic.1.4.12778

Douillard, F. P., Ribbera, A., Jarvinen, H. M., Kant, R., Pietila, T. E., Randazzo, C., et al. (2013). Comparative genomic and functional analysis of *Lactobacillus casei* and *Lactobacillus rhamnosus* strains marketed as probiotics. *Appl. Environ. Microbiol.* 79, 1923–1933. doi: 10.1128/AEM.0346712

Duangjitcharoen, Y., Kantachote, D., Prasitpuripreecha, C., Peerajan, S., and Chaiyasut, C. (2014). Selection and characterisation of probiotic lactic acid bacteria with heterocyclic amine binding and nitrosamine degradation properties. *J. Appl. Pharm. Sci.* 4, 014–023. doi: 10.7324/JAPS.2014.40703

Duncan, S. H., Lobley, G. E., Holtrop, G., Ince, J., Johnstone, A. M., Louis, P., et al. (2008). Human colonic microbiota associated with diet, obesity and weight loss. *Int. J. Obes. (Lond.)* 32, 1720–1724. doi: 10.1038/ijo.2008.155

Eaton, K. A., Honkala, A., Auchtung, T. A., and Britton, R. A. (2011). Probiotic *Lactobacillus reuteri* ameliorates disease due to enterohemorrhagic *Escherichia coli* in germfree mice. *Infect. Immun.* 79, 185–191. doi: 10.1128/IAI.00880-10

Ebel, B., Lemetais, G., Beney, L., Cachon, R., Sokol, H., Langella, P., et al. (2014). Impact of probiotics on risk factors for cardiovascular diseases. *Crit. Rev. Food Sci. Nutr.* 54, 175–189. doi: 10.1080/10408398.2011.579361

Eck, P., and Friel, J. (2013). Should probiotics be considered as vitamin supplements? *Vitam. Miner.* 3, e124. doi: 10.4172/vms.1000e124

EFSA. (2008). 2008 Annual report on pesticide residues according to Article 32 of Regulation (EC) No 396/2005. *EFSA J. 2010.* 8:1646.

Eutamene, H., Lamine, F., Chabo, C., Theodorou, V., Rochat, F., Bergonzelli, G. E., et al. (2007). Synergy between *Lactobacillus paracasei* and its bacterial products to counteract stress-induced gut permeability and sensitivity increase in rats. *J. Nutr.* 137, 1901–1907.

Evans, N. P., Misyak, S. A., Schmelz, E. M., Guri, A. J., Hontecillas, R., and Bassaganya-Riera, J. (2010). Conjugated linoleic acid ameliorates inflammation-induced colorectal cancer in mice through activation of PPARgamma. *J. Nutr.* 140, 515–521. doi: 10.3945/jn.109.115642

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., et al. (2013). Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9066–9071. doi: 10.1073/pnas.1219451110

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 doi: 10.1152/ajpgi.90227.2008

Ewaschuk, J. B., Walker, J. W., Diaz, H., and Madsen, K. L. (2006). Bioproduction of conjugated linoleic acid by probiotic bacteria occurs *in vitro* and *in vivo* in mice. *J. Nutr.* 136, 1483–1487.

Falentin, H., Deutsch, S. M., Jan, G., Loux, V., Thierry, A., Parayre, S., et al. (2010). The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1T, a hardy actinobacterium with food and probiotic applications. *PLoS ONE* 5:e11748. doi: 10.1371/journal.pone.0011748

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

Faridnia, F., Hussin, A. S., Saari, N., Mustafa, S., Yee, L. Y., and Manap, M. Y. (2010). *In vitro* binding of mutagenic heterocyclic aromatic amines by *Bifidobacterium pseudocatenulatum* G4. *Benef. Microbes* 1, 149–154. doi: 10.3920/BM2009.0035

Fasseas, M. K., Fasseas, C., Mountzouris, K. C., and Syntichaki, P. (2013). Effects of *Lactobacillus salivarius*, *Lactobacillus reuteri*, and *Pediococcus acidilactici* on the nematode *Caenorhabditis elegans* include possible antitumor activity. *Appl. Microbiol. Biotechnol.* 97, 2109–2118. doi: 10.1007/s00253-012-4357-9

Ferreira, A. B., De Oliveira, M. N., Freitas, F. S., Alfenas-Zerbini, P., Da Silva, D. F., De Queiroz, M. V., et al. (2013). Increased expression of *c/p* genes in *Lactobacillus delbrueckii* UFV H2b20 exposed to acid stress and bile salts. *Benef. Microbes* 4, 367–374. doi: 10.3920/BM2013.0022

Ferreira, C. L., Grześkowiak, L., Collado, M. C., and Salminen, S. (2011). *In vitro* evaluation of *Lactobacillus gasseri* strains of infant origin on adhesion and aggregation of specific pathogens. *J. Food Prot.* 74, 1482–1487. doi: 10.4315/0362-028X.JFP-11-074

Fitzpatrick, L. R., Small, J. S., Greene, W. H., Karpa, K. D., and Keller, D. (2011). *Bacillus coagulans* GBI-30 (BC30) improves indices of *Clostridium difficile*-induced colitis in mice. *Gut Pathog.* 3, 16. doi: 10.1186/1757-4749-3-16

Fleming, A., Jankowski, J., and Goldsmith, P. (2010). *In vivo* analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: a feasibility study. *Inflamm. Bowel Dis.* 16, 1162–1172. doi: 10.1002/ibd.21200

Foligne, B., Deutsch, S. M., Breton, J., Cousin, F. J., Dewulf, J., Samson, M., et al. (2010a). Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced *in vitro* and in *vivo*. *Appl. Environ. Microbiol.* 76, 8259–8264. doi: 10.1128/AEM.01976-10

Foligne, B., Dewulf, J., Vandekerckove, P., Pignede, G., and Pot, B. (2010b). Probiotic yeasts: anti-inflammatory potential of various non-pathogenic strains in experimental colitis in mice. *World J. Gastroenterol.* 16, 2134–2145. doi: 10.3748/wjg.v16.i17.2134

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., et al. (2007). Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. *World J. Gastroenterol.* 13, 236–243. doi: 10.3748/wjg.v13.i2.236

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., and Gil, A. (2013). Sources, isolation, characterisation, and evaluation of probiotics. *Br. J. Nutr.* 109(Suppl. 2), S35–S50. doi: 10.1017/S0007114512004011

Foroni, E., Serafini, F., Amidani, D., Turroni, F., He, F., Bottacini, F., et al. (2011). Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb. Cell Fact.* 10(Suppl. 1), S16. doi: 10.1186/1475-2859-10-S1-S16

Fujii, T., Ingham, C., Nakayama, J., Beerthuyzen, M., Kunuki, R., Molenaar, D., et al. (2008). Two homologous Agr-like quorum-sensing systems cooperatively control adherence, cell morphology, and cell viability properties in *Lactobacillus plantarum* WCFS1. *J. Bacteriol.* 190, 7655–7665. doi: 10.1128/JB.01489

Fujiwara, D., Inoue, S., Wakabayashi, H., and Fujii, T. (2004). The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance. *Int. Arch. Allergy Immunol.* 135, 205–215. doi: 10.1159/000081305

Fukuda, S., Toh, H., Taylor, T. D., Ohno, H., and Hattori, M. (2012). Acetate-producing bifidobacteria protect the host from enteropathogenic infection via carbohydrate transporters. *Gut Microbes* 3, 449–454. doi: 10.4161/gmic.21214

Furrie, E. (2005). Probiotics and allergy. *Proc. Nutr. Soc.* 64, 465–469. doi: 10.1079/PNS2005466

Gagnon, M., Zihler, A., Chassard, C., and Lacroix, C. (2011). "Ecology of probiotics and enteric protection," in *Probiotic Bacteria and Enteric Infections*, eds J. J. Malago, J. F. J. G. Koninkx, and R. Marinsek-Logar (Netherlands: Springer), 65–85. doi: 10.1007/978-94-007-0386-5_3

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 3, 710–720. doi: 10.1038/nri1180

García-Cayuela, T., Korany, A. M., Bustos, I., Gómez De Cadiñanos, L. P., Requena, T., Peláez, C., et al. (2014). Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. *Food Res. Int.* 57, 44–50. doi: 10.1016/j.foodres.2014.01.010

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

Gepts, W., and Lecompte, P. M. (1981). The pancreatic islets in diabetes. *Am. J. Med.* 70, 105–115. doi: 10.1016/0002-9343(81)90417-4

Gilad, O., Svensson, B., Viborg, A. H., Stuer-Lauridsen, B., and Jacobsen, S. (2011). The extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* BB-12 reveals proteins with putative roles in probiotic effects. *Proteomics* 11, 2503–2514. doi: 10.1002/pmic.201000716

Gilbert, J. A., Krajmalnik-Brown, R., Porazinska, D. L., Weiss, S. J., and Knight, R. (2013). Toward effective probiotics for autism and other neurodevelopmental disorders. *Cell* 155, 1446–1448. doi: 10.1016/j.cell.2013.11.035

Gill, H., Grover, S., Batish, V., and Gill, P. (2009). "Immunological effects of probiotics and their significance to human health," in *Prebiotics and Probiotics Science and Technology*, eds D. Charalampopoulos and R. Rastall (New York: Springer), 901–948. doi: 10.1007/978-0-387-79058-9_23

Gioacchini, G., Giorgini, E., Olivotto, I., Maradonna, F., Merrifield, D. L., and Carnevali, O. (2014). The influence of probiotics on zebrafish *Danio rerio* innate immunity and hepatic stress. *Zebrafish* 11, 98–106. doi: 10.1089/zeb.2013.0932

Goldin, B. R., and Gorbach, S. L. (1984). Alterations of the intestinal microflora by diet, oral antibiotics, and *Lactobacillus*: decreased production of free amines from aromatic nitro compounds, azo dyes, and glucuronides. *J. Natl. Cancer Inst.* 73, 689–695.

Gorbach, S. L., and Goldin, B. R. (1992). Nutrition and the gastrointestinal microflora. *Nutr. Rev.* 50, 378–381. doi: 10.1111/j.1753-4887.1992.tb02485.x

Grompone, G., Martorell, P., Llopis, S., Gonzalez, N., Genoves, S., Mulet, A. P., et al. (2012). Anti-inflammatory *Lactobacillus rhamnosus* CNCM I-3690 strain protects against oxidative stress and increases lifespan in *Caenorhabditis elegans*. *PLoS ONE* 7:e52493. doi: 10.1371/journal.pone.0052493

Gueimonde, M., and Collado, M. C. (2012). Metagenomics and probiotics. *Clin. Microbiol. Infect.* 18(Suppl. 4), 32–34. doi: 10.1111/j.1469-0691.2012.03873.x

Gueimonde, M., Garrigues, C., Van Sinderen, D., De Los Reyes-Gavilan, C. G., and Margolles, A. (2009). Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. *Appl. Environ. Microbiol.* 75, 3153–3160. doi: 10.1128/AEM.00172-09

Guo, X. H., Kim, J. M., Nam, H. M., Park, S. Y., and Kim, J. M. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. *Anaerobe* 16, 321–326. doi: 10.1016/j.anaerobe.2010.03.006

Halász, A., Baráth, Á., Simon-Sarkadi, L., and Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* 5, 42–49. doi: 10.1016/0924-2244(94)90070-1

Halttunen, T., Salminen, S., and Tahvonen, R. (2007). Rapid removal of lead and cadmium from water by specific lactic acid bacteria. *Int. J. Food Microbiol.* 114, 30–35. doi: 10.1016/j.ijfoodmicro.2006.10.040

Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner, D., et al. (2011). Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. *BMC Microbiol.* 11:63. doi: 10.1186/1471-2180-11-63

Hamon, E., Horvatovich, P., Marchioni, E., Aoude-Werner, D., and Ennahar, S. (2014). Investigation of potential markers of acid resistance in *Lactobacillus plantarum* by comparative proteomics. *J. Appl. Microbiol.* 116, 134–144. doi: 10.1111/jam.12339

Hapfelmeier, S., and Hardt, W. D. (2005). A mouse model for *S. typhimurium*-induced enterocolitis. *Trends Microbiol.* 13, 497–503. doi: 10.1016/j.tim.2005.08.008

Harty, D. W., Oakey, H. J., Patrikakis, M., Hume, E. B., and Knox, K. W. (1994). Pathogenic potential of lactobacilli. *Int. J. Food Microbiol.* 24, 179–189. doi: 10.1016/0168-1605(94)90117-1

Haukioja, A. (2010). Probiotics and oral health. *Eur. J. Dent.* 4, 348–355.

He, Q., Wang, L., Wang, F., Wang, C., Tang, C., Li, Q., et al. (2013). Microbial fingerprinting detects intestinal microbiota dysbiosis in Zebrafish models with chemically-induced enterocolitis. *BMC Microbiol.* 13:289. doi: 10.1186/1471-2180-13-289

Helm, R. M., and Burks, A. W. (2002). Animal models of food allergy. *Curr. Opin. Allergy Clin. Immunol.* 2, 541–546. doi: 10.1097/01.all.0000044541.45448.bb

Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W. Z., Strowig, T., et al. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 482, 179–185. doi: 10.1038/nature10809

Hickson, M. (2011). Probiotics in the prevention of antibiotic-associated diarrhoea and *Clostridium difficile* infection. *Therap. Adv. Gastroenterol.* 4, 185–197. doi: 10.1177/1756283X11399115

Hildebrandt, M. A., Hoffmann, C., Sherrill-Mix, S. A., Keilbaugh, S. A., Hamady, M., Chen, Y. Y., et al. (2009). High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology* 137, 1716.e1-2 – 1724.e1-2. doi: 10.1053/j.gastro.2009.08.042

Hsiao, E. Y., Mcbride, S. W., Hsien, S., Sharon, G., Hyde, E. R., Mccue, T., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155, 1451–1463. doi: 10.1016/j.cell.2013.11.024

Hughes, D. B., and Hoover, D. G. (1995). Viability and enzymatic activity of bifidobacteria in milk. *J. Dairy Sci.* 78, 268–276. doi: 10.3168/jds.S0022-0302(95)76634-6

Hummel, K. P., Dickie, M. M., and Coleman, D. L. (1966). Diabetes, a new mutation in the mouse. *Science* 153, 1127–1128. doi: 10.1126/science.153.3740.1127

Ichinohe, T., Pang, I. K., Kumamoto, Y., Peaper, D. R., Ho, J. H., Murray, T. S., et al. (2011). Microbiota regulates immune defense against respiratory tract influenza a virus infection. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5354–5359. doi: 10.1073/pnas.1019378108

Ingalls, A. M., Dickie, M. M., and Snell, G. D. (1950). Obese, a new mutation in the house mouse. *J. Hered.* 41, 317–318

Ito, M., Kobayashi, K., and Nakahata, T. (2008). "NOD/Shi-*scid IL2rγ*^{null} (NOG) mice more appropriate for humanized mouse models," in *Humanized Mice*, eds T. Nomura, T. Watanabe, and S. Habu (Berlin: Springer), 53–76.

Izquierdo, E., Horvatovich, P., Marchioni, E., Aoude-Werner, D., Sanz, Y., and Ennahar, S. (2009). 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus plantarum*, a first step toward early selection of probiotics based on bacterial biomarkers. *Electrophoresis* 30, 949–956. doi: 10.1002/elps.200800399

Jacobi, C. A., Grundler, S., Hsieh, C. J., Frick, J. S., Adam, P., Lamprecht, G., et al. (2012). Quorum sensing in the probiotic bacterium *Escherichia coli* Nissle 1917 (Mutaflor) - evidence that furanosyl borate diester (AI-2) is influencing the cytokine expression in the DSS colitis mouse model. *Gut Pathog.* 4:8. doi: 10.1186/1757-4749-4-8

Jacobsen, C. N., Rosenfeldt Nielsen, V., Hayford, A. E., Moller, P. L., Michaelsen, K. F., Paerregaard, A., et al. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the

colonization ability of five selected strains in humans. *Appl. Environ. Microbiol.* 65, 4949–4956.

Jena, P. K., Trivedi, D., Thakore, K., Chaudhary, H., Giri, S. S., and Seshadri, S. (2013). Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. *Microbiol. Immunol.* 57, 407–416. doi: 10.1111/1348-0421.12054

Jin, J., Zhang, B., Guo, H., Cui, J., Jiang, L., Song, S., et al. (2012). Mechanism analysis of acid tolerance response of *Bifidobacterium longum* subsp. *longum* BBMN 68 by gene expression profile using RNA-sequencing. *PLoS ONE* 7:e50777. doi: 10.1371/journal.pone.0050777

Johnson-Henry, K. C., Nadjafi, M., Avitzur, Y., Mitchell, D. J., Ngan, B. Y., Galindo-Mata, E., et al. (2005). Amelioration of the effects of *Citrobacter rodentium* infection in mice by pretreatment with probiotics. *J. Infect. Dis.* 191, 2106–2117. doi: 10.1086/430318

Joint FAO/WHO Working Group. (2002). *Report on Drafting Guidelines for the Evaluation of Probiotics in Food: Guidelines for the Evaluation of Probiotics in Food.* London, ON: FAO/WHO.

Jones, R. M., Luo, L., Ardita, C. S., Richardson, A. N., Kwon, Y. M., Mercante, J. W., et al. (2013). Symbiotic lactobacilli stimulate gut epithelial proliferation *via* Nox-mediated generation of reactive oxygen species. *EMBO J.* 32, 3017–3028. doi: 10.1038/emboj.2013.224

Kang, M.-S., Kim, B.-G., Chung, J., Lee, H.-C., and Oh, J.-S. (2006). Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds. *J. Clin. Periodontol.* 33, 226–232. doi: 10.1111/j.1600-051X.2006.00893.x

Kankainen, M., Paulin, L., Tynkkynen, S., Von Ossowski, I., Reunanen, J., Partanen, P., et al. (2009). Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 106, 17193–17198. doi: 10.1073/pnas.0908876106

Kataoka, S., Satoh, J., Fujiya, H., Toyota, T., Suzuki, R., Itoh, K., et al. (1983). Immunologic aspects of the nonobese diabetic (NOD) mouse. Abnormalities of cellular immunity. *Diabetes* 32, 247–253. doi: 10.2337/diab.32.3.247

Kato, I., Endo-Tanaka, K., and Yokokura, T. (1998). Suppressive effects of the oral administration of *Lactobacillus casei* on type II collagen-induced arthritis in DBA/1 mice. *Life Sci.* 63, 635–644. doi: 10.1016/S0024-3205(98)00315-4

Kaur, S., Vaishnavi, C., Ray, P., Kochhar, R., and Prasad, K. K. (2010). Effect of biotherapeutics on cyclosporin-induced *Clostridium difficile* infection in mice. *J. Gastroen. Hepatol.* 25, 832–838. doi: 10.1111/j.1440-1746.2009.06135.x

Kaushik, J. K., Kumar, A., Duary, R. K., Mohanty, A. K., Grover, S., and Batish, V. K. (2009). Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PLoS ONE* 4:e8099. doi: 10.1371/journal.pone.0008099

Kechaou, N., Chain, F., Gratadoux, J. J., Blugeon, S., Bertho, N., Chevalier, C., et al. (2013). Identification of one novel candidate probiotic *Lactobacillus plantarum* strain active against influenza virus infection in mice by a large-scale screening. *Appl. Environ. Microbiol.* 79, 1491–1499. doi: 10.1128/AEM.03075-12

Kikuchi, Y., Kunitoh-Asari, A., Hayakawa, K., Imai, S., Kasuya, K., Abe, K., et al. (2014). Oral administration of *Lactobacillus plantarum* strain AYA enhances IgA secretion and provides survival protection against influenza virus infection in mice. *PLoS ONE* 9:e86416. doi: 10.1371/journal.pone.0086416

Kim, H. J., Kim, Y. J., Lee, S. H., Yu, J., Jeong, S. K., and Hong, S. J. (2014). Effects of *Lactobacillus rhamnosus* on allergic march model by suppressing Th2, Th17, and TSLP responses via $CD4^+CD25^+Foxp3^+$ Tregs. *Clin. Immunol.* 153, 178–186. doi: 10.1016/j.clim.2014.04.008

Kim, H. S., and Gilliland, S. E. (1983). *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *J. Dairy Sci.* 66, 959–966. doi: 10.3168/jds.S0022-0302(83)81887-6

Kim, J. F., Jeong, H., Yu, D. S., Choi, S. H., Hur, C. G., Park, M. S., et al. (2009). Genome sequence of the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* AD011. *J. Bacteriol.* 191, 678–679. doi: 10.1128/JB.01515-08

Kim, J. Y., Park, B. K., Park, H. J., Park, Y. H., Kim, B. O., and Pyo, S. (2013). Atopic dermatitis-mitigating effects of new *Lactobacillus* strain, *Lactobacillus sakei* probio 65 isolated from Kimchi. *J. Appl. Microbiol.* 115, 517–526. doi: 10.1111/jam.12229

Kim, K. H., and Park, H. S. (2003). Dietary supplementation of conjugated linoleic acid reduces colon tumor incidence in DMH-treated rats by increasing apoptosis with modulation of biomarkers. *Nutrition* 19, 772–777. doi: 10.1016/S0899-9007(03)00098-4

Kinoshita, H., Imoto, S., Suda, Y., Ishida, M., Watanabe, M., Kawai, Y., et al. (2013). Proposal of screening method for intestinal mucus adhesive lactobacilli using the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Anim. Sci. J.* 84, 150–158. doi: 10.1111/j.1740-0929.2012.01054.x

Kirjavainen, P. V., Ouwehand, A. C., Isolauri, E., and Salminen, S. J. (1998). The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol. Lett.* 167, 185–189. doi: 10.1111/j.1574-6968.1998.tb13226.x

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

Knight, A. (2007). Animal experiments scrutinised: systematic reviews demonstrate poor human clinical and toxicological utility. *ALTEX* 24, 320–325.

Konstantinov, S. R., Smidt, H., De Vos, W. M., Bruijns, S. C., Singh, S. K., Valence, F., et al. (2008). S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19474–19479. doi: 10.1073/pnas.0810305105

Koponen, J., Laakso, K., Koskenniemi, K., Kankainen, M., Savijoki, K., Nyman, T. A., et al. (2012). Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG. *J. Proteomics* 75, 1357–1374. doi: 10.1016/j.jprot.2011.11.009

Koskenniemi, K., Laakso, K., Koponen, J., Kankainen, M., Greco, D., Auvinen, P., et al. (2011). Proteomics and transcriptomics characterization of bile stress response in probiotic *Lactobacillus rhamnosus* GG. *Mol. Cell. Proteomics* 10, M110.002741. doi: 10.1074/mcp.M110.002741

Kostic, A. D., Howitt, M. R., and Garrett, W. S. (2013). Exploring host-microbiota interactions in animal models and humans. *Genes Dev.* 27, 701–718. doi: 10.1101/gad.212522.112

Krasse, P., Carlsson, B., Dahl, C., Paulsson, A., Nilsson, A., and Sinkiewicz, G. (2006). Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*. Swed. *Dent. J.* 30, 55–60.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., et al. (2002). Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.* 72, 215–224. doi: 10.1016/S0168-1605(01)00674-2

Kumar, M., Kumar, A., Nagpal, R., Mohania, D., Behare, P., Verma, V., et al. (2010). Cancer-preventing attributes of probiotics: an update. *Int. J. Food Sci. Nutr.* 61, 473–496. doi: 10.3109/09637480903455971

Kwon, H. K., Kim, G. C., Kim, Y., Hwang, W., Jash, A., Sahoo, A., et al. (2013). Amelioration of experimental autoimmune encephalomyelitis by probiotic mixture is mediated by a shift in T helper cell immune response. *Clin. Immunol.* 146, 217–227. doi: 10.1016/j.clim.2013.01.001

Lahtinen, S. J., Jalonen, L., Ouwehand, A. C., and Salminen, S. J. (2007). Specific *Bifidobacterium* strains isolated from elderly subjects inhibit growth of *Staphylococcus aureus*. *Int. J. Food Microbiol.* 117, 125–128. doi: 10.1016/j.ijfoodmicro.2007.02.023

Lahteinen, T., Malinen, E., Koort, J. M., Mertaniemi-Hannus, U., Hankimo, T., Karikoski, N., et al. (2010). Probiotic properties of *Lactobacillus* isolates originating from porcine intestine and feces. *Anaerobe* 16, 293–300. doi: 10.1016/j.anaerobe.2009.08.002

Laparra, J. M., and Sanz, Y. (2009). Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium. *Lett. Appl. Microbiol.* 49, 695–701. doi: 10.1111/j.1472-765X.2009.02729.x

Lavermicocca, P., Valerio, F., Lonigro, S. L., Di Leo, A., and Visconti, A. (2008). Antagonistic activity of potential probiotic lactobacilli against the ureolytic pathogen *Yersinia enterocolitica*. *Curr. Microbiol.* 56, 175–181. doi: 10.1007/s00284-007-9069-5

Lebeer, S., Claes, I., Tytgat, H. L., Verhoeven, T. L., Marien, E., Von Ossowski, I., et al. (2012). Functional analysis of *Lactobacillus rhamnosus* GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. *Appl. Environ. Microbiol.* 78, 185–193. doi: 10.1128/AEM.06192-11

Lebeer, S., Claes, I. J. J., Verhoeven, T. L. A., Vanderleyden, J., and De Keersmaecker, S. C. J. (2011). Exopolysaccharides of *Lactobacillus rhamnosus* GG form a protective shield against innate immune factors in the intestine. *Microb. Biotechnol.* 4, 368–374. doi: 10.1111/j.1751-7915.2010.00199.x

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. R.* 72, 728–764. doi: 10.1128/MMBR.00017

Lee, H. Y., Park, J. H., Seok, S. H., Baek, M. W., Kim, D. J., Lee, K. E., et al. (2006). Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated

linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochim. Biophys. Acta* 1761, 736–744. doi: 10.1016/j.bbalip.2006.05.007

Lee, J., Kim, Y., Yun, H. S., Kim, J. G., Oh, S., and Kim, S. H. (2010). Genetic and proteomic analysis of factors affecting serum cholesterol reduction by *Lactobacillus acidophilus* A4. *Appl. Environ. Microbiol.* 76, 4829–4835. doi: 10.1128/AEM.02892

Lee, J. W., Shin, J. G., Kim, E. H., Kang, H. E., Yim, I. B., Kim, J. Y., et al. (2004). Immunomodulatory and antitumor effects *in vivo* by the cytoplasmic fraction of *Lactobacillus casei* and *Bifidobacterium longum*. *J. Vet. Sci.* 5, 41–48.

Leist, M., and Hartung, T. (2013). Inflammatory findings on species extrapolations: humans are definitely no 70-kg mice. *Arch. Toxicol.* 87, 563–567. doi: 10.1007/s00204-013-1038-0

Le Leu, R. K., Brown, I. L., Hu, Y., Bird, A. R., Jackson, M., Esterman, A., et al. (2005). A synbiotic combination of resistant starch and *Bifidobacterium lactis* facilitates apoptotic deletion of carcinogen-damaged cells in rat colon. *J. Nutr.* 135, 996–1001.

Le Marechal, C., Peton, V., Ple, C., Vroland, C., Jardin, J., Briard-Bion, V., et al. (2014). Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J. Proteomics.* 113, 447–461. doi: 10.1016/j.jprot.2014.07.018

Le Roy, T., Llopis, M., Lepage, P., Bruneau, A., Rabot, S., Bevilacqua, C., et al. (2013). Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. *Gut* 62, 1787–1794. doi: 10.1136/gutjnl-2012-303816

Lessman, C. A. (2011). The developing zebrafish (*Danio rerio*): a vertebrate model for high-throughput screening of chemical libraries. *Birth Defects Res. C Embryo Today* 93, 268–280. doi: 10.1002/bdrc.20212

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Lieschke, G. J., and Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* 8, 353–367. doi: 10.1038/nrg2091

Lye, H. S., Rusul, G., and Liong, M. T. (2010). Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. *J. Dairy Sci.* 93, 1383–1392. doi: 10.3168/jds.2009-2574

Macho Fernandez, E., Pot, B., and Grangette, C. (2011). Beneficial effect of probiotics in IBD: are peptidogycan and *NOD2* the molecular key effectors? *Gut Microbes* 2, 280–286. doi: 10.4161/gmic.2.5.18255

Mack, D. R., Ahrne, S., Hyde, L., Wei, S., and Hollingsworth, M. A. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. *Gut* 52, 827–833. doi: 10.1136/gut.52.6.827

Mackenzie, D. A., Jeffers, F., Parker, M. L., Vibert-Vallet, A., Bongaerts, R. J., Roos, S., et al. (2010). Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of *Lactobacillus reuteri*. *Microbiology* 156, 3368–3378. doi: 10.1099/mic.0.043265-0

Mainville, I., Arcand, Y., and Farnworth, E. R. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *Int. J. Food Microbiol.* 99, 287–296. doi: 10.1016/j.ijfoodmicro.2004.08.020

Malik, S., Petrova, M. I., Claes, I. J., Verhoeven, T. L., Busschaert, P., Vaneechoutte, M., et al. (2013). The highly autoaggregative and adhesive phenotype of the vaginal *Lactobacillus plantarum* strain CMPG5300 is sortase dependent. *Appl. Environ. Microbiol.* 79, 4576–4585. doi: 10.1128/AEM.00926-13

Mandlik, A., Swierczynski, A., Das, A., and Ton-That, H. (2008). Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. *Trends Microbiol.* 16, 33–40. doi: 10.1016/j.tim.2007.10.010

Mariadason, J. M., Catto-Smith, A., and Gibson, P. R. (1999). Modulation of distal colonic epithelial barrier function by dietary fibre in normal rats. *Gut* 44, 394–399. doi: 10.1136/gut.44.3.394

Marteau, P., Minekus, M., Havenaar, R., and Huis In't Veld, J. H. J. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J. Dairy Sci.* 80, 1031–1037. doi: 10.3168/jds.S0022-0302(97)76027-2

Martin, F. P., Wang, Y., Sprenger, N., Yap, I. K., Rezzi, S., Ramadan, Z., et al. (2008). Top-down systems biology integration of conditional prebiotic modulated transgenomic interactions in a humanized microbiome mouse model. *Mol. Syst. Biol.* 4, 205. doi: 10.1038/msb.2008.40

Matos, R. C., and Leulier, F. (2014). Lactobacilli-host mutualism: learning on the fly. *Microb. Cell Fact.* 13(Suppl. 1), S6. doi: 10.1186/1475-2859-13-S1-S6

Matsumoto, S., Hara, T., Nagaoka, M., Mike, A., Mitsuyama, K., Sako, T., et al. (2009). A component of polysaccharide peptidoglycan complex on *Lactobacillus* induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer. *Immunology* 128, e170–e180. doi: 10.1111/j.1365-2567.2008.02942.x

Matsuzaki, T., Yokokura, T., and Azuma, I. (1985). Anti-tumour activity of *Lactobacillus casei* on Lewis lung carcinoma and line-10 hepatoma in syngeneic mice and guinea pigs. *Cancer Immunol. Immunother.* 20, 18–22. doi: 10.1007/BF00199768

Matsuzaki, T., Yokokura, T., and Mutai, M. (1988). Antitumor effect of intrapleural administration of *Lactobacillus casei* in mice. *Cancer Immunol. Immunother.* 26, 209–214. doi: 10.1007/BF00199931

McKay, D. M., Philpott, D. J., and Perdue, M. H. (1997). Review article: *in vitro* models in inflammatory bowel disease research-a critical review. *Aliment. Pharmacol. Ther.* 11(Suppl. 3), 70–80. doi: 10.1111/j.1365-2036.1997.tb00811.x

Meijerink, M., Van Hemert, S., Taverne, N., Wels, M., De Vos, P., Bron, P. A., et al. (2010). Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. *PLoS ONE* 5:e10632. doi: 10.1371/journal.pone.0010632

Mennigen, R., and Bruewer, M. (2009). Effect of probiotics on intestinal barrier function. *Ann. N. Y. Acad. Sci.* 1165, 183–189. doi: 10.1111/j.1749-6632.2009.04059.x

Miettinen, M., Matikainen, S., Vuopio-Varkila, J., Pirhonen, J., Varkila, K., Kurimoto, M., et al. (1998). Lactobacilli and Streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. *Infect. Immun.* 66, 6058–6062.

Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., et al. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Appl. Microbiol. Biotechnol.* 53, 108–114. doi: 10.1007/s002530051622

Miquel, S., Martín, R., Rossi, O., Bermúdez-Humarán, L. G., Chatel, J. M., Sokol, H., et al. (2013). *Faecalibacterium prausnitzii* and human intestinal health. *Curr. Opin. Microbiol.* 16, 255–261. doi: 10.1016/j.mib.2013.06.003

Mitsuma, T., Odajima, H., Momiyama, Z., Watanabe, K., Masuguchi, M., Sekine, T., et al. (2008). Enhancement of gene expression by a peptide p(CHWPR) produced by *Bifidobacterium lactis* BB-12. *Microbiol. Immunol.* 52, 144–155. doi: 10.1111/j.1348-0421.2008.00022.x

Molly, K., Woestyne, M. V., and Smet, I. D. (1994). Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. *Microb. Ecol. Health Dis.* 7, 191–200. doi: 10.3109/08910609409141354

Moorehead, R. J., Hoper, M., and Mckelvey, S. T. (1987). Assessment of ornithine decarboxylase activity in rectal mucosa as a marker for colorectal adenomas and carcinomas. *Br. J. Surg.* 74, 364–365. doi: 10.1002/bjs.1800740513

Moser, A. R., Luongo, C., Gould, K. A., Mcneley, M. K., Shoemaker, A. R., and Dove, W. F. (1995). Apc^{Min}: a mouse model for intestinal and mammary tumorigenesis. *Eur. J. Cancer* 31A, 1061–1064. doi: 10.1016/0959-8049(95)00181-H

Moslehi-Jenabian, S., Gori, K., and Jespersen, L. (2009). AI-2 signalling is induced by acidic shock in probiotic strains of *Lactobacillus* spp. *Int. J. Food Microbiol.* 135, 295–302. doi: 10.1016/j.ijfoodmicro.2009.08.011

Moslehi-Jenabian, S., Vogensen, F. K., and Jespersen, L. (2011). The quorum sensing *luxS* gene is induced in *Lactobacillus acidophilus* NCFM in response to *Listeria monocytogenes*. *Int. J. Food Microbiol.* 149, 269–273. doi: 10.1016/j.ijfoodmicro.2011.06.011

Muller, J. A., Ross, R. P., Fitzgerald, G. F., and Stanton, C. (2009). "Manufacture of probiotic bacteria," in *Prebiotics and Probiotics Science and Technology*, eds D. Charalampopoulos and R. Rastall (New York: Springer), 725–759. doi: 10.1007/978-0-387-79058-9_18

Munoz-Provencio, D., Rodriguez-Diaz, J., Collado, M. C., Langella, P., Bermudez-Humaran, L. G., and Monedero, V. (2012). Functional analysis of the *Lactobacillus casei* BL23 sortases. *Appl. Environ. Microbiol.* 78, 8684–8693. doi: 10.1128/AEM.02287-12

Nadal, I., Santacruz, A., Marcos, A., Warnberg, J., Garagorri, J. M., Moreno, L. A., et al. (2009). Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. *Int. J. Obes. (Lond.)* 33, 758–767. doi: 10.1038/ijo.2008.260

Nicholson, J. K., Holmes, E., and Wilson, I. D. (2005). Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* 3, 431–438. doi: 10.1038/nrmicro1152

Nybom, S. M., Salminen, S. J., and Meriluoto, J. A. (2008). Specific strains of probiotic bacteria are efficient in removal of several different cyanobacterial toxins from solution. *Toxicon* 52, 214–220. doi: 10.1016/j.toxicon.2008.04.169

Oakey, H. J., Harty, D. W., and Knox, K. W. (1995). Enzyme production by lactobacilli and the potential link with infective endocarditis. *J. Appl. Bacteriol.* 78, 142–148. doi: 10.1111/j.1365-2672.1995.tb02834.x

Oehlers, S. H., Flores, M. V., Hall, C. J., Swift, S., Crosier, K. E., and Crosier, P. S. (2011). The inflammatory bowel disease (IBD) susceptibility genes *NOD1* and *NOD2* have conserved anti-bacterial roles in zebrafish. *Dis. Model. Mech.* 4, 832–841. doi: 10.1242/dmm.006122

O'Mahony, L., Feeney, M., O'halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., et al. (2001). Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. *Aliment. Pharmacol. Ther.* 15, 1219–1225. doi: 10.1046/j.1365-2036.2001.01027.x

Orrhage, K. M., Annas, A., Nord, C. E., Brittebo, E. B., and Rafter, J. J. (2002). Effects of lactic acid bacteria on the uptake and distribution of the food mutagen Trp-P-2 in mice. *Scand. J. Gastroenterol.* 37, 215–221. doi: 10.1080/003655202753416902

Ouwehand, A. C., Kirjavainen, P. V., Grönlund, M. M., Isolauri, E., and Salminen, S. J. (1999). Adhesion of probiotic micro-organisms to intestinal mucus. *Int. Dairy J.* 9, 623–630. doi: 10.1016/S0958-6946(99)00132-6

Pan, X., Yang, Y., and Zhang, J.-R. (2014). Molecular basis of host specificity in human pathogenic bacteria. *Emerg. Microbes Infect.* 3:e23. doi: 10.1038/emi.2014.23

Papadimitriou, C. G., Vafopoulou-Mastrojiannaki, A., Silva, S. V., Gomes, A.-M., Malcata, F. X., and Alichanidis, E. (2007). Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity. *Food Chem.* 105, 647–656. doi: 10.1016/j.foodchem.2007.04.028

Park, M. R., Yun, H. S., Son, S. J., Oh, S., and Kim, Y. (2014). Short communication: development of a direct *in vivo* screening model to identify potential probiotic bacteria using *Caenorhabditis elegans*. *J. Dairy Sci.* 97, 6828–6834. doi: 10.3168/jds.2014-8561

Parkes, G. C., Sanderson, J. D., and Whelan, K. (2009). The mechanisms and efficacy of probiotics in the prevention of *Clostridium difficile*-associated diarrhoea. *Lancet Infect. Dis.* 9, 237–244. doi: 10.1016/S1473-3099(09)70059-3

Perea Velez, M., Verhoeven, T. L., Draing, C., Von Aulock, S., Pfitzenmaier, M., Geyer, A., et al. (2007). Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG. *Appl. Environ. Microbiol.* 73, 3595–3604. doi: 10.1128/AEM.02083

Pisano, M. B., Viale, S., Conti, S., Fadda, M. E., Deplano, M., Melis, M. P., et al. (2014). Preliminary evaluation of probiotic properties of *Lactobacillus* strains isolated from Sardinian dairy products. *Biomed Res. Int.* 2014:286390. doi: 10.1155/2014/286390

Pokusaeva, K., Fitzgerald, G. F., and Van Sinderen, D. (2011). Carbohydrate metabolism in bifidobacteria. *Genes Nutr.* 6, 285–306. doi: 10.1007/s12263-010-0206-6

Pompei, A., Cordisco, L., Amaretti, A., Zanoni, S., Matteuzzi, D., and Rossi, M. (2007). Folate production by bifidobacteria as a potential probiotic property. *Appl. Environ. Microbiol.* 73, 179–185. doi: 10.1128/AEM.01763

Pool-Zobel, B. L., Neudecker, C., Domizlaff, I., Ji, S., Schillinger, U., Rumney, C., et al. (1996). *Lactobacillus*- and *Bifidobacterium*-mediated antigenotoxicity in the colon of rats. *Nutr. Cancer* 26, 365–380. doi: 10.1080/01635589609514492

Price, C. E., Reid, S. J., Driessen, A. J., and Abratt, V. R. (2006). The *Bifidobacterium longum* NCIMB 702259^T *ctr* gene codes for a novel cholate transporter. *Appl. Environ. Microbiol.* 72, 923–926. doi: 10.1128/AEM.72.1.923-926.2006

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Rastmanesh, R. (2011). High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. *Chem. Biol. Interact.* 189, 1–8. doi: 10.1016/j.cbi.2010.10.002

Ravel, J., Blaser, M., Braun, J., Brown, E., Bushman, F., Chang, E., et al. (2014). Human microbiome science: vision for the future, Bethesda, MD, July 24 to 26, 2013. *Microbiome* 2:16. doi: 10.1186/2049-2618-2-16

Resta-Lenert, S., and Barrett, K. E. (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). *Gut* 52, 988–997. doi: 10.1136/gut.52.7.988

Richmond, J. (2000). The 3Rs – past, present and future. *Scand. J. Lab. Anim. Sci.* 27, 84–92.

Rieu, A., Aoudia, N., Jego, G., Chluba, J., Yousfi, N., Briandet, R., et al. (2014). The biofilm mode of life boosts the anti-inflammatory properties of *Lactobacillus*. *Cell. Microbiol.* 16, 1836–1853. doi: 10.1111/cmi.12331

Rijkers, G. T., Bengmark, S., Enck, P., Haller, D., Herz, U., Kalliomaki, M., et al. (2010). Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. *J. Nutr.* 140, 671S–676S. doi: 10.3945/jn.109.113779

Rousseaux, C., Thuru, X., Gelot, A., Barnich, N., Neut, C., Dubuquoy, L., et al. (2007). *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat. Med.* 13, 35–37. doi: 10.1038/nm1521

Ruas-Madiedo, P., Gueimonde, M., Fernandez-Garcia, M., De Los Reyes-Gavilan, C. G., and Margolles, A. (2008). Mucin degradation by *Bifidobacterium* strains isolated from the human intestinal microbiota. *Appl. Environ. Microbiol.* 74, 1936–1940. doi: 10.1128/AEM.02509-07

Ruiz, L., Zomer, A., O'Connell-Motherway, M., Van Sinderen, D., and Margolles, A. (2012). Discovering novel bile protection systems in *Bifidobacterium breve* UCC2003 through functional genomics. *Appl. Environ. Microbiol.* 78, 1123–1131. doi: 10.1128/AEM.06060-11

Russel, W. M. S., and Burch, R. L. (1959). *The Principles of Humane Experimental Technique*. London: Universities Federation for Animal Welfare, Wheathampstead.

Ryan, S. M., Fitzgerald, G. F., and Van Sinderen, D. (2005). Transcriptional regulation and characterization of a novel β-fructofuranosidase-encoding gene from *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 71, 3475–3482. doi: 10.1128/AEM.71.7.3475-3482.2005

Sakata, T., Kojima, T., Fujieda, M., Takahashi, M., and Michibata, T. (2003). Influences of probiotic bacteria on organic acid production by pig caecal bacteria *in vitro*. *Proc. Nutr. Soc.* 62, 73–80. doi: 10.1079/PNS2002211

Salminen, S., Ouwehand, A., Benno, Y., and Lee, Y. K. (1999). Probiotics: how should they be defined? *Trends Food Sci. Technol.* 10, 107–110. doi: 10.1016/S0924-2244(99)00027-8

Sanchez, B., Champomier-Verges, M. C., Anglade, P., Baraige, F., De Los Reyes-Gavilan, C. G., Margolles, A., et al. (2005). Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *J. Bacteriol.* 187, 5799–5808. doi: 10.1128/JB.187.16.5799-5808.2005

Sanchez, B., Champomier-Verges, M. C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., et al. (2007). Adaptation and response of *Bifidobacterium animalis* subsp. *lactis* to bile: a proteomic and physiological approach. *Appl. Environ. Microbiol.* 73, 6757–6767. doi: 10.1128/AEM.00637-07

Sanchez, B., De Los Reyes-Gavilan, C. G., and Margolles, A. (2006). The F_1F_0-ATPase of *Bifidobacterium animalis* is involved in bile tolerance. *Environ. Microbiol.* 8, 1825–1833. doi: 10.1111/j.1462-2920.2006.01067.x

Sanchez, B., Ruiz, L., De Los Reyes-Gavilan, C. G., and Margolles, A. (2008). Proteomics of stress response in *Bifidobacterium*. *Front. Biosci.* 13:6905.

Sanders, M. E., Akkermans, L. M., Haller, D., Hammerman, C., Heimbach, J., Hormannsperger, G., et al. (2010). Safety assessment of probiotics for human use. *Gut Microbes* 1, 164–185. doi: 10.4161/gmic.1.3.12127

Saulnier, D. M., Santos, F., Roos, S., Mistretta, T. A., Spinler, J. K., Molenaar, D., et al. (2011). Exploring metabolic pathway reconstruction and genome-wide expression profiling in *Lactobacillus reuteri* to define functional probiotic features. *PLoS ONE* 6:e18783. doi: 10.1371/journal.pone.0018783

Scaldaferri, F., Gerardi, V., Lopetuso, L. R., Del Zompo, F., Mangiola, F., Boskoski, I., et al. (2013). Gut microbial flora, prebiotics, and probiotics in IBD: their current usage and utility. *Biomed. Res. Int.* 2013, 435268. doi: 10.1155/2013/435268

Schlee, M., Wehkamp, J., Altenhoefer, A., Oelschlaeger, T. A., Stange, E. F., and Fellermann, K. (2007). Induction of human β-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. *Infect. Immun.* 75, 2399–2407. doi: 10.1128/IAI.01563

Sekine, K., Toida, T., Saito, M., Kuboyama, M., Kawashima, T., and Hashimoto, Y. (1985). A new morphologically characterized cell wall preparation (whole peptidoglycan) from *Bifidobacterium infantis* with a higher efficacy on the regression of an established tumor in mice. *Cancer Res.* 45, 1300–1307.

Sela, D. A., Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., et al. (2008). The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18964–18969. doi: 10.1073/pnas.0809584105

Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3507–3512. doi: 10.1073/pnas.1222878110

Shanks, N., Greek, R., and Greek, J. (2009). Are animal models predictive for humans? *Philos. Ethics Humanit. Med.* 4, 2. doi: 10.1186/1747-5341-4-2

Shimada, Y., Watanabe, Y., Wakinaka, T., Funeno, Y., Kubota, M., Chaiwangsri, T., et al. (2014). α-*N*-Acetylglucosaminidase from *Bifidobacterium bifidum* specifically hydrolyzes α-linked *N*-acetylglucosamine at nonreducing terminus of *O*-glycan on gastric mucin. *Appl. Microbiol. Biotechnol.* doi: 10.1007/s00253-014-6201-x [Epub ahead of print].

Siniscalco, D., and Antonucci, N. (2013). Involvement of dietary bioactive proteins and peptides in autism spectrum disorders. *Curr. Protein Pept. Sci.* 14, 674–679. doi: 10.2174/1389203711209070602

Smet, I. D., Hoorde, L. V., Saeyer, N. D., Woestyne, M. V., and Verstraete, W. (1994). *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. *Microb. Ecol. Health Dis.* 7, 315–329.

Smits, H. H., Engering, A., Van Der Kleij, D., De Jong, E. C., Schipper, K., Van Capel, T. M., et al. (2005). Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J. Allergy Clin. Immunol.* 115, 1260–1267. doi: 10.1016/j.jaci.2005.03.036

Sodhi, C. P., Neal, M. D., Siggers, R., Sho, S., Ma, C., Branca, M. F., et al. (2012). Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology* 143, 708–718, e701–e705. doi: 10.1053/j.gastro.2012.05.053

Songisepp, E., Kals, J., Kullisaar, T., Mändar, R., Hütt, P., Zilmer, M., et al. (2005). Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers. *Nutr. J.* 4, 22–22. doi: 10.1186/1475-2891-4-22

Steinberg, R. S., Silva, L. C., Souza, T. C., Lima, M. T., De Oliveira, N. L., Vieira, L. Q., et al. (2014). Safety and protective effectiveness of two strains of *Lactobacillus* with probiotic features in an experimental model of salmonellosis. *Int. J. Environ. Res. Public Health* 11, 8755–8776. doi: 10.3390/ijerph110908755

Strus, M., Kucharska, A., Kukla, G., Brzychczy-Wloch, M., Maresz, K., and Heczko, P. B. (2005). The *in vitro* activity of vaginal *Lactobacillus* with probiotic properties against *Candida*. *Infect. Dis. Obstet. Gynecol.* 13, 69–75. doi: 10.1080/10647440400028136

Sturme, M. H., Nakayama, J., Molenaar, D., Murakami, Y., Kunugi, R., Fujii, T., et al. (2005). An *agr*-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. *J. Bacteriol.* 187, 5224–5235. doi: 10.1128/JB.187.15.5224-5235.2005

Suardi, E., Crippa, F., and D'arminio Monforte, A. (2013). Probiotics in the prevention of antibiotic-associated diarrhea in adults. *Int. J. Probiotics Prebiotics* 8, 41–44.

Sun, X., Wang, H., Zhang, Y., Chen, K., Davis, B., and Feng, H. (2011). Mouse relapse model of *Clostridium difficile* infection. *Infect. Immun.* 79, 2856–2864. doi: 10.1128/IAI.01336-10

Tagg, J. R., and McGiven, A. R. (1971). Assay system for bacteriocins. *Appl. Microbiol.* 21, 943.

Tan, Q., Xu, H., Aguilar, Z. P., Peng, S., Dong, S., Wang, B., et al. (2013). Safety assessment and probiotic evaluation of *Enterococcus faecium* YF5 isolated from sourdough. *J. Food Sci.* 78, M587–M593. doi: 10.1111/1750-3841.12079

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

Tomaro-Duchesneau, C., Jones, M. L., Shah, D., Jain, P., Saha, S., and Prakash, S. (2014). Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an *in vitro* investigation. *Biomed Res. Int.* 2014, 380316. doi: 10.1155/2014/380316

Topping, D. L., and Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* 81, 1031–1064.

Turnbaugh, P. J., Backhed, F., Fulton, L., and Gordon, J. I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* 3, 213–223. doi: 10.1016/j.chom.2008.02.015

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

Turpin, W., Humblot, C., Noordine, M. L., Thomas, M., and Guyot, J. P. (2012). *Lactobacillaceae* and cell adhesion: genomic and functional screening. *PLoS ONE* 7:e38034. doi: 10.1371/journal.pone.0038034

Turroni, F., Bottacini, F., Foroni, E., Mulder, I., Kim, J. H., Zomer, A., et al. (2010a). 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., Foroni, E., O'connell Motherway, M., Bottacini, F., Giubellini, V., Zomer, A., et al. (2010b). Characterization of the serpin-encoding gene of *Bifidobacterium breve* 210B. *Appl. Environ. Microbiol.* 76, 3206–3219. doi: 10.1128/AEM.02938

Turroni, F., Milani, C., Van Sinderen, D., and Ventura, M. (2011). Genetic strategies for mucin metabolism in *Bifidobacterium bifidum* PRL2010: an example of possible human-microbe co-evolution. *Gut Microbes* 2, 183–189. doi: 10.4161/gmic.2.3.16105

Turroni, F., Serafini, F., Foroni, E., Duranti, S., O'connell Motherway, M., Taverniti, V., et al. (2013). Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host interactions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11151–11156. doi: 10.1073/pnas.1303897110

Uccello, M., Malaguarnera, G., Basile, F., D'agata, V., Malaguarnera, M., Bertino, G., et al. (2012). Potential role of probiotics on colorectal cancer prevention. *BMC Surg.* 12(Suppl. 1):S35. doi: 10.1186/1471-2482-12-S1-S35

Upadrasta, A., Stanton, C., Hill, C., Fitzgerald, G., and Ross, R. P. (2011). "Improving the stress tolerance of probiotic cultures: recent trends and future directions," in *Stress Responses of Lactic Acid Bacteria*, eds E. Tsakalidou and K. Papadimitriou (New York: Springer), 395–438.

van Baarlen, P., Troost, F. J., Van Hemert, S., Van Der Meer, C., De Vos, W. M., De Groot, P. J., et al. (2009). Differential NF-κB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2371–2376. doi: 10.1073/pnas.0809919106

van Bokhorst-van de Veen, H., Lee, I. C., Marco, M. L., Wels, M., Bron, P. A., and Kleerebezem, M. (2012). Modulation of *Lactobacillus plantarum* gastrointestinal robustness by fermentation conditions enables identification of bacterial robustness markers. *PLoS ONE* 7:e39053. doi: 10.1371/journal.pone.0039053

Van den Abbeele, P., Roos, S., Eeckhaut, V., Mackenzie, D. A., Derde, M., Verstraete, W., et al. (2012). Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. *Microb. Biotechnol.* 5, 106–115. doi: 10.1111/j.1751-7915.2011.00308.x

van Hemert, S., Meijerink, M., Molenaar, D., Bron, P. A., De Vos, P., Kleerebezem, M., et al. (2010). Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells. *BMC Microbiol.* 10:293. doi: 10.1186/1471-2180-10-293

Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.-B., et al. (2008). Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. *Trends Food Sci. Technol.* 19, 102–114. doi: 10.1016/j.tifs.2007.07.013

Vastano, V., Salzillo, M., Siciliano, R. A., Muscariello, L., Sacco, M., and Marasco, R. (2014). The E1 beta-subunit of pyruvate dehydrogenase is surface-expressed in *Lactobacillus plantarum* and binds fibronectin. *Microbiol. Res.* 169, 121–127. doi: 10.1016/j.micres.2013.07.013

Ventura, M., Jankovic, I., Walker, D. C., Pridmore, R. D., and Zink, R. (2002). Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Appl. Environ. Microbiol.* 68, 6172–6181. doi: 10.1128/AEM.68.12.6172-6181.2002

Verdu, E. F., and Collins, S. M. (2004). Microbial-gut interactions in health and disease. Irritable bowel syndrome. *Best Pract. Res. Clin. Gastroenterol.* 18, 315–321. doi: 10.1016/j.bpg.2003.11.003

Vesterlund, S., Paltta, J., Karp, M., and Ouwehand, A. C. (2005). Adhesion of bacteria to resected human colonic tissue: quantitative analysis of bacterial adhesion and viability. *Res. Microbiol.* 156, 238–244. doi: 10.1016/j.resmic.2004.08.012

Viaud, S., Saccheri, F., Mignot, G., Yamazaki, T., Daillere, R., Hannani, D., et al. (2013). The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* 342, 971–976. doi: 10.1126/science.1240537

Villena, J., Salva, S., Aguero, G., and Alvarez, S. (2011). Immunomodulatory and protective effect of probiotic *Lactobacillus casei* against *Candida albicans* infection in malnourished mice. *Microbiol. Immunol.* 55, 434–445. doi: 10.1111/j.1348-0421.2011.00334.x

Vinderola, C. G., Medici, M., and Perdigon, 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

Vogel, S. N. (2012). How discovery of Toll-mediated innate immunity in *Drosophila* impacted our understanding of TLR signaling (and vice versa). *J. Immunol.* 188, 5207–5209. doi: 10.4049/jimmunol.1201050

Wang, C., Wang, J., Gong, J., Yu, H., Pacan, J. C., Niu, Z., et al. (2011). Use of *Caenorhabditis elegans* for preselecting *Lactobacillus* isolates to control *Salmonella typhimurium*. *J. Food Prot.* 74, 86–93. doi: 10.4315/0362-028X.JFP-10-155

Wang, L., Cao, H., Liu, L., Wang, B., Walker, W. A., Acra, S. A., et al. (2014). Activation of epidermal growth factor receptor mediates mucin production stimulated by p40, a *Lactobacillus rhamnosus* GG-derived protein. *J. Biol. Chem.* 289, 20234–20244. doi: 10.1074/jbc.M114.553800

Waters, C. M., and Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* 21, 319–346. doi: 10.1146/annurev.cellbio.21.012704.131001

Weiss, G., and Jespersen, L. (2010). Transcriptional analysis of genes associated with stress and adhesion in *Lactobacillus acidophilus* NCFM during the passage through an *in vitro* gastrointestinal tract model. *J. Mol. Microbiol. Biotechnol.* 18, 206–214. doi: 10.1159/000316421

Westermann, C., Zhurina, D., Baur, A., Shang, W., Yuan, J., and Riedel, C. (2012). Exploring the genome sequence of *Bifidobacterium bifidum* S17 for potential players in host-microbe interactions. *Symbiosis* 58, 191–200. doi: 10.1007/s13199-012-0205-z

Wollowski, I., Rechkemmer, G., and Pool-Zobel, B. L. (2001). Protective role of probiotics and prebiotics in colon cancer. *Am. J. Clin. Nutr.* 73, 451s–455s.

Wu, R., Sun, Z., Wu, J., Meng, H., and Zhang, H. (2010). Effect of bile salts stress on protein synthesis of *Lactobacillus casei* Zhang revealed by 2-dimensional gel electrophoresis. *J. Dairy Sci.* 93, 3858–3868. doi: 10.3168/jds.2009-2967

Wu, X., Vallance, B. A., Boyer, L., Bergstrom, K. S., Walker, J., Madsen, K., et al. (2008). *Saccharomyces boulardii* ameliorates *Citrobacter rodentium*-induced colitis through actions on bacterial virulence factors. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G295–G306. doi: 10.1152/ajpgi.00173.2007

Yadav, H., Jain, S., and Sinha, P. R. (2008). The effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* on gastropathic consequences in diabetic rats. *J. Med. Food* 11, 62–68. doi: 10.1089/jmf.2006.136

Yan, F., Cao, H., Cover, T. L., Whitehead, R., Washington, M. K., and Polk, D. B. (2007). Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* 132, 562–575. doi: 10.1053/j.gastro.2006.11.022

Yang, F., Wang, J., Li, X., Ying, T., Qiao, S., Li, D., et al. (2007). 2-DE and MS analysis of interactions between *Lactobacillus fermentum* I5007 and intestinal epithelial cells. *Electrophoresis* 28, 4330–4339. doi: 10.1002/elps.200700166

Yoshida, E., Sakurama, H., Kiyohara, M., Nakajima, M., Kitaoka, M., Ashida, H., et al. (2012). *Bifidobacterium longum* subsp. *infantis* uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. *Glycobiology* 22, 361–368. doi: 10.1093/glycob/cwr116

Zacarias, M. F., Reinheimer, J., Forzani, L., Grangette, C., and Vinderola, G. (2014). Mortality and translocation assay to study the protective capacity of *Bifidobacterium lactis* INL1 against *Salmonella* Typhimurium infection in mice. *Benef. Microbes* 5, 427–436. doi: 10.3920/BM2013.0086

Zheng, Y., Lu, Y., Wang, J., Yang, L., Pan, C., and Huang, Y. (2013). Probiotic properties of *Lactobacillus* strains isolated from Tibetan kefir grains. *PLoS ONE* 8:e69868. doi: 10.1371/journal.pone.0069868

Zoumpopoulou, G., Foligne, B., Christodoulou, K., Grangette, C., Pot, B., and Tsakalidou, E. (2008). *Lactobacillus fermentum* ACA-DC 179 displays probiotic potential *in vitro* and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and *Salmonella* infection in murine models. *Int. J. Food Microbiol.* 121, 18–26. doi: 10.1016/j.ijfoodmicro.2007.10.013

Zyrek, A. A., Cichon, C., Helms, S., Enders, C., Sonnenborn, U., and Schmidt, M. A. (2007). Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCζ redistribution resulting in tight junction and epithelial barrier repair. *Cell Microbiol.* 9, 804–816. doi: 10.1111/j.1462-5822.2006.00836.x

Conflict of Interest Statement: 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.

Received: 07 December 2014; paper pending published: 30 December 2014; accepted: 17 January 2015; published online: 17 February 2015.

*Citation: Papadimitriou K, Zoumpopoulou G, Foligné B, Alexandraki V, Kazou M, Pot B and Tsakalidou E (2015) Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. Front. Microbiol. **6**:58. doi: 10.3389/fmicb.2015.00058*

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 September 2015
doi: 10.3389/fmicb.2015.00952

The vaginal isolate *Lactobacillus paracasei* LPC-S01 (DSM 26760) is suitable for oral administration

Silvia Balzaretti[1], Valentina Taverniti[1], Greta Rondini[1], Giorgio Marcolegio[1], Mario Minuzzo[2], Maria C. Remagni[1†], Walter Fiore[3], Stefania Arioli[1] and Simone Guglielmetti[1]*

[1] Division of Food Microbiology and Bioprocessing, Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Milano, Italy, [2] Department of Biosciences, Università degli Studi di Milano, Milano, Italy, [3] Sofar S.p.A., Trezzano Rosa, Italy

Bacterial vaginosis is one of the most common urogenital diseases affecting women in reproductive age. The administration of probiotics as vaginal suppository has been proposed as a strategy to cure this condition and reduce its recurrence. Nonetheless, also oral consumption of probiotics, which is a more practical route of administration, proved to be an efficient strategy. In this perspective, we studied *Lactobacillus paracasei* LPC-S01 (DSM 26760), a human vaginal isolate included in commercial probiotic preparations for topical use, in order to assess if this bacterium can also perform as gastrointestinal probiotic. Comparative genomics revealed the presence of several accessory genes suggesting that LPC-S01 is a niche-generalist member of its species. According to a procedure conventionally used to predict the probiotic potential, we demonstrated that the probiotic properties of strain LPC-S01, with respect to those of the well-known probiotic references *L. paracasei* Shirota and DG, are equal for the bile tolerance and the reduction of NF-κB activation in Caco-2 cells, or superior for the tolerance to gastric juice and the adhesion to Caco-2 epithelial cells. We then demonstrated that LPC-S01 is susceptible to antibiotics indicated by EFSA and does not produce biogenic amines. Finally, a double-blind cross-over pilot intervention trial on healthy human volunteers showed that, after a 7-days oral consumption of capsules containing about 24 billion live cells, the fecal cell concentrations of strains LPC-S01 and DG (evaluated by qPCR) were not dissimilar. Specifically, both probiotics' cell concentrations were above the detection limit for an average of 5 days from the end of the treatment, corresponding to a mean number of evacuations of 7 ± 2. Taken together, these data demonstrate that the vaginal isolate *L. paracasei* LPC-S01 possesses safety and functional properties that may support its use as probiotic to be administered *per os* for potential intestinal as well as vaginal applications.

Keywords: lactobacilli, probiotic, Caco-2 adhesion, NF-κB, *in vivo* trial, gastrointestinal persistence

OPEN ACCESS

Edited by:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Marcela Carina Audisio, Instituto de Investigaciones para la Industria Química-CONICET, Argentina
Amit Kumar Tyagi, The University of Texas MD Anderson Cancer Center, USA

****Correspondence:***
Simone Guglielmetti, Division of Food Microbiology and Bioprocessing, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via Celoria 2, Milano 20133, Italy
simone.guglielmetti@unimi.it

†Present Address:
Maria C. Remagni, Sacco S.r.l., Cadorago, Italy

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

Received: *30 April 2015*
Accepted: *28 August 2015*
Published: *15 September 2015*

Citation:
Balzaretti S, Taverniti V, Rondini G, Marcolegio G, Minuzzo M, Remagni MC, Fiore W, Arioli S and Guglielmetti S (2015) The vaginal isolate Lactobacillus paracasei LPC-S01 (DSM 26760) is suitable for oral administration. Front. Microbiol. 6:952. doi: 10.3389/fmicb.2015.00952

Introduction

The genus *Lactobacillus* is a taxonomically broad and heterogeneous group of Gram positive bacteria, which has important industrial applications as fermented food starter and probiotic adjuncts. Particularly, lactobacilli are the bacteria most frequently employed as probiotics, i.e., "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Inside the *Lactobacillus* genus, the *L. casei* group of species (i.e., *L. casei*, *L. paracasei*, and *L. rhamnosus*) includes some of the most conventional and well-characterized probiotic strains, such as *L. paracasei* Shirota (considered the first probiotic ever; Nanno et al., 2011), and *L. rhamnosus* GG (Szajewska and Chmielewska, 2013).

Strains of the *L. paracasei* species have been isolated from several diverse ecological niches such as raw milk, plants, fermented artisanal products (fermented milk, cheese, sourdough bread starter, and fermented vegetables), and the intestinal tracts and reproductive systems of humans and animals (Cai et al., 2007). The remarkable ecological adaptability of *L. paracasei* to diverse habitats can be plausibly explained by very frequent accumulation of indels and genome rearrangements, which originated a high level of genotypic and phenotypic diversity in the species (Cai et al., 2007; Smokvina et al., 2013). Given that most effects of probiotics are strain-specific (Azaïs-Braesco et al., 2010), the intrinsic high heterogeneity existing among *L. paracasei* strains makes this species an optimal source for the selection of novel candidate probiotic strains possessing unique technological and health-promoting traits.

So far, lactobacilli have been predominantly investigated and explicitly proposed as probiotics for their potential beneficial roles on the gastrointestinal tract and its microbiota (Guglielmetti et al., 2011; Ferrario et al., 2014). Nevertheless, the interest for the application of lactobacilli beyond the gut is constantly increasing, leading to the development of new categories of probiotic products that target oral cavity (Guglielmetti et al., 2010a,b; Taverniti et al., 2012; Wescombe et al., 2012), skin (Krutmann, 2009), stomach (Johnson-Henry et al., 2004), urinary tract (Borchert et al., 2008), and vaginal mucosa (Borges et al., 2014).

Interestingly, it was demonstrated that orally administered lactobacilli can be re-isolated from the vagina, presumably as a consequence of the migration from the rectum via perineum (Vásquez et al., 2005). Consistently, bacteria colonizing the vaginal mucosa (both commensals and vaginosis-associated microbes) have been isolated from the rectum and the mouth, suggesting that gut and oral cavity act as extravaginal reservoirs of vaginal microbiota bacteria (van de Wijgert et al., 2014). Therefore, it appears plausible that the oral administration of probiotic bacteria may potentially influence the vaginal microbiota through two possible mechanisms: (i) modification of the intestinal microbiota (e.g., by reducing potentially harmful bacteria and increasing endogenous lactobacilli); (ii) direct migration to the vaginal mucosa via the gastrointestinal route. Furthermore, the possibility to benefit vaginal health by administering vaginal probiotics *per os* has the additional advantage of favoring the accomplishment of long-term treatments. However, in order to make this strategy effective, selected bacteria must possess properties supporting their survival and activities both in the gastro-intestinal tract and vaginal mucosa. In this perspective, we assessed if *L. paracasei* LPC-S01 (DSM 26760) (a strain originally isolated from the vaginal mucosa of a healthy woman and included in probiotic preparations for vaginal use marketed in Europe) can also perform as a conventional gastrointestinal probiotic. To this aim, we generated a draft genome of strain LPC-S01 and performed a comparative genomic analysis against other *L. paracasei* reference strains. In addition, we included LPC-S01 in several *in vitro* tests conventionally employed to establish the intestinal probiotic potential of microbial strains. Finally, we carried out a double-blind cross-over pilot intervention trial in comparison with the well-known intestinal probiotic strain *L. paracasei* DG (Ferrario et al., 2014) in order to assess the ability of LPC-S01 to transiently persist in the gastrointestinal tract of healthy adults.

Materials and Methods

Bacterial Strains and Culture Conditions

Unless differently specified, lactobacilli were grown at 37°C in De Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI) for 24 h. *L. paracasei* LPC-S01 and DG were isolated from the commercial products PreGyn® and Enterolactis® Plus (Sofar S.p.A.), respectively; *L. paracasei* strain Shirota was isolated from Yakult fermented milk; strain FMBr3 was isolated from Raschera artisanal Italian cheese.

Genome Sequencing, Sequence Annotation, and Comparative Analysis

The draft genome sequences of *L. paracasei* LPC-S01, DG, and FMBr3 were obtained through Ion Torrent PGM (Life Technologies, Germany) according to a previously described protocol (Milani et al., 2013) by GenProbio Ltd. The raw sequence data were assembled using MIRA v.3.9 (http://www.chevreux.org/projects_mira.html), applying default parameters recommended for Ion Torrent data processing. Initial automated annotation of the *L. paracasei* genomes was performed using RAST, combined with BLASTX. Results of the gene-finder program were combined manually with data from BLASTP analysis against a non-redundant protein database provided by the National Center for Biotechnology Information (NCBI). LPC-S01 draft genome sequence was compared with other *L. paracasei* genome sequences by means of BLAST Ring Image Generator (BRIG; Alikhan et al., 2011). The DNA sequences presented in this study have been deposited in the EMBL database under the accession numbers LN846896 (putative phospho-β-galactosidase operon), LN846897 (putative taurine ABC-type transport and metabolization operon), LN846898 (putative ABC-type Fe3+ transport system), LN846899 (putative nucleotide transport and metabolism operon), LN846900 (putative EPS2 region), and LN846901 (multi-transport region for the uptake of sugars and other small molecules).

Bacterial Resistance to Simulated Gastric Juices and Bile

One hundred microliters of *L. paracasei* culture containing 10^8–10^9 CFU/ml were transferred to 6 ml of simulated gastric juice at pH 2 (1 g/l DIFCO peptone, 100 mM KCl, 500 U/ml pepsin) or simulated gastric juice at pH 3 (125 mM NaCl, 45 mM $NaHCO_3$, 7 mM KCl, 500 U/ml pepsin). Cells in 0.1 M phosphate buffer pH 7 were used as control. After 90 min of incubation at 37°C, viability was monitored by plating on MRS agar. Afterwards, cell suspension was neutralized by adding 1 M phosphate buffer pH 8 and centrifuged at 10,000 × g for 12 min. Bacterial cell pellet was then suspended in 5 ml bile solution (1 g/l peptone, 0.3% Difco Oxgall in 0.1 M phosphate buffer at pH 6.5). Cells in 0.1 M phosphate buffer pH 6.5 in the presence of 1 g/l peptone were used as control. The number of viable cells was determined after 3 h of incubation at 37°C by plating on MRS agar. Plates were incubated under anaerobic conditions and colonies were counted after 48 h. The ability to grow in presence of bile was also investigated by inoculating bacteria in MRS broth supplemented with increasing concentrations of Oxgall (from 0.0625 to 9.6%). Growth was assessed by measuring the optical density at 600 nm.

Bacterial Adhesion to Caco-2 Cell Line

The adhesion of *L. paracasei* strains to Caco-2 (ATCC HTB-37) cell layer was assessed as previously described (Guglielmetti et al., 2008). In brief, Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine and incubated at 37°C in an atmosphere of 95% air and 5% carbon dioxide. For adhesion experiments, fully differentiated Caco-2 were used (i.e., 15 days after confluence). Approximately 2×10^8 cells for each bacterial strain (determined microscopically with Neubauer Improved counting chamber; Marienfeld GmbH, Lauda-Königshofen, Germany) were incubated with a monolayer of Caco-2 cells for 1h at 37°C. Monolayers were washed three times with phosphate-buffered saline pH 7.3 (PBS) to release unbound bacteria and incubated with 3 ml of methanol for 8 min at room temperature to fix cells. Afterwards, cells were stained with 3 mL of Giemsa stain solution (1:20) (Carlo Erba, Milano, Italy) and left 30 min at room temperature in the dark. Finally, monolayers were washed three times with PBS, dried in an incubator for 1 h, and examined microscopically (magnification, 400×) under oil immersion. All experiments were performed in duplicate.

Inhibition of Pathogens

We investigated the ability of *L. paracasei* strains to secret inhibitor molecules against pathogens by disc diffusion assay. *Lactobacillus plantarum* strain WHE 92 was used as a positive control for bacteriocin production (Ennahar et al., 1996). We used seven indicator microorganisms: *Salmonella enterica* MIMms, *Listeria monocytogenes* FMB4b, *Escherichia coli* VE7108, *Streptococcus pyogenes* emm TYPE 77, *Pseudomonas aeruginosa* FMBpa18, *Staphylococcus aureus* FMBDR, and *Candida albicans* ATCC MYA2876. Indicator bacteria were cultivated in Brain Heart Infusion (BHI) agar medium (Oxoid S.p.A., Milan, Italy), supplemented with 0.3% yeast extract and 1% glucose (ygBHI), whereas *C. albicans* was cultivated on Sabouraud dextrose agar (Oxoid S.p.A., Basingstoke, UK). Three different fractions for each *Lactobacillus* strain (tester bacteria) were used: (i) broth culture at stationary growth phase, (ii) cell-free broth (obtained by centrifugation at 10,000 × g for 10 min and sterilization with a 0.22 μm syringe filter), and (iii) cell-free broth neutralized with NaOH to pH 7.4. Sterile MRS was used as negative control. The assay was carried out as follows: 0.1 ml from an over-night culture of the indicator strains were plated on ygBHI (or Sabouraud) agar in order to obtain a confluent growth after incubation. Sterile paper discs (Whatman™ Grade AA 9 mm disc, Maidstone, UK) were dipped into three different preparations from tester bacteria and subsequently positioned on the Petri dishes inoculated with the indicator strain. Plates were kept at 4°C for 2 h and then incubated at 37°C. The presence of an inhibition halo was checked after 24 or 48 h.

Fermentation Profile

Carbon source fermentation was determined in a 96-well microtiter plate in a final volume of 200 μl with a basal CHL medium at pH 6.3 (Bio-Merieux, Montelieu-Vercieu, France) containing bromocresol purple as pH indicator, and the desired filter-sterilized carbohydrate at a final concentration of 0.5% (w/v). The 42 different substrates tested (Supplementary Table 1) were from Sigma-Aldrich (St. Louis, MO, USA), with the exception of fructooligosaccharides (FOS) which were from Actilight® (Giulio Gross S.p.A., Trezzano sul Naviglio, Italy). Cells from an over-night culture of *L. paracasei* were collected by centrifugation, washed with PBS, and used to inoculate the liquid medium in microtiter wells (1/100 inoculation). Plates were examined for color change (from purple to yellow) after 24 and 48 h incubation at 37°C.

NF-κB Activation Assay

The activation nuclear factor κB (NF-κB) was studied by means of a recombinant Caco-2 cell line stably transfected with vector pNiFty2-Luc (InvivoGen, Labogen, Rho, Italy) as in Taverniti et al. (2013). In brief, recombinant Caco-2 monolayers (approximately 3×10^5 cells/well), cultivated in the presence of 50 μg/ml zeocin, were washed with 0.1 M Tris-HCl buffer (pH 8.0) and then incubated with 3.5×10^8 cells of *L. paracasei* suspended in fresh DMEM containing 100 mM HEPES (pH 7.4), resulting in a MOI of approximately 1000. In a different set of experiments, Caco-2 monolayers were incubated with 0.1 ml of cell-free broth obtained by centrifugation and filter-sterilization from a stationary-phase culture of *L. paracasei*. Stimulation was conducted by adding 2 ng/ml of IL-1β. After incubation at 37°C for 4 h, the samples were treated and the bioluminescence was measured as described by Stuknyte et al. (2011). Two independent experiments were conducted in triplicate for each condition.

Susceptibility to Antibiotics and Biogenic Amines Production

The minimal inhibitory concentrations (MICs) of *L. paracasei* have been determined using a micro-dilution method in LSM

broth (ISO-Sensitest broth, Oxoid supplemented with 10% v/v MRS Difco) as described in the ISO10932 IDF 223 document and recommended by EFSA (2012). The data are reported as average of two independent assays. The ability to produce cadaverine, tyramine, histamine and putrescine respectively from L-lysine, tyrosine disodium salt, L-histidine monohydrochloride and L-ornithine monohydrochloride was investigated by a qualitative test, according to the method proposed by Bover-Cid and Holzapfel (1999). All amino acids were purchased from Sigma. Strains were inoculated (1%) in MRS broth containing 0.1% of each amino acid precursor and incubated at 37°C for 18 h. The qualitative test to evaluate decarboxylase activity was performed in an agar medium specially formulated (0.5% tryptone, 0.5% yeast extract, 0.5% meat extract, 0.25% NaCl, 0.05% glucose, 0.1% Tween 80, 0.02% MgSO4, 0.005% MnSO4, 0.004% FeSO4, 0.2% ammonium citrate, 0.001% thiamine, 0.2% K2PO4, 0.01% CaCO3, 0.005% pyridoxal phosphate, 1% amino acid precursor, 0.006% bromocresol purple, 2% agar; pH 5.3). 10 μL of each culture were placed on the medium containing the same amino acid precursor. The plates were incubated at 37°C for 4 days. Positive test is indicated by the color change and by aminoacid precipitation around the corresponding spot (for tyramine only).

Human Intervention Trial

The study protocol was approved by the Research Ethics Committee of the Università degli Studi di Milano (opinion no. 37/12, December 2012). Written informed consent was obtained from all subjects before recruitment. The intervention study consisted of a randomized, double blind, cross-over pilot trial with two parallel groups (Supplementary Figure 1). Each volunteer was asked to participate to 6 visits: before run-in period (visit V0); before the first treatment (V1); after the first treatment (V2); before the second treatment (V3); after the second treatment (V4), and at the end of the trial (V5) (Supplementary Figure 1). After a 4-week pre-recruitment phase, volunteers have been randomized to receive 1 capsule daily of *L. paracasei* LPC-S01 (product A, 5 subjects) or *L. paracasei* DG (product B, 6 subjects) every day for 1 week, in addition to their habitual diet. After a 2 week wash-out period, the volunteers received a daily capsule of the other product for 1 week. Volunteers received directions to keep the products at room temperature and to avoid exposure to heat sources. Volunteers received also oral and written instructions to consume the capsule during the morning, while drinking natural water, at least 15 min before breakfast; alternatively, volunteers were allowed to consume capsule in the evening at least 3 h after the last meal of the day. The two probiotic preparations (provided by Sofar S.p.A., Trezzano Rosa, Italy) consisted of a gelatin capsule containing about 24 billion viable cells of the bacterial strain *L. paracasei* DG (CNCM I-1572) and LPC-S01 (DSM 26760), respectively; silicon dioxide and magnesium stearate were also added inside capsules as antiagglomerants. Capsules were delivered to participants in metal boxes sealed with a plastic cap containing desiccant salts. From the last day of consumption of the probiotic capsules, for the following 7 days, the volunteers provided one stool sample per day. Furthermore, participants provided a fecal sample at visits V1, V3, and V5 (Supplementary Figure 1). The sample was collected in a sterile plastic pot no more than 24 h before visit. Volunteers were asked to preserve the fecal sample at room temperature until delivery to the laboratory, according to the recommendations on storage conditions of intestinal microbiota matter in metagenomic analysis provided by Cardona et al. (2012). During the trial period, the participants compiled a weekly diary (including a Bristol stool chart) of their bowel habits.

Fecal DNA Extraction and qPCR

Stools were stored at −80°C until DNA extraction, performed by means of QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Before the extraction, the sample was homogenized in a Bead Beater Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) after addition of 0.45 g 0.5 mm glass beads. Subsequently, the extraction proceeded consistently with manufacturer's recommendations. Real-Time quantitative (qPCR) protocols were adopted for the quantification of *L. paracasei* DG in fecal metagenomic DNA (targeting the glycosyltransferase gene *welF*, with primers rtWELFf, 5′-TACTAAAGAAATTAGCTTTTGT-3′ and rtWELFr, 5′-AGTAATGTCTGCATCCTCCA-3′; Ferrario et al., 2014) and LPC-S01(targeting an hypothetical protein coding sequence with primers qS01a-F, 5′-TGGAAGAGACCCTGCGAA-3′ and qS01a-R, 5′-GAGGTTGATTCACAAACCGTGC-3′; this study). These two genes were selected because they resulted unique to the respective strain as revealed by a search in the GenBank nucleotide database. A gradient PCR was initially performed to standardize the qPCR conditions. qPCR amplifications were carried out in a final volume of 15 μl containing 7.5 μl of EvaGreen® Supermix and 0.5 μM of each primer. We used 100 ng of fecal DNA template in each reaction. Samples were amplified with the following programs: for rtWELF primers, initial hold at 95°C for 3 min, and 44 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s; for qS01a primers, initial hold at 95°C for 3 min, and 40 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 30 s. Melting curves were analyzed to confirm the specificity of the amplification products. To generate the standard curves with spiked feces for DG and LPC-S01 detection, a 200 mg matrix of fecal sample was added with 1×10^9 bacterial cells and DNA was extracted as reported above. Six-fold dilution series of isolated DNA were prepared and used in qPCR reactions. The equation of the derived standard curves was used to calculate the correlation between Ct values and the concentration of bacterial cells per g of feces.

Results

Comparative Genomics Revealed the Indels of Several Chromosomal Regions in *L. paracasei* LPC-S01

We generated a draft genome sequence of *L. paracasei* LPC-S01 consisting of 17 contigs, which in total contained 3.02 Mbp. Comparative genomic analysis was performed on strain LPC-S01 and the following six *L. paracasei* strains: the type strain of the species (*L. paracasei* JCM 8130^T), the probiotic strains DG and Shirota, a strain isolated from Raschera Italian

artisanal cheese (FMBr3), and two other *L. paracasei* strains whose complete genome is available in GenBank (strains 8700:2 and N1115) (**Figure 1**). When we used *L. paracasei* LPC-S01 as reference genome in the comparative analysis, we identified 14 genomic islands with putatively known biological functions, which have not been found in the genomes of type strain JCM 8130^T or other *L. paracasei* strains. These chromosomal islands, which discriminate LPC-S01 genome from the others, include 7 mobile genetic elements (e.g., prophages, phage remnants, and integrated plasmids; **Figure 1**), a transposon containing a phospho-β-galactosidase operon (region "a" in **Figure 1**; **Figure 2A**), a putative taurine transport and metabolization operon (b; **Figure 2B**), a large sugars transport/metabolization island (c; **Figure 2C**), an ABC-type Fe^{3+} transport system (d; **Figure 2D**), and a putative nucleotide transport and metabolism operon (e; **Figure 2E**). We also found in the genome of strain LPC-S01 two regions putatively involved in exopolysaccharide (EPS) synthesis. Specifically, one of these EPS regions (namely, EPS2; **Figure 1**) was found exclusively in the genome of *L. paracasei* LPC-S01 and is constituted of 14 putative genes, including six genes potentially coding for glycosyl transferases. Notably, BLASTN search revealed inside EPS2 a region of about 8 kb encompassing eight putative genes, which did not find any significant match in the GenBank database (genes from A to H in **Figure 3**). In subsequent comparative analyses, we used the genome of the other *L. paracasei* strains as reference to identify missing chromosomal islands in the genome of strain LPC-S01. Largely most of the genomic regions that are absent in LPC-S01 genome refer to mobile genetic elements, DNA restriction-modification systems, and EPS biosynthesis regions (data not shown).

In vitro Assays Showed That *L. paracasei* LPC-S01possesses Marked Acid Tolerance and Noticeable Adhesion Ability

The probiotic potential of strain *L. paracasei* LPC-S01 was assessed through *in vitro* experiments including *L. paracasei* DG and *L. paracasei* Shirota as reference probiotic strains. The potential ability to survive the gastro-intestinal transit was assessed in simulated gastric juices at pH 3 or 2 (90 min incubation), followed by 3 h incubation in bile (Oxgall) solution. All strains showed high tolerance to pH 3; cell viability, in fact, was not significantly reduced after 4.5 h (Supplementary Figure 2). Conversely, pH 2 determined a drastic reduction of the viability of all tested strains (**Figure 4**). However, *L. paracasei* LPC-S01 displayed the highest tolerance, which was significantly higher than that of the reference strain *L. paracasei* Shirota (**Figure 4**).

Growing concentrations of Oxgall were added to the MRS broth to assess the ability to grow in presence of bile. For all tested

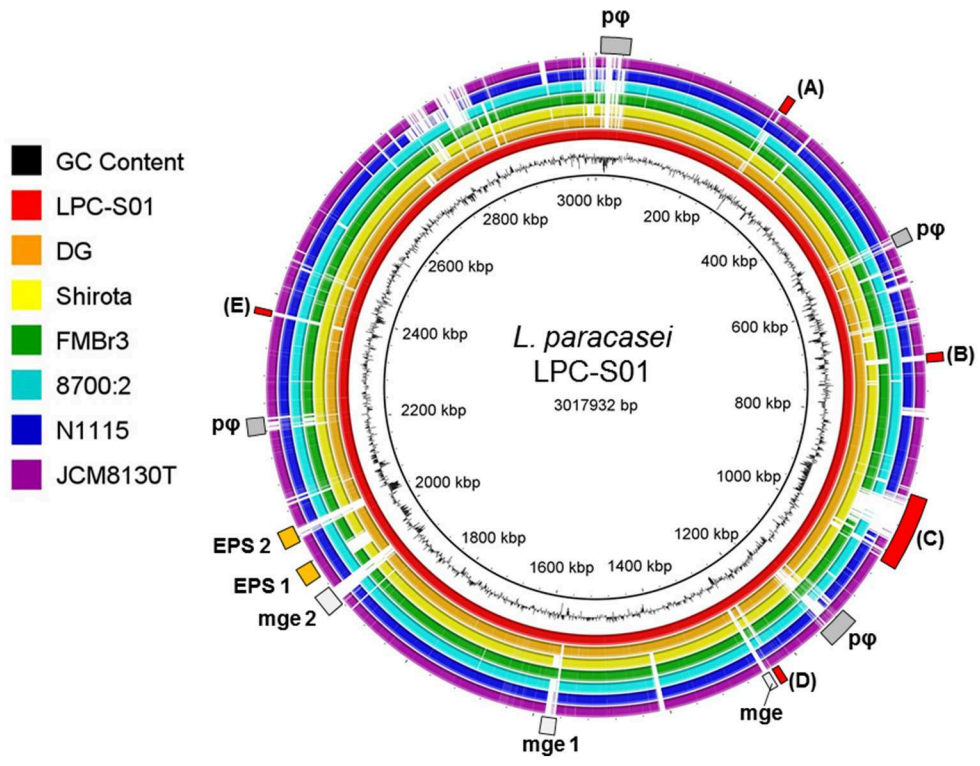


FIGURE 1 | Comparative genomic analysis of *Lactobacillus paracasei* LPC-S01 (reference genome) with the genome sequences of other six *L. paracasei* strains. Gray rectangles indicate mobile genetic elements. pφ, prophage-related regions. mge, mobile genetic element; mge 1, putative integrated plasmid; mge 2, putative mobile genetic element containing a restriction-modification system. EPS, putative exopolysaccharide coding operon. Letters flanking red rectangles refers to panels of **Figure 2**.

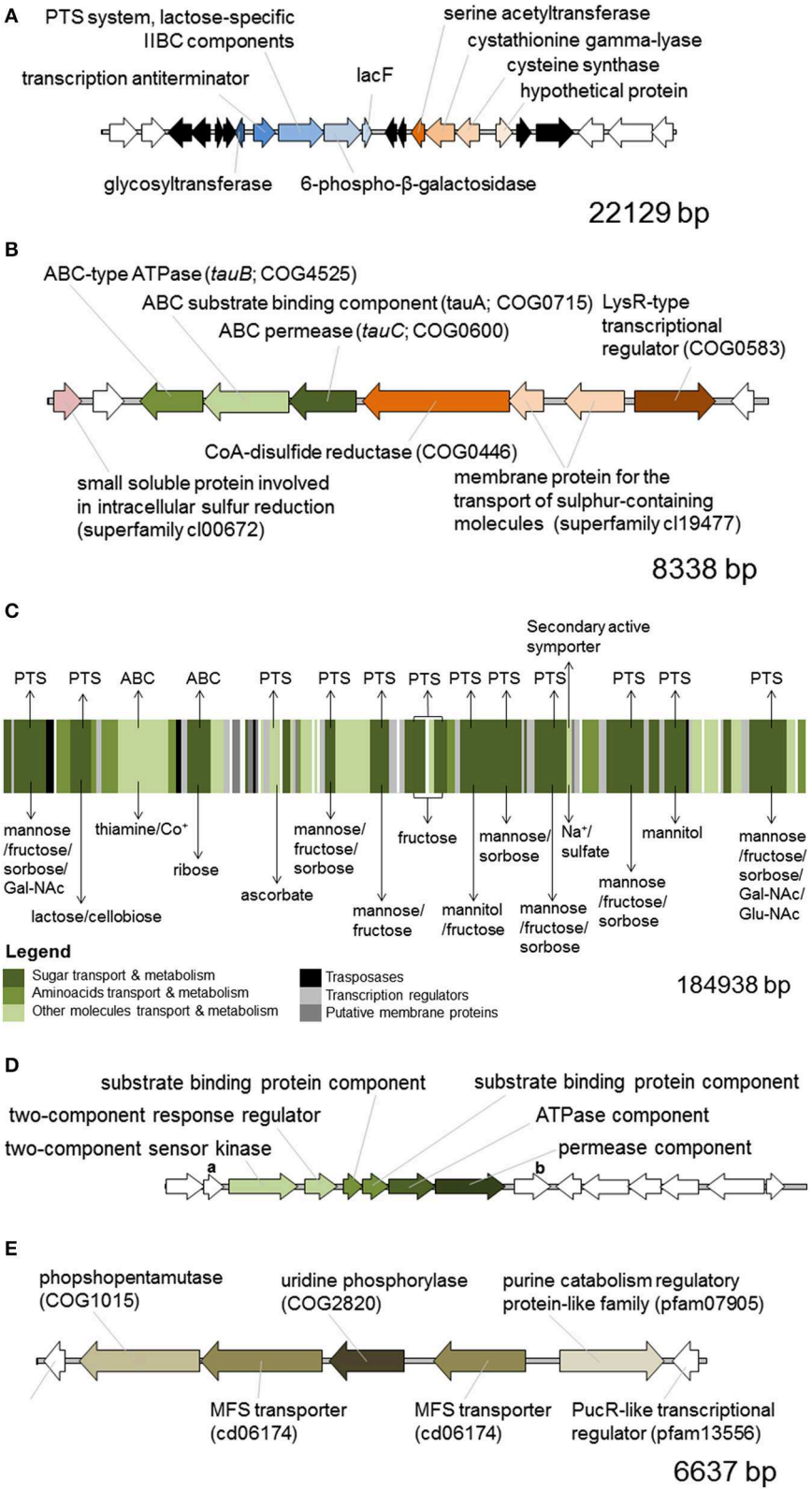


FIGURE 2 | *In silico* predicted functional organization of the chromosomal regions of *Lactobacillus paracasei* LPS-S01 that discriminates LPC-S01 from the others strains included in the comparative genomic analysis depicted in Figure 1. Letters in the panels of this figure refer to the letters flanking red

(Continued)

FIGURE 2 | Continued
rectangles in **Figure 1**. **(A)** Putative phospho-β-galactosidase operon. In black are indicated transposase-associated putative genes. In white are indicated putative genes that are also present in the genome of the other *L. paracasei* strains considered for comparative genomic analysis. **(B)** Putative taurine ABC-type transport and metabolization operon. **(C)** Multi-transport region for the uptake of sugars and other small molecules. The membrane transport mechanism is indicated above the picture, whereas the transported molecule is indicated below. ABC, ATP binding cassette transport system, PTS, phosphotransferase transport system. Gal-NAc, N-acetyl galactosamine; Glu-NAc, N-acetyl glucosamine; Co^{+}, cobalt ions. **(D)** Putative ABC-type Fe^{3+} transport system. Gene "a," putative acetyltransferase (COG0456) coding gene; gene "b," putative gene coding for an uncharacterized protein (DegV family, COG1307). **(E)** Putative nucleotide transport and metabolism operon. MFS, major facilitator superfamily. In white are indicated putative genes that are also present in the genome of all the other *L. paracasei* strains considered.

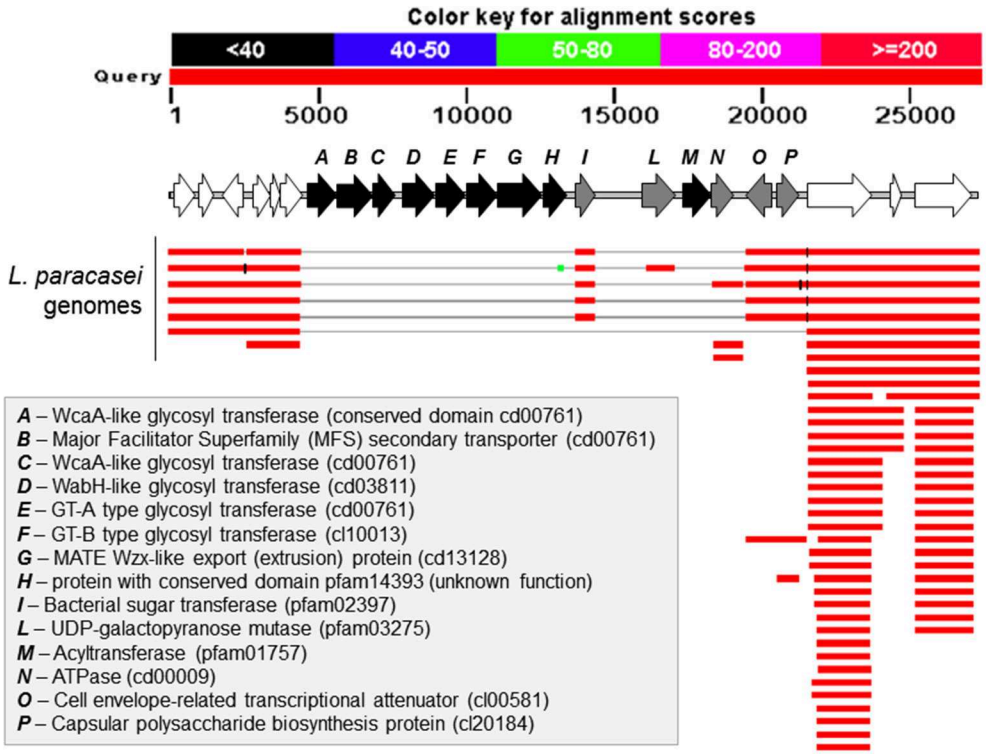


FIGURE 3 | BLASTN search results for the region EPS2 from *Lactobacillus paracasei* LPC-S01, which putatively codes for the enzymes involved in the synthesis of an exopolysaccharide (EPS). The figure has been obtained adding a picture of the putative EPS operon over the graphic representation of the BLASTN output. In white are indicated genes outside the putative EPS operon.

strains, we observed a dose-dependent inhibition of the bacterial growth, which was completely arrested at Oxgall concentrations higher than 1%. In specific, Shirota displayed the best tolerance to bile, even though not statistically significant (Supplementary Figure 3).

Subsequently, the ability of bacterial strains to adhere to the Caco-2 epithelial cell layer was tested. Only LPC-S01 displayed a marked adhesive phenotype, corresponding to an adhesion index (i.e., bacterial cells per 100 Caco-2 cells) of more than 2000 (**Figure 5A**). Also strain DG displayed adhesion ability, whereas strain Shirota was unable to adhere on Caco-2 cells, as observed in previous studies (Botes et al., 2008; Guglielmetti et al., 2008).

Caco-2 cell layer was also used to explore the immunomodulatory properties of *L. paracasei* strains by testing the effect of the microorganisms on NF-κB activation in presence of the pro-inflammatory cytokine IL-1β. To this aim, we employed a reporter cell line obtained by transfecting Caco-2 cells with a luciferase reporter vector induced by active NF-κB (Guglielmetti et al., 2010a). For all three *L. paracasei* strains, both bacterial cells and cell-free neutralized broths were able to significantly decrease the NF-κB-dependent production of bioluminescence. Particularly, we found that the highest ability to reduce NF-κB activation was exerted by DG and LPC-S01 cells, and by broth from Shirota culture (**Figure 5B**).

We also tested the ability of the bacteria under investigation to produce inhibitor molecules against pathogens by disc diffusion assay carried out by using cell-free MRS broths cultures. With the only exception of *L. plantarum* WHE 92 against *L. monocytogenes*, none of the neutralized broths was able to prevent pathogens' growth, suggesting that, plausibly, the only inhibitory molecules produced by tested *L. paracasei* strains were organic acids (Data not shown).

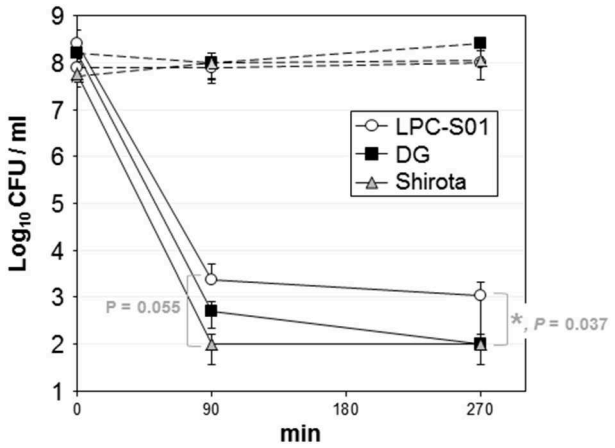


FIGURE 4 | Tolerance of *Lactobacillus paracasei* strains to simulated gastric juice at pH 2 (90 min incubation) and bile (180 min incubation). Dashed lines refer to controls (i.e., bacterial cells incubated in phosphate buffer, pH 6.5). Data are reported as the number of viable bacterial cells (CFU) plotted on a semi-logarithmic diagram. Vertical bars at each point refer to standard deviation calculated on three independent experiments conducted in duplicate. Asterisk between strain LPC-S01 and Shirota at the end of the experiment indicates statistically significant difference according to two-tailed unpaired Student's t test (*$P < 0.05$).

Afterwards, the *in vitro* probiotic properties of LPC-S01 and reference strains were assessed by testing their ability to produce acid from 44 different carbohydrate sources. The fermentation patterns of *L. paracasei* strains were very similar. In detail, the three tested strains fermented cellobiose, FOS, fructose, galactose, glucose, inulin, maltulose, mannitol, mannose, ribose, salicine, sorbose, sucrose, trehalose, and turanose (Supplementary Table 1).

In conclusion, the *in vitro* experiments carried out in this study showed that strain *L. paracasei* LPC-S01 possesses potential probiotic capabilities to be oral administered comparable to those of the probiotic commercial strains *L. paracasei* DG and Shirota. Notably, the survival rate of LPC-S01 to simulated gastrointestinal transit at pH 2 was significantly higher than that of the reference probiotic strain Shirota; in addition, strain LPC-S01 displayed marked adhesion ability, corresponding to an adhesion index higher than 2000.

We also studied the antibiotic resistance of *L. paracasei* LPC-S01by the microdilution assay recommended by International Organization for Standardization (ISO, 2010) with reference to the EFSA breakpoints for *L. casei*/*paracasei* (EFSA, 2012). The MICs for strain LPC-S01 was below the breakpoints for all tested antibiotics (**Table 1**). Finally, the safety of *L. paracasei* strains was also estimated by assessing the bacterial decarboxylation of amino acids, which generates biogenic amines in food (Deepika Priyadarshani and Rakshit, 2014). According to our experiments, none of the tested *L. paracasei* strains produced histamine, tyramine, putrescine and cadaverine (Data not shown), which are among the most common biogenic amines found in food products (Naila et al., 2010).

L. paracasei LPC-S01 Persists in the Gut of Healthy Adults

The ability of *L. paracasei* LPC-S01 to colonize the human gastrointestinal tract was tested with a pilot intervention trial based on a double-blind, randomized, cross-over design, in comparison with the probiotic strain *L. paracasei* DG. We enrolled 11 healthy adult volunteers, who were asked to consume for 1 week a capsule per day containing LPC-S01 or DG cells. Out of 11 enrolled volunteers, 8 concluded the study. Three volunteers dropped out of the study due to non-conformity ($n = 1$) and protocol violation (missed probiotic consumption; $n = 2$) (Supplementary Figure 1). Capsules, which contained about 24 billion CFU of *L. paracasei*, were well tolerated by all participants and no adverse events were reported. In order to quantify *L. paracasei* cells in the fecal samples all over the trial, we used qPCR with primers targeting gene *welF* (specific for strain DG; Ferrario et al., 2014) and a gene coding for a hypothetical protein (specific for strain LPC-S01; primers designed in this study). According to repeated measure ANOVA, we did not find significant difference in the persistence of the two *L. paracasei* strains (**Figure 6**). Specifically, the concentrations of LPC-S01 and DG cells were above the qPCR detection limits for an average of 5 days from the end of treatment; in addition, *L. paracasei* cells were not detectable in the fecal samples after 7 ± 2 evacuations from the last capsule consumption (**Figure 6A**).

Discussion

Bacterial vaginosis (BV) is one of the most common urogenital diseases affecting women of reproductive age (Allsworth and Peipert, 2007). BV is typically associated to the disruption of the healthy vaginal microbiota, which is normally dominated by lactobacilli (Ma et al., 2012). Due to the dominant role of dysbiosis in the onset of BV, probiotics, which are regularly used to prevent and treat dysbiosis in the intestine, have been proposed as a strategy to cure this urogenital condition and reduce its recurrence (Bodean et al., 2013). Several studies documented the beneficial effects on BV of viable *Lactobacillus* cells directly introduced in the vagina as suppository (Patel et al., 2008; Donders et al., 2010). Nonetheless, also the oral administration of probiotics has been demonstrated to be an efficient strategy to alleviate BV symptoms and prevent recurrence (Reid, 2006; Bodean et al., 2013). In fact, it was proposed that ingested lactobacilli, similarly to colonic pathogenic bacteria, once excreted from rectum, may ascend to the vagina through perineum (Reid et al., 2001; Bastani et al., 2012). Then, once in the vagina, lactobacilli create a protecting barrier against the translocation from the gut of possible detrimental bacteria such as *Prevotella* spp. or *E. coli*, and, at the same time, limit the possible migration of uropathogens from vagina to the bladder (Reid et al., 2001).

The oral administration of probiotics is a more practical route than their direct introduction in the vagina, and can also provide consumers with the gastrointestinal health benefits typically associated with the consumption *per os*. In this context, this study aimed to the identification of a probiotic strain

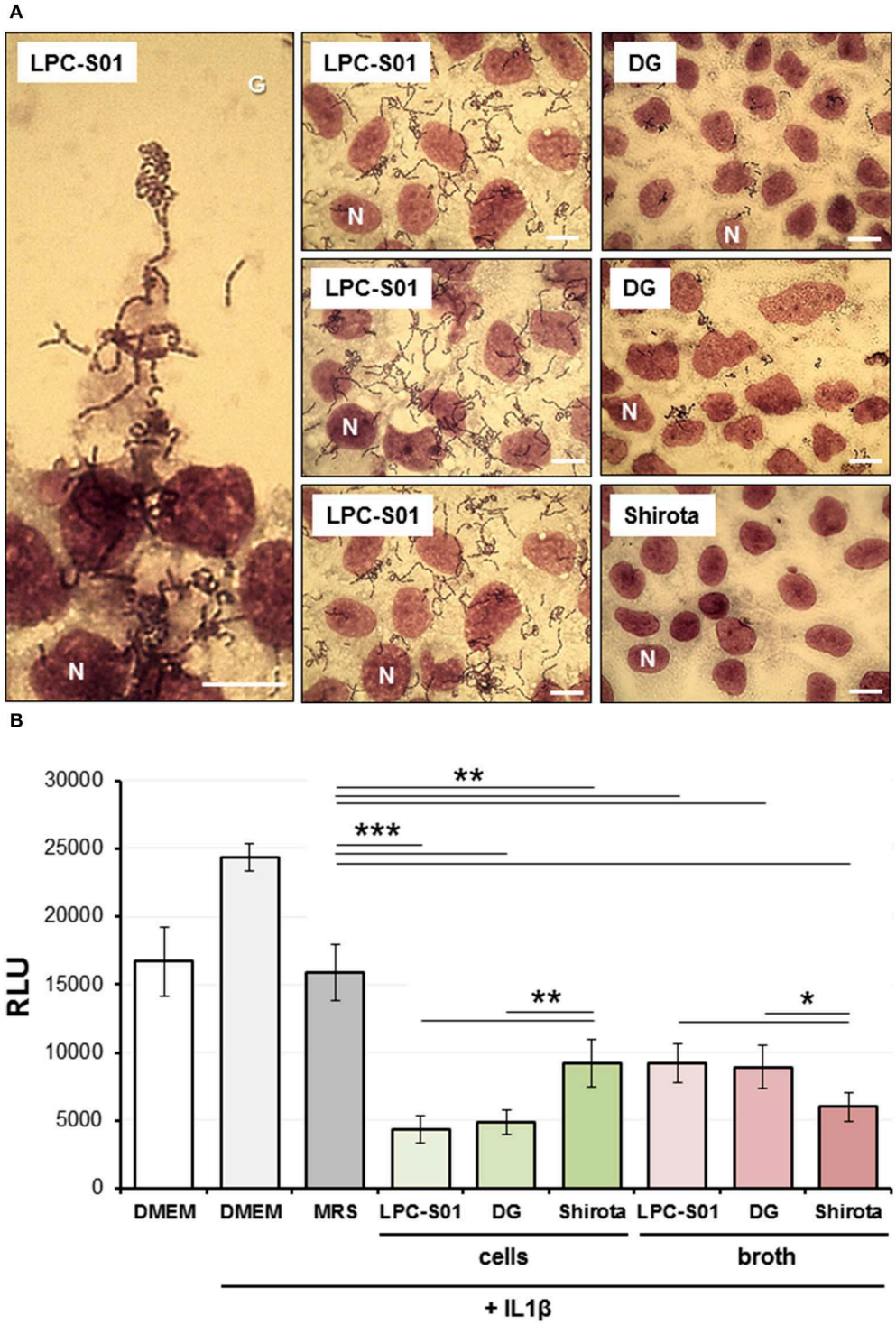


FIGURE 5 | Experiments on human epithelial colorectal Caco-2 cell layer. (A) Adhesion of *Lactobacillus paracasei* strains to the Caco-2 epithelial cell layer as observed with Giemsa staining under a light microscope. Bars, 25 μm. One Caco-2 nucleus for each layer is indicated with the letter N. LPC-S01 adhesion was specific to Caco-2 cells: no adhesion was detected on the cover glass underlying Caco-2 cells (G; top part of the panel on the left). **(B)** Effect of *L. paracasei* strains on Caco-2 cells stably transfected with an NF-κB/luciferase reporter vector, in the presence of 5 ng/ml of IL-1β. Data in the histograms are the means (± standard deviations) from two independent experiments conducted in triplicate. RLU, relative luminescence units. Asterisks indicate statistically significant differences according to two-tailed unpaired Student's *t*-test: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

TABLE 1 | Antibiotic sensitivity of *Lactobacillus paracasei* LPC-S01 determined according to the microdilution assay recommended by EFSA (2012).

	EFSA breakpoint	LPC-S01
Ampicillin	2	2
Vancomycin	not required	>256
Gentamicin	32	32
Kanamycin	64	32
Streptomycin	Not required	32
Erythromycin	1	0.5
Clindamycin	1	0.062
Tetracycline	4	2
Chloramphenicol	4	4
Amoxicillin	Not indicated	1

Data are reported as the lower concentration (mg/l) inhibiting bacterial growth (minimal inhibitory concentration, MIC).

possessing a wide range of properties, which may support its (i) industrial production, (ii) gastrointestinal administration, and (iii) vaginal colonization. To this aim, we focused our attention on *Lactobacillus paracasei*, a species characterized by marked ecological flexibility and extensive genetic diversity, which is widely used in industry (Cai et al., 2007). Particularly, we studied strain *L. paracasei* LPC-S01, which was originally isolated from the vagina of a healthy adult and has had a several-years history of commercial use as a vaginal probiotic for topical application in Europe. Specifically, the experiments carried out in this study were designed to evaluate if *L. paracasei* LPC-S01 has probiotic properties that can support its administration *per os*.

At first, the draft genome of LPC-S01 was analyzed by comparative genomics. We found the presence of numerous accessory genes (i.e., unique to this particular strain) that suggests the niche-generalists nature of LPC-S01; such potential ability to adapt to a wide range of environmental conditions is evidenced, for instance, by the presence of large chromosomal regions including genes for the utilization of numerous sugars (**Figure 2C**). Similar regions are also present in the genome of the probiotic strains DG and Shirota, which are of human intestinal origin. On the contrary, it was proposed that the adaptation of *L. paracasei* to the nutrient-rich milk environment had been gone along with extensive decay of genes involved in carbohydrate utilization (Broadbent et al., 2012). Accordingly, we found the lack of genes for sugar transport and metabolism in the chromosome of the dairy strains *L. paracasei* FMBr3 and N1115 compared to LPC-S01, DG, and Shirota strains (**Figure 1**).

The *in vitro* characterization of *L. paracasei* LPC-S01 was conducted in accordance with a procedure conventionally used to predict the probiotic potential of a microbial strain; such process consists in the assessment of sensitivity to acidity and bile, adhesion ability, pathogen inhibition, sugar fermentation profile, and immunomodulatory properties. In order to better predict the probiotic capabilities of LPC-S01, the reference strains Shirota and DG were also included in the study. *L. paracasei* Shirota is one of the most intensively studied probiotics (van den Nieuwboer et al., 2015; Wang et al., 2015) that has been used in the production of the fermented milk Yakult since 1935. *L. paracasei* DG is a bacterial strain with demonstrated probiotic properties (D'Incà et al., 2011; Tursi et al., 2013; Ferrario et al., 2014), which is included in Enterolactis®, one the most popular probiotic supplements in Italy. We observed that the *in vitro* performances of LPC-S01 were basically very similar to those of the reference strains. One exception was the significantly higher ability of LPC-S01 to survive in simulated gastric juice at pH 2 compared to *L. paracasei* Shirota, which is an acid-tolerant bacterial strain with demonstrated survivability to human gastrointestinal transit (Tuohy et al., 2007; Wang et al., 2015). In addition, LPC-S01 showed a much higher ability to adhere to Caco-2 epithelial cell layer than DG and Shirota. Bacterial adhesion properties mainly depend on the molecules exposed at the external cell surface (Guglielmetti et al., 2008; Polak-Berecka et al., 2014). Genome analysis revealed the presence in LPC-S01 of putative exopolysaccharide coding region (EPS2) containing a cluster of five genes which are not present in the other known *L. paracasei* genomes. Therefore, EPS2 could potentially support the synthesis of a peculiar exopolysaccharide molecule, which could plausibly contribute to the adhesion ability of the LPC-S01. Exopolysaccharides have been proposed to promote *Lactobacillus* adhesion (Ren et al., 2014), although, more frequently, the presence of a polysaccharide capsule has been shown to reduce adhesion by masking cell surface *Lactobacillus* adhesins determinants (Lebeer et al., 2012; Horn et al., 2013; Dertli et al., 2015), which are most commonly proteins (Lebeer et al., 2012; Polak-Berecka et al., 2014), (lipo)teichoic acids (Granato et al., 1999), and/or fatty acids (Polak-Berecka et al., 2014).

The acid tolerance and adhesion properties of LPC-S01 suggest that this bacterium might tolerate and adapt to the gastrointestinal environment. To test this hypothesis, we performed an intervention trial, in which we compared the gut persistence of *L. paracasei* LPC-S01 with that of strain DG. Following a daily consumption of 24 billion viable cells for 1 week, we estimated that strain LPC-S01 reached a concentration of about 10^8 cells per g of feces, persisting above the detection limit (10^5 cells per g of feces) for about 5 days, which is not significantly dissimilar to the persistence of the reference *L. paracasei* DG, a strain demonstrated to colonize the human gut of healthy adults and induce specific modifications in the microbiota composition (De Vecchi et al., 2008; Ferrario et al., 2014). Our data on *L. paracasei* persistence are in agreement with other studies, which observed that the persistence of *L. paracasei* Shirota in the gastrointestinal tract of healthy adults is lower than 1 week after cessation of the probiotic ingestion (Tuohy et al., 2007; Wang et al., 2015). The persistence of a certain probiotic strain in the gut plausibly depends on the evacuation frequency. In our study, we found persistence of strains LPC-S01 and DG as average for 7 evacuations. No data correlating the day of persistence with the number of evacuations were found in literature.

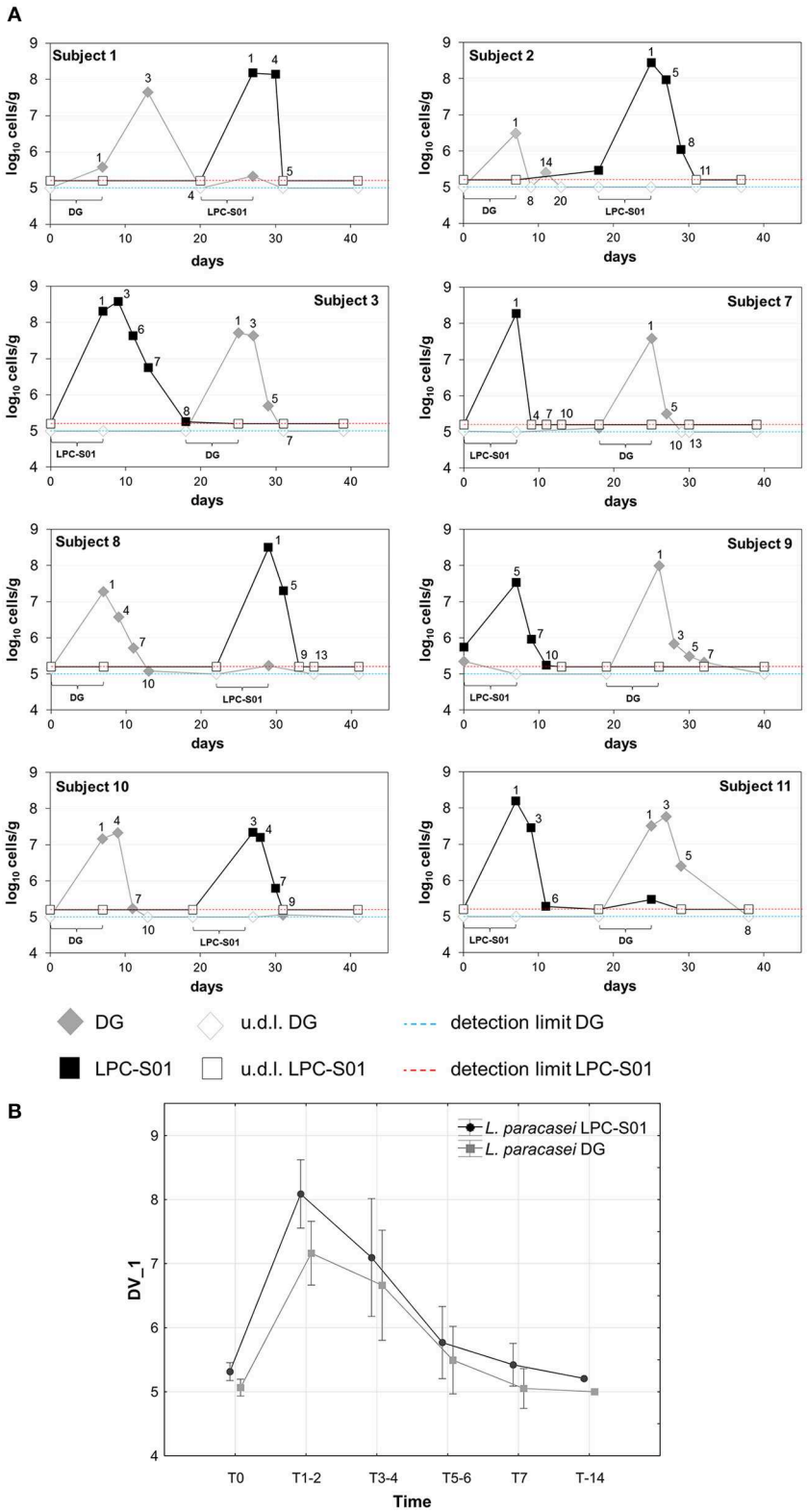


FIGURE 6 | Persistence of *Lactobacillus paracasei* in the gastrointestinal tract of healthy adults. (A) Bacterial concentration of *L. paracasei* DG and LPC-S01 in the fecal samples collected during the study per single participant. **(B)** Average change in the fecal concentration of *L. paracasei* cells after the probiotic-intake period. Vertical bars denote 0.95 confidence intervals. Current effect according to repeated measures ANOVA performed to determine the statistical significance of the treatment × time interaction: $F_{(5,\ 65)} = 0.794$; $p = 0.558$.

Conclusions

The results of this study can be summarized as follows:

(i) according to comparative genomic analysis, *L. paracasei* LPC-S01 possesses the genetic features of a niche-generalist member of its species;

(ii) *in vitro* tests evidenced that the probiotic properties of strain LPC-S01, with respect to those of the well-known probiotic references L. paracasei Shirota and DG, are equal for the bile tolerance and the reduction of NF-κB activation in Caco-2 cells, or superior for the tolerance to gastric juice and the adhesion to Caco-2 epithelial cells (Shirota is unable to adhere on Caco-2-cells);

(iii) *L. paracasei* LPC-S01 is a safe bacterial strain for human consumption, which does not contain any acquired antibiotic resistance, does not produce biogenic amines and can be administered in high number (24 billion CFU) to healthy people without adverse events;

(iv) when administered as capsule containing 24 billion CFU, *L. paracasei* LPC-S01 transiently colonizes the gastrointestinal tract of the host, persisting for at least 5 days after the end of a 7-days oral consumption (corresponding to about 7 evacuations) and therefore displaying colonizing performances not dissimilar to those of the probiotic commercial strain *L. paracasei* DG.

In conclusion, this study demonstrates that the vaginal isolate *L. paracasei* LPC-S01 possesses safety and functional properties that may support its use as probiotic to be administered *per os* for potential intestinal as well as vaginal applications. In this perspective, it would be of interest to carry out a clinical trial consisting of the oral administration to healthy adult women of *L. paracasei* LPC-S01 cells, in order to verify the potential ability of this probiotic bacterium to (i) modulate the intestinal and vaginal microbiota, and (ii) colonize the human vagina via gastrointestinal route.

Acknowledgments

This work was financially supported by Sofar S.p.A. (grant cod.16693). We thank Valentina Balzaretti for her assistance during manuscript revision.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00952

References

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Allsworth, J. E., and Peipert, J. F. (2007). Prevalence of bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. *Obstet. Gynecol.* 109, 114–120. doi: 10.1097/01.AOG.0000247627.84791.91

Azaïs-Braesco, V., Bresson, J. L., Guarner, F., and Corthier, G. (2010). Not all lactic acid bacteria are probiotics,...but some are. *Br. J. Nutr.* 103, 1079–1081. doi: 10.1017/S0007114510000723

Bastani, P., Homayouni, A., Ziyadi, S., and Tabrizian, V. G. (2012). "Dairy probiotic foods and bacterial vaginosis: a review on mechanism of action," in *Probiotics*, ed E. C. Rigobelo (Croatia: Intech publishing), 445–456.

Bodean, O., Munteanu, O., Cirstoiu, C., Secara, D., and Cirstoiu, M. (2013). Probiotics: a helpful additional therapy for bacterial vaginosis. *J. Med. Life* 6, 434–436.

Borchert, D., Sheridan, L., Papatsoris, A., Faruquz, Z., Barua, J. M., Junaid, I., et al. (2008). Prevention and treatment of urinary tract infection with probiotics: review and research perspective. *Indian J. Urol.* 24, 139–144. doi: 10.4103/0970-1591.40604

Borges, S., Silva, J., and Teixeira, P. (2014). The role of lactobacilli and probiotics in maintaining vaginal health. *Arch. Gynecol. Obstet.* 289, 479–489. doi: 10.1007/s00404-013-3064-9

Botes, M., Loos, B., van Reenen, C. A., and Dicks, L. M. (2008). Adhesion of the probiotic strains Enterococcus mundtii ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. *Arch. Microbiol.* 190, 573–584. doi: 10.1007/s00203-008-0408-0

Bover-Cid, S., and Holzapfel, W. H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.* 53, 33–41. doi: 10.1016/S0168-1605(99)00152-X

Broadbent, J. R., Neeno-Eckwall, E. C., Stahl, B., Tandee, K., Cai, H., Morovic, W., et al. (2012). Analysis of the Lactobacillus casei supragenome and its influence in species evolution and lifestyle adaptation. *BMC Genomics* 13:533. doi: 10.1186/1471-2164-13-533

Cai, H., Rodríguez, B. T., Zhang, W., Broadbent, J. R., and Steele, J. L. (2007). Genotypic and phenotypic characterization of Lactobacillus casei strains isolated from different ecological niches suggests frequent recombination and niche specificity. *Microbiology* 153, 2655–2665. doi: 10.1099/mic.0.2007/006452-0

Cardona, S., Eck, A., Cassellas, M., Gallart, M., Alastrue, C., Dore, J., et al. (2012). Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiol.* 12:158. doi: 10.1186/1471-2180-12-158

Deepika Priyadarshani, W. M., and Rakshit, S. K. (2014). Growth and biogenic amine (histamine and tyramine) potential of probiotic *Lactobacillus casei* in skim milk. *Am. J. Food Technol.* 9, 69–79. doi: 10.3923/ajft.2014.69.79

Dertli, E., Mayer, M. J., and Narbad, A. (2015). Impact of the exopolysaccharide layer on biofilms, adhesion and resistance to stress in *Lactobacillus johnsonii* FI9785. *BMC Microbiol.* 15:18. doi: 10.1186/s12866-015-0347-2

De Vecchi, E., Nicola, L., Zanini, S., and Drago, L. (2008). *In vitro* screening of probiotic characteristics of some Italian products. *J. Chemother.* 20, 341–347. doi: 10.1179/joc.2008.20.3.341

D'Incà, R., Barollo, M., Scarpa, M., Grillo, A. R., Brun, P., Vettorato, M. G., et al. (2011). Rectal administration of *Lactobacillus casei* DG modifies flora composition and Toll-like receptor expression in colonic mucosa of patients with mild ulcerative colitis. *Dig. Dis. Sci.* 56, 1178–1187. doi: 10.1007/s10620-010-1384-1

Donders, G. G., Van Bulck, B., Van de Walle, P., Kaiser, R. R., Pohlig, G., Gonser, S., et al. (2010). Effect of lyophilized lactobacilli and 0.03 mg estriol (Gynoflor®) on vaginitis and vaginosis with disrupted vaginal microflora: a multicenter, randomized, single-blind, active-controlled pilot study. *Gynecol. Obstet. Invest.* 70, 264–272. doi: 10.1159/000314016

EFSA. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.* 10:2740. doi: 10.2903/j.efsa.2012.2740

Ennahar, S., Aoude-Werner, D., Sorokine, O., Van Dorsselaer, A., Bringel, F., Hubert, J. C., et al. (1996). Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. *Appl. Environ. Microbiol.* 62, 4381–4387.

FAO/WHO. (2002). *Report of a Joint FAO/WHO Expert Consultation on Guidelines for the Evaluation of Probiotics in Food*. London, ON: World Health Organization and Food and Agriculture Organization of the United Nations.

Ferrario, C., Taverniti, V., Milani, C., Fiore, W., Laureati, M., De Noni, I., et al. (2014). Modulation of fecal Clostridiales bacteria and butyrate by probiotic intervention with *Lactobacillus paracasei* DG varies among healthy adults. *J. Nutr.* 144, 1787–1796. doi: 10.3945/jn.114.197723

Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., et al. (1999). Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Appl. Environ. Microbiol.* 65, 1071–1077.

Guglielmetti, S., Mora, D., Gschwender, M., and Popp, K. (2011). Randomised clinical trial: *Bifidobacterium bifidum* MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life - a double-blind, placebo-controlled study. *Aliment. Pharm. Therap.* 33, 1123–1132. doi: 10.1111/j.1365-2036.2011.04633.x

Guglielmetti, S., Tamagnini, I., Mora, D., Minuzzo, M., Scarafoni, A., Arioli, S., et al. (2008). Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. *Appl. Environ. Microb.* 74, 4695–4702. doi: 10.1128/AEM.00124-08

Guglielmetti, S., Taverniti, V., Minuzzo, M., Arioli, S., Stuknyte, M., Karp, M., et al. (2010a). Oral bacteria as potential probiotics for the pharyngeal mucosa. *Appl. Environ. Microbiol.* 76, 3948–3958. doi: 10.1128/AEM.00109-10

Guglielmetti, S., Taverniti, V., Minuzzo, M., Arioli, S., Zanoni, I., Stuknyte, M., et al. (2010b). A dairy bacterium displays *in vitro* probiotic properties for the pharyngeal mucosa by antagonizing group A streptococci and modulating the immune response. *Infect. Immun.* 78, 4734–4743. doi: 10.1128/IAI.00559-10

Horn, N., Wegmann, U., Dertli, E., Mulholland, F., Collins, S. R., Waldron, K. W., et al. (2013). Spontaneous mutation reveals influence of exopolysaccharide on *Lactobacillus johnsonii* surface characteristics. *PLoS ONE* 8:e59957. doi: 10.1371/journal.pone.0059957

ISO. (2010). *Milk and Milk Products - Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-enterococcal Lactic acid Bacteria (LAB)*. Geneva: International Organization for Standardization 10932:2010 (IDF 223:2010).

Johnson-Henry, K. C., Mitchell, D. J., Avitzur, Y., Galindo-Mata, E., Jones, N. L., and Sherman, P. M. (2004). Probiotics reduce bacterial colonization and gastric inflammation in *H. pylori*-infected mice. *Dig. Dis. Sci.* 49, 1095–1102. doi: 10.1023/B:DDAS.0000037794.02040.c2

Krutmann, J. (2009). Pre- and probiotics for human skin. *J. Dermatol. Sci.* 54, 1–5. doi: 10.1016/j.jdermsci.2009.01.002

Lebeer, S., Claes, I., Tytgat, H. L., Verhoeven, T. L., Marien, E., von Ossowski, I., et al. (2012). Functional analysis of *Lactobacillus rhamnosus* GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. *Appl. Environ. Microbiol.* 78, 185–193. doi: 10.1128/AEM.06192-11

Ma, B., Forney, L. J., and Ravel, J. (2012). Vaginal microbiome: rethinking health and disease. *Annu. Rev. Microbiol.* 66, 371–389. doi: 10.1146/annurev-micro-092611-150157

Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G. A., 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

Naila, A., Flint, S., Fletcher, G., Bremer, P., and Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *J. Food Sci.* 75, R139–R150. doi: 10.1111/j.1750-3841.2010.01774.x

Nanno, M., Kato, I., Kobayashi, T., and Shida, K. (2011). Biological effects of probiotics: what impact does *Lactobacillus casei* Shirota have on us? *Int. J. Immunopathol. Pharmacol.* 24, 45S–50S.

Patel, Y., Gopalan, S., Bagga, R., Sharma, M., Chopra, S., and Sethi, S. (2008). A randomized trial comparing a polyherbal pessary (a complementary and alternative medicine) with Ginlac-V pessary (containing clotrimazole, tinidazole and lactobacilli) for treatment of women with symptomatic vaginal discharge. *Arch. Gynecol. Obstet.* 278, 341–347. doi: 10.1007/s00404-008-0568-9

Polak-Berecka, M., Wasko, A., Paduch, R., Skrzypek, T., and Sroka-Bartnicka, A. (2014). The effect of cell surface components on adhesion ability of *Lactobacillus rhamnosus*. *Antonie Van Leeuwenhoek* 106, 751–762. doi: 10.1007/s10482-014-0245-x

Reid, G. (2006). Probiotics to prevent the need for, and augment the use of antibiotics. *Can. J. Infect. Dis. Med. Microbiol.* 17, 291–295.

Reid, G., Beuerman, D., Heinemann, C., and Bruce, A. W. (2001). Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. *FEMS Immunol. Med. Microbiol.* 32, 37–41. doi: 10.1111/j.1574-695X.2001.tb00531.x

Ren, D., Li, C., Qin, Y., Yin, R., Du, S., Ye, F., et al. (2014). *In vitro* evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. *Anaerobe* 30, 1–10. doi: 10.1016/j.anaerobe.2014.07.004

Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., et al. (2013). *Lactobacillus paracasei* comparative genomics: towards species pan-genome definition and exploitation of diversity. *PLoS ONE* 8:e68731. doi: 10.1371/journal.pone.0068731

Stuknyte, M., De Noni, I., Guglielmetti, S., Minuzzo, M., and Mora, D. (2011). Potential immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria. *Int. Dairy J.* 21, 763–769. doi: 10.1016/j.idairyj.2011.04.011

Szajewska, H., and Chmielewska, A. (2013). Growth of infants fed formula supplemented with *Bifidobacterium lactis* Bb12 or *Lactobacillus* GG: a systematic review of randomized controlled trials. *BMC Pediatr.* 13:185. doi: 10.1186/1471-2431-13-185

Taverniti, V., Minuzzo, M., Arioli, S., Junttila, I., Hämäläinen, S., Turpeinen, H., et al. (2012). *In vitro* functional and immunomodulatory properties of the *Lactobacillus helveticus* MIMLh5-*Streptococcus salivarius* ST3 association that are relevant to the development of a pharyngeal probiotic product. *Appl. Environ. Microbiol.* 78, 4209–4216. doi: 10.1128/AEM.00325-12

Taverniti, V., Stuknyte, M., Minuzzo, M., Arioli, S., De Noni, I., Scabiosi, C., et al. (2013). S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. *Appl. Environ. Microbiol.* 79, 1221–1231. doi: 10.1128/AEM.03056-12

Tuohy, K. M., Pinart-Gilberga, M., Jones, M., Hoyles, L., McCartney, A. L., and Gibson, G. R. (2007). Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. *J. Appl. Microbiol.* 102, 1026–1032. doi: 10.1111/j.1365-2672.2006.03154.x

Tursi, A., Brandimarte, G., Elisei, W., Picchio, M., Forti, G., Pianese, G., et al. (2013). Randomised clinical trial: mesalazine and/or probiotics in maintaining remission of symptomatic uncomplicated diverticular disease—a double-blind, randomised, placebo-controlled study. *Aliment. Pharmacol. Ther.* 38, 741–751. doi: 10.1111/apt.12463

van den Nieuwboer, M., Klomp-Hogeterp, A., Verdoorn, S., Metsemakers-Brameijer, L., Vriend, T. M., Claassen, E., et al. (2015). Improving the bowel habits of elderly residents in a nursing home using probiotic fermented milk. *Benef. Microbes* 17, 1–8. doi: 10.3920/bm2014.0113

van de Wijgert, J. H., Borgdorff, H., Verhelst, R., Crucitti, T., Francis, S., Verstraelen, H., et al. (2014). The vaginal microbiota: what have we learned after a decade of molecular characterization? *PLoS ONE* 9:e105998. doi: 10.1371/journal.pone.0105998

Vásquez, A., Ahrné, S., Jeppsson, B., and Molin, G. (2005). Oral administration of *Lactobacillus* and *Bifidobacterium* strains of intestinal and vaginal origin to healthy human females: re-isolation from faeces and vagina. *Microb. Ecol. Health Dis.* 17, 15–20. doi: 10.1080/08910600510031376

Wang, R., Chen, S., Jin, J., Ren, F., Li, Y, Qiao, Z., et al. (2015). Survival of Lactobacillus casei strain Shirota in the intestines of healthy Chinese adults. *Microbiol Immunol.* 59, 268–276. doi: 10.1111/1348-0421.12249

Wescombe, P. A., Hale, J. D., Heng, N. C., and Tagg, J. R. (2012). Developing oral probiotics from *Streptococcus salivarius*. *Future Microbiol.* 7, 1355–1371. doi: 10.2217/fmb.12.113

Conflict of Interest Statement: Research was financially supported by Sofar S.p.A., which is the company that produces and sells the probiotics products employed in the intervention study. WF is an employee of Sofar S.p.A. The other 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 ARTICLE
published: 03 September 2014
doi: 10.3389/fmicb.2014.00467

Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2

F. Rodríguez-Gómez†, V. Romero-Gil†, P. García-García, A. Garrido-Fernández and Francisco N. Arroyo-López*

Department of Food Biotechnology, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Seville, Spain

Edited by:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Chrysoula C. Tassou, National Agricultural Research Foundation, Greece
Aldo Corsetti, University of Teramo, Italy

***Correspondence:**
Francisco N. Arroyo-López, Department of Food Biotechnology, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Avda. Padre García Tejero 4, 41012 Seville, Spain
e-mail: fnarroyo@cica.es

†F. Rodríguez-Gómez and V. Romero-Gil have contributed equally to this work.

Dairy products are currently the main carriers of probiotic microorganisms to the human body. However, the development of new matrices for probiotic delivery is convenient for intolerant to milk (or its derivatives) and those requiring low-cholesterol diet consumers. The present work focused on the fortification of previously fermented green Spanish style olives with the autochthonous putative probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. The fortification was carried out by inoculating the bacteria into the packing brines using Manzanilla fruits from three different processes: (i) spontaneously fermented (F1), (ii) fermented using *L. pentosus* TOMC-LAB2 as starter (F2), and (iii) spontaneously fermented and then thermally treated (F3). Data showed that all inoculated treatments had higher population levels (5.49, 4.41, and 6.77 $\log_{10}$ cfu/cm^2) than their respective controls (1.66, 4.33, and 0.0 $\log_{10}$ cfu/cm^2, for F1, F2, and F3 treatments, respectively). The presence of *L. pentosus* TOMC-LAB2 on olive surface was confirmed by rep-PCR, with a recovery frequency at the end of the shelf life (200 days) of 52.6, 57.9, and 100.0% for F1, F2, and F3 treatments, respectively. Thus, results obtained in this work show the ability of this microorganism to survive under packing conditions for long period of times as well as to colonize the olive surface which is the food finally ingested by consumers. This opens the possibility for the development of a new and simply probiotic fortified olive product.

Keywords: fortification, *Lactobacillus pentosus*, olive packing, probiotic vegetables, table olives

INTRODUCTION

Table olive is one of the most important fermented vegetable of the food industry in the western countries, especially in the Mediterranean basin, with Spain, Turkey, Egypt, Italy, and Greece as the main producers. The International Olive Oil Council (IOC) estimated that the last consolidate balance of table olive production (2011/2012 season) was above 2.4 million tons (International Olive Council [IOC], 2014). Approximately 60% of this amount was processed according to green Spanish-style table olives, which implies a lye treatment of fruits (1.8–2.5% NaOH, w/v), followed by washing and brining (10–11% NaCl, w/v) where fruits undergo a typical lactic acid fermentation (Garrido-Fernández et al., 1997).

Lactic acid bacteria (LAB) exhibit diverse characteristics with potential benefits on human health. Among others, the following favorable effects have been documented: improvement of food digestibility and lactose assimilation, modulation of the immune response, reduction of hypercholesterolemia, prevention of intestinal infections, cancer, food allergies, and constipation (Champagne and Gardner, 2005). To these favorable aspects, we have to add that table olives might also be considered as a functional food itself because of their high content in antioxidant compounds, vitamins, dietary fiber, and anticancer compounds (Garrido Fernández et al., 2001; Reyes-Zurita et al., 2009).

Fermented vegetables are nowadays being considered as a splendid source and vehicle of probiotic microorganisms (Peres et al., 2012). Probiotic vegetables have the potential to attract more consumers who demand functional products, since vegetables provide to those who are intolerant to milk and its derivatives, or require low-cholesterol diets, access to new probiotic formulations (Lavermicocca et al., 2010; Peres et al., 2012). Packed green Spanish-style table olives can be thermally treated for preservation, but they can also be stabilized simply by physicochemical characteristics, being in this case considered as ready-to-eat product directly consumed without any prior cooking. This makes them very convenient carriers of beneficial microorganisms to the human body.

Ranadhera et al. (2010) considered that the type of food carrier plays an essential role in buffering the probiotic during its passage throughout the gastrointestinal tract, regulating their colonization or interacting with other microorganisms to alter functionality. Lavermicocca et al. (2005) used table olives as a vehicle to incorporate exogenous probiotic bacteria species into the human body. Particularly, one strain of *Lactobacillus rhamnosus* was remained invariant until the end of the experiment and showed a good recovery after fermentation. *L. paracasei* IMPC2.1 successfully colonized the olive surface and dominate the natural LAB population until the end of the fermentation (De Bellis et al., 2010), making the product a suitable carrier for its delivery to humans. Recently, Argyri et al. (2013a) and Bautista-Gallego et al. (2013) have isolated diverse LAB strains from table olive processing with promising characteristics for their use as probiotic agent.

Usually, the development of probiotic table olives has been focused on the application of these microorganisms as starter cultures at the onset of fermentation. A recent study showed that the inoculation with multifunctional lactobacilli starters led to a decrease in the *Enterobacteriaceae* populations and to higher LAB and yeast populations as well as to a faster acidification of the brines (Rodríguez-Gómez et al., 2013). Argyri et al. (2014) and Blana et al. (2014) also evaluated with promising results diverse potential probiotic LAB strains, originally isolated from olive fermentation, as starter cultures in table olive fermentation.

On the contrary, the present study is completely novel because is focused on the application of autochthonous putative probiotic microorganisms isolated from olive processing not during fermentation, but at the moment of packing closing with the aim of: (i) the development of a new and easy method for the fortification of table olives with potential probiotic, (ii) studying the survival of bacteria under the packing conditions to determine shelf life of the functional product.

MATERIALS AND METHODS

OLIVE PACKING CONDITIONS

The fruits used in the present study were of the Manzanilla variety (*Olea europaea pomiformis*), previously fermented according to the green Spanish-style (Garrido-Fernández et al., 1997). To mimic industrial packing conditions, after 6 months of fermentation (from October 2010 to March 2011), 175 g of olives were introduced into A314 jars (314 mL volume, 75 mm diameter × 103.5 mm high) and covered with 145 mL of new fresh brine. This brine had the adequate concentrations of salt and lactic acid to reach 5.5% NaCl and 0.6% titratable acidity, expressed as lactic acid, in the equilibrium. The final pH was, approximately, 3.8 ± 0.1.

FORTIFICATION OF OLIVES

Packed olives were fortified with overnight cultures of strain *L. pentosus* TOMC-LAB2 (henceforth LAB2), previously selected because of their potential probiotic characteristics (Bautista-Gallego et al., 2013), ability of adhesion to olive epidermis (Arroyo-López et al., 2012), good performance as starter in previous fermentation trials performed at laboratory scale (Rodríguez-Gómez et al., 2013), and high survival to simulated *in vitro* human digestions (0.74%). Inoculum was grown until early stationary phase and then an aliquot of the suspension was added to the packing brines to reach an initial inoculum level of approximately 6.5 $\log_{10}$ cfu mL^{-1}. The addition was essayed using three different types of fruits:

(i) F1 treatment, fruits obtained from a spontaneous olive fermentation. It included a control (F1), packaged without LAB addition, and olives fortified with LAB2 strain (F1-F).

(ii) F2 treatment, fruits previously fermented using LAB2 as starter culture. It included a control (F2), packaged without further LAB addition, and olives reinforced with the same strain (F2-F) at packing closing.

(iii) F3 treatment, fruits spontaneously fermented and then subjected to a thermal treatment by immersion in a water bath at 85°C for 5 min. It included a control (F3), packaged without further addition of LAB, and fruits fortified with LAB2 strain after cooling of brines (F3-F).

Each treatment was carried out in duplicate, making a total of 12 packing containers which were kept during the entire process at the Instituto de la Grasa pilot plant (CSIC, Seville, Spain) at room temperature (variable from 18°C in March/2011 to 31°C September/2011). The experiment was monitored for 200 days.

MICROBIOLOGICAL ANALYSES OF FRUITS

Olive samples, or their decimal dilutions, were plated using a Spiral Plating System model dwScientific (Don Whitley Scientific Ltd., Shipley, UK) on the media described below at 0, 38, 80, and 200 days of packing. Plates were counted using a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system. *Enterobacteriaceae* were counted on VRBD (Crystal-violet Neutral-Red bile glucose)-agar (Merck, Darmstadt, Germany), LAB on MRS (de Man, Rogosa and Sharpe)-agar (Oxoid) supplemented with 0.02% (w/v) sodium azide (Sigma, St. Louis, MO, USA), and yeasts on YM (yeast-malt-peptone-glucose) agar (Difco™, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate as selective agents for yeasts. Plates were incubated at 37°C for 24 h (*Enterobacteriaceae*) or 30°C for 48 h (yeasts and LAB).

To determine the number of microorganisms adhered to the olive epidermis, the protocol developed by Böckelmann et al. (2003) was slightly adapted to the specific characteristics of table olives. Briefly, two fruits from each packing container were randomly taken at different sampling times and washed for 1 h with 250 mL of a sterile PBS buffer solution (8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, pH finally adjusted to 4.7 with HCl 1 M) to remove non-adhering cells. Then, olives were transferred to 50 mL of a PBS solution added of the following enzymes: 14.8 mg L^{-1} lipase (L3126), 12.8 mg L^{-1} β-galactosidase (G-5160) and 21 μL L^{-1} α-glucosidase (G-0660) (Sigma–Aldrich, St. Louis, MO, USA). To achieve biofilm disintegration and removal of the adhered cells, the fruits were incubated at 30°C in this enzyme cocktail with slight shaking (150 rpm). After 12 h, the olives were removed and the resulting suspension was centrifuged at 9,000 × *g* for 10 min at 4°C. Finally, the pellet was re-suspended in 2 mL of PBS and spread onto the different culture media described above. Olive microbial counts were expressed as $\log_{10}$ cfu/cm^2, using the formula of a prolate spheroid for the approximate calculus of olive surface from the longitudinal and transverse axes of fruits (Weisstein, 2013). For the Manzanilla fruits used in the present study, the average area was 10.99 ± 1.01 cm^2 and the weight 4.08 ± 0.46 g.

CHARACTERIZATION OF THE LACTIC ACID BACTERIA POPULATION

For characterization of the lactobacilli population, repetitive bacterial DNA element fingerprinting analysis (rep-PCR) with primer GTG_5 was followed using the protocol described in Gevers et al. (2001). The PCR reaction in a final volume of 25 μL contained: 5 μL of 5x MyTaq reaction buffer (5 mM dNTPs and 15 mM $MgCl_2$), 0.1 μL of My Taq DNA polymerase (Bioline reactives, UK), 1 μL GTG_5 primer (25 μm), 13.9 μL desionized H_20, and

5 μL DNA (~20 ng/μL). PCR amplification was carried out in a thermal cycler (Master Cycler Pro, Eppendorf) with the following program: 95°C for 5 min as initial step, plus 30 cycles at: (1) 95°C for 30 s, (2) annealing at 40°C for 30 s, and (3) 65°C for 8 min, with a final step of 65°C for 16 min to conclude the amplification. This methodology was used to determine the recovery frequency of the inoculated strain at the end of packing. Firstly, the repeatability of the technique was determined using the LAB2 strain as internal control, obtaining a 86.9% ± 3.4% of similarity for this bacteria in ten different gels (**Figure 1**). Then, diverse isolates were randomly picked from each treatment at the end of experiment (200 days), making a total of 88 lactobacilli to analyze. They were named with the name of treatment (F1, F2, or F3) and with a "F" in the case of fortified/reinforced olives. Their pattern profiles of bands (from 100 up to 3,000 bp) were compared with the strain used to fortify the treatments (LAB2). For this purpose, PCR products were electrophoresed in a 2% agarose gel and visualized under ultraviolet light by staining with ethidium bromide. The resulting fingerprints were digitally captured and analyzed with the BioNumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). The similarity among digitalized profiles was calculated using the Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm and the automatic calibration tool for the determination of the optimization and curve smoothing parameters.

STATISTICAL ANALYSIS

Analysis of variance was performed by means of the one-way ANOVA module of Statistica 7.1 software to check for significant differences among treatments. For this purpose, a *post hoc* comparison was applied by means of the Scheffé test.

RESULTS AND DISCUSSION

After fermentation (6 months), three different types of fruits where placed in new fresh brine and inoculated with a putative probiotic bacteria (LAB2) just before closing the containers. Initial physicochemical conditions (pH, salt, free, and combined acidity) were stable during the whole period of packing (data not shown). Below, the main results obtained for the changes in the microbial populations and the presence of the inoculated strain after 200 days of shelf life are shown.

EVOLUTION OF MICROBIAL POPULATIONS THROUGH PACKING

Diverse works have reported that the survival of microorganisms through table olive packing is considerable. Argyri et al. (2013b) showed that microbial population after 40 days of Halkidiki green table olive packing was above 7 and 5 $\log_{10}$ cfu g^{-1} for LAB and yeasts, respectively. Bautista-Gallego et al. (2011) reported that after 60 days of Aloreña directly brined packing, the counts for LAB and yeasts were above 5 and 6 $\log_{10}$ cfu mL^{-1}, respectively. For this reason, it can be expected that probiotic bacteria could also survive for long period of time under packing conditions, and thereby, be used for fortification purposes.

Figure 2 shows the evolution of the LAB and yeasts population on olive surface for fruits spontaneously fermented (F1) and fortified (F1-F) with LAB2 strain at the moment of packing. A clear and statistical significant difference between the changes in both LAB populations in the two types of treatments was noticed (**Figure 2A**). In the case of F1 fruits, a marked decline of population was observed through packing, while in fortified fruits (F1-F), LAB counts were always higher and practically constant during the shelf life. Thereby, at the end of the studied period (200 days), the population for fortified fruits was 5.5 against 1.7 $\log_{10}$ cfu/cm^2 for non-fortified fruits. In the case of yeast population (**Figure 2B**), the evolution between both treatments was similar but with slight differences. A marked decline of yeast population was observed through packing in both cases, from the initial 5.8 to 1.3 (F1-F) or 0.0 (F1) $\log_{10}$ cfu/cm^2. Then, yeast survival was also slightly (but statistical significant) favored by the addition of the bacteria.

Figure 3 shows the evolution of the LAB and yeast populations on olive surface for fruits previously fermented using LAB2 as starter culture, packaged just as they were processed (F2) or reinforced with the same strain just before closing of containers (F2-F). The changes in the LAB population was very similar (no statistical differences) between reinforced and non-reinforced fruits, with a progressive reduction from the initial 7.0 to around 4.4 $\log_{10}$ cfu/cm^2 at 80 days. Later on, the population remained,

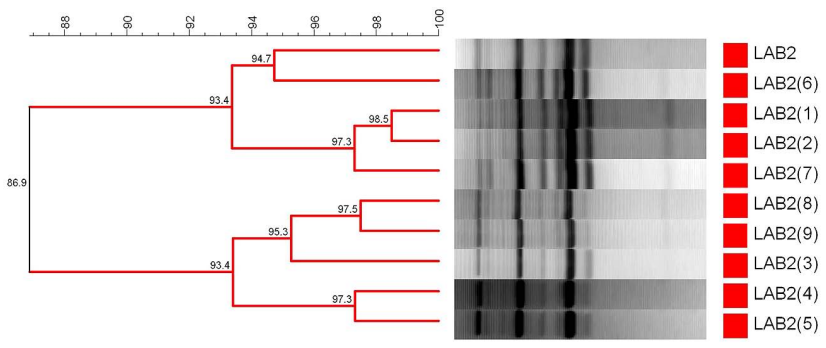


FIGURE 1 | Dendrogram generated after cluster analysis of the digitalized fingerprints of *L. pentosus* TOMC-LAB2 obtained from 10 different running to determine the repeatability of the rep-PCR technique (86.9%) with primer GTG_5. Clustering parameters: 0.5% optimization and 0.0 % curve smoothing.

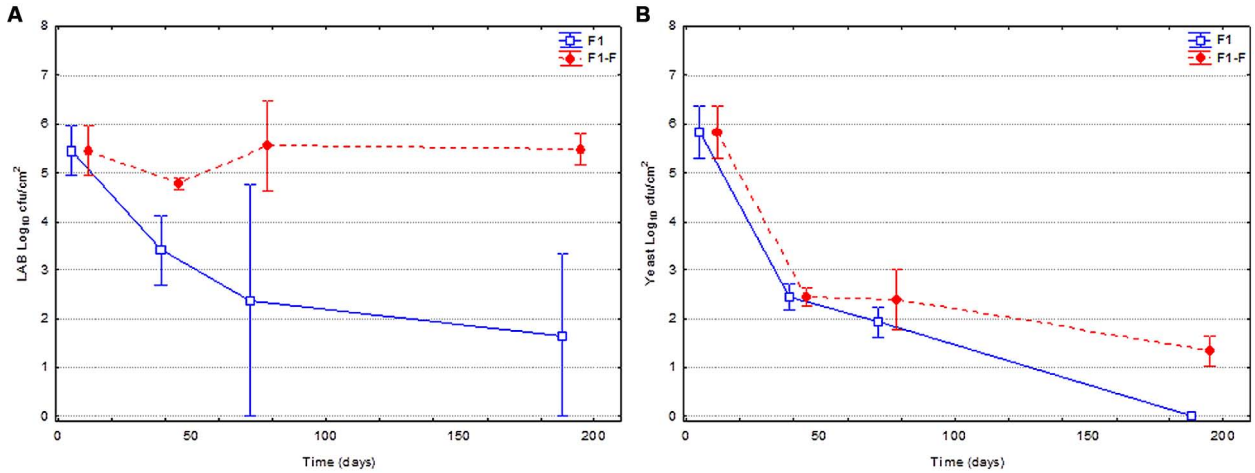


FIGURE 2 | Changes in the LAB (A) and yeast (B) populations on olive surface during shelf life. Control (F1) corresponded to just packaged spontaneously fermented fruits, while fortified (F1-F) were those inoculated with *L. pentosus* TOMC-LAB2 strain.

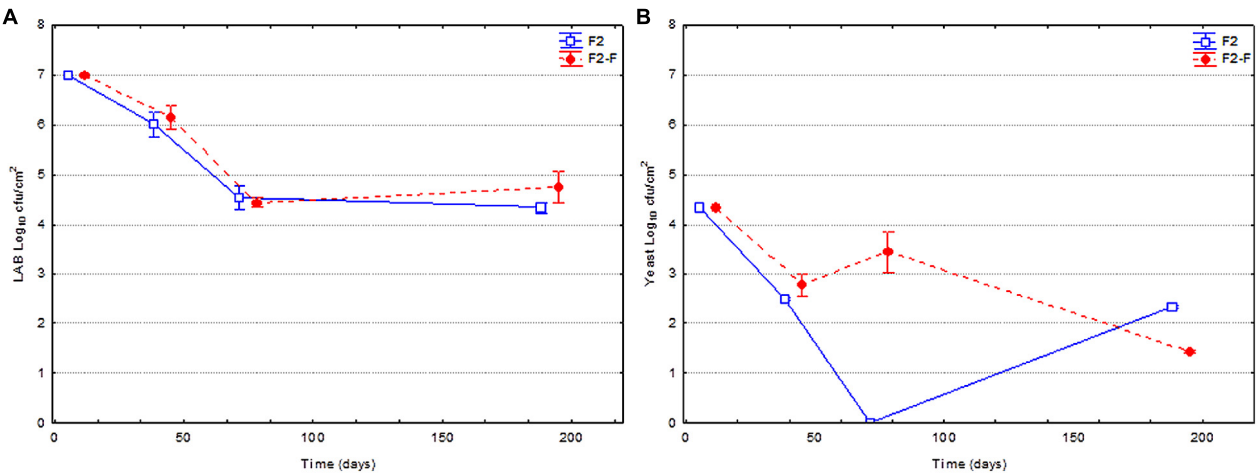


FIGURE 3 | Changes in the LAB (A) and yeast (B) populations on olive surface through shelf life. Fruits used in this experiment had been previously fermented using *L. pentosus* TOMC-LAB2 as starter culture, and just packaged (F2) or reinforced (F2-F) with the same strain at the moment of closing containers.

in practice, constant until the end of experiment. The evolution of the yeast population was very similar as in the previous case, with a clear decline through packing period (from the initial 4.3 to around 2.0 $\log_{10}$ cfu/cm^2 at the end of study), although a higher population was observed during most of the shelf life.

Figure 4 shows the evolution of the LAB and yeast populations on olive surface for fruits spontaneously fermented and thermally treated before packing. As commented, some fruits were packed without LAB addition (F3), while others were fortified with LAB2 strain (F3-F). Apparently, the thermal treatment (85°C for 5 min) was very efficient for killing the vegetative cells of both LAB and yeasts adhered to the olive surface, and no microbial cells were detected, except in the treatments inoculated with LAB2 strain (**Figure 4A**). In this case, the LAB added to the brine was able to colonize the olive surface in less than 38 days. In fact, the population increase from 0.0 to approximately 6.8 $\log_{10}$ cfu/cm^2 was very fast and persisted up to the end of the shelf life.

Finally, *Enterobacteriaceae* were not detected in any of the packing treatments assayed through shelf life. This is in agreement with the low pH obtained in the packing conditions (pH $<$ 4.0), which is a very inhibitory factor for the survival of this group of microorganism (Garrido-Fernández et al., 1997), but also due to the lack of appropriate nutrients because the sugars were previously exhausted during fermentation process (data not shown).

Therefore, data obtained in this work show the ability of the LAB2 strain to move from packing brine onto the fruits and to colonize the olive surface, which was especially evident in the case of F3-F treatment. This is in agreement with results obtained recently by Arroyo-López et al. (2012) and Domínguez-Manzano

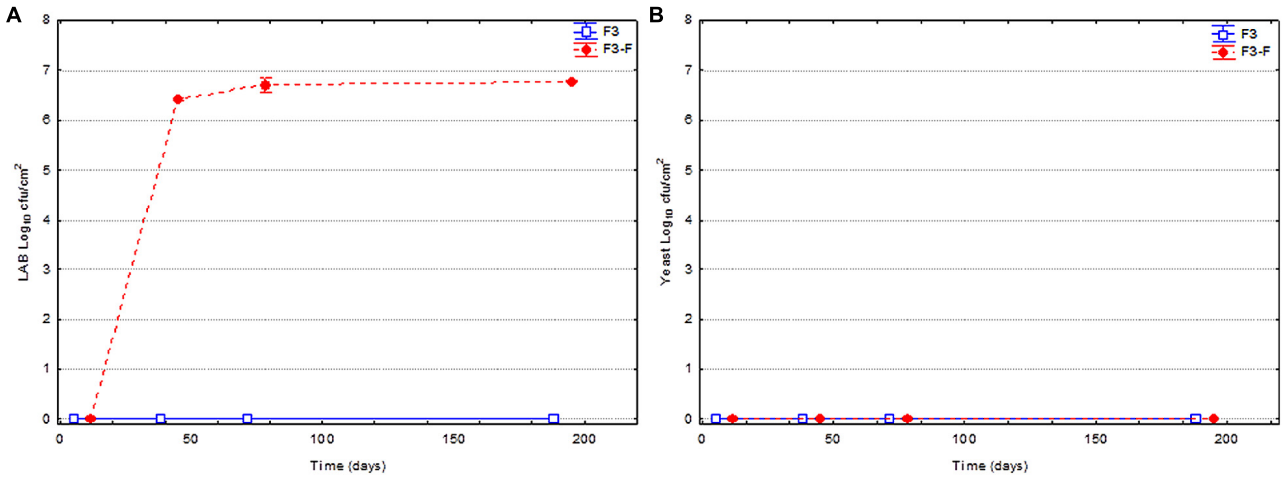


FIGURE 4 | Changes in the LAB (A) and yeast (B) populations on olive surface through shelf life. Olives were spontaneously fermented and treated with heat (85°C during 5 min). Part of fruits were just packed after thermal treatment (F3), while others were fortified (F3-F) with *L. pentosus* TOMC-LAB2 strain at the moment of closing containers.

et al. (2012) who demonstrated that diverse *L. plantarum* and *L. pentosus* strains were able to establish polymicrobial communities with yeasts on the surface of olives processed according to green Spanish-style. Peres et al. (2012) mention diverse types of technological processes (microencapsulation, vacuum, and immersion impregnation, cell immobilization, etc.) to fortify plant matrices with probiotic microorganism. In this work, the simply preparation of a packing brine with a concentration of $\sim$ 6.5 $\log_{10}$ cfu mL^{-1} of LAB2 strain, resulted in a single and easy method for the fortification of olives with the desire bacteria by means of a surface adhesion mechanism.

PRESENCE OF THE INOCULATED STRAIN AT THE END OF SHELF LIFE

The presence of the inoculated LAB2 strain at the end of packing was corroborated by molecular methods (rep-PCR) using a total of 88 lactobacilli isolates obtained from F1, F2, F3, and their respective fortified/reinforced treatments.

Figure 5 shows the dendrogram generated using the patterns profile of 29 LAB isolates randomly obtained from epidermis of F1 (10) and F1-F (19) fruits plus the inoculated LAB2 strain. The cluster analysis showed that 10/19 isolates obtained from the fortified F1 treatment shared a 87.4% similarity with the inoculated LAB2 strain, which was above repeatability of the technique (86.8%). Thereby, LAB2 strain showed in F1-F treatment a recovery frequency of 52.6%, while 9/19 isolates (47.4%) where grouped in different clusters together with many of the isolates obtained from the spontaneous and non-fortified F1 treatment.

Figure 6 shows the dendrogram generated using the patterns profile of 39 lactobacilli isolates randomly obtained from epidermis of F2 (20) and F2-F (19) fruits plus the inoculated LAB2 strain. The cluster analysis showed that 11/19 isolates obtained from the reinforced F2-F treatment shared a 88.8% similarity with the inoculated LAB2 strain, which again was above repeatability of the technique. Thus, LAB2 strain showed in the reinforced treatment a recovery frequency of 57.9% at 200 days of packing, while 8/19 isolates (42.1%) where grouped in different clusters with many of the isolates obtained from the non-reinforced F2 treatment. It is remarkable to verify that, in the case of no inoculation, it was not possible to recover the profile of the LAB2 strain from the F2 treatment even when this microorganism was used as starter culture at the onset of fermentation. Thus, a reinforcement step with this strain at the beginning of packing to assure its presence during shelf life it is always advisable.

Finally, **Figure 7** shows the dendrogram generated using the patterns profile of 20 lactobacilli isolates randomly obtained from olive epidermis of F3-F treatments plus the inoculated LAB2 strain. In this case, because of the thermal treatment, no LAB isolates were obtained from F3 treatment. The cluster analysis showed that 20/20 isolates obtained from the F3-F treatment shared a 86.5% similarity with the inoculated LAB2 strain, which is in the limit of repeatability of the technique. Thereby, LAB2 strain showed in the fortified treatment a recovery frequency of 100.0% at 200 days, which is logical because, in this case, the survival of other microorganism was prevented by the thermal treatment.

Taking into account the average surface values obtained for the Manzanilla fruits used in the present study (11 cm^2), a total of $\sim$ 10^8 LAB cells could be ingested by consumers who eat only one fortified olive with LAB2 strain after thermal treatment. Data could also be converted to cfu g^{-1} taking into account the average weight of fruits (4.08 g). Probiotic microorganisms have to be taken regularly and to sufficiently high levels (at least 10^6 cfu mL^{-1} per daily dose) to avoid washout, and to assure that their benefits will be accrued in a sustained manner (Peres et al., 2012). The Spanish Association of Processors and Exporters (ASEMESA, 2014) considers 7 olives (about 25 g of olive flesh) as a satisfactory daily intake. Olives thermally treated and fortified with

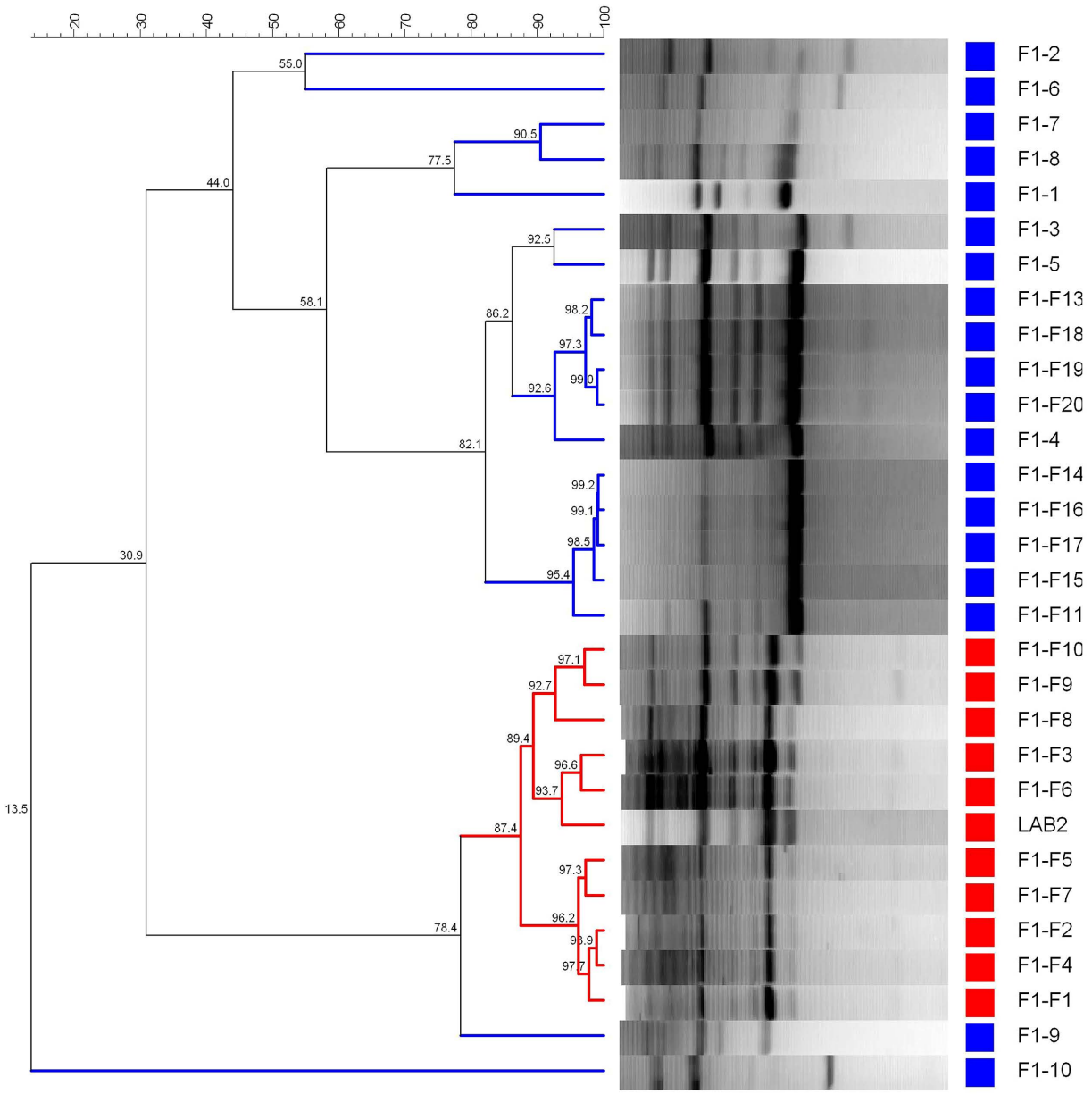


FIGURE 5 | Dendrogram generated after cluster analysis of the digitalized rep-PCR fingerprints with primer GTG_5 of 29 lactobacilli isolates obtained from fruits spontaneously fermented. Part of fruits were just packaged (F1, control) or fortified with *L. pentosus* TOMC-LAB2 strain (F1-F), while 1–20 is the number for the isolates obtained within each treatment. Clustering parameters: 0.5% optimization and 0.0% curve smoothing.

LAB2 strain clearly exceed the number of recommended probiotic microorganism. Pasteurization of Spanish green table olives using glass and tin as packing material is usually carried out by industry to enlarge the shelf life of the product using a minimum amount of preservatives (Garrido-Fernández et al., 1997). Thus, this type of product could be a convenient carrier of probiotic to the human body because of the high number of microorganisms that potentially could be ingested by consumers with a larger shelf life compared to dairy product.

CONCLUSION

Data obtained in this work show that fortification of table olives with *L. pentosus* TOMC-LAB2 is possible because of the considerable survival of this microorganism during shelf life at room temperature without chill chain. Among the different types of fortification assayed, a thermal treatment after fermentation to eliminate microorganism and later inoculation with the bacteria is desired because of the high counts and recovery frequency of the added LAB bacteria obtained at the end of the packing period. However, sensorial studies should be also carried out to determine

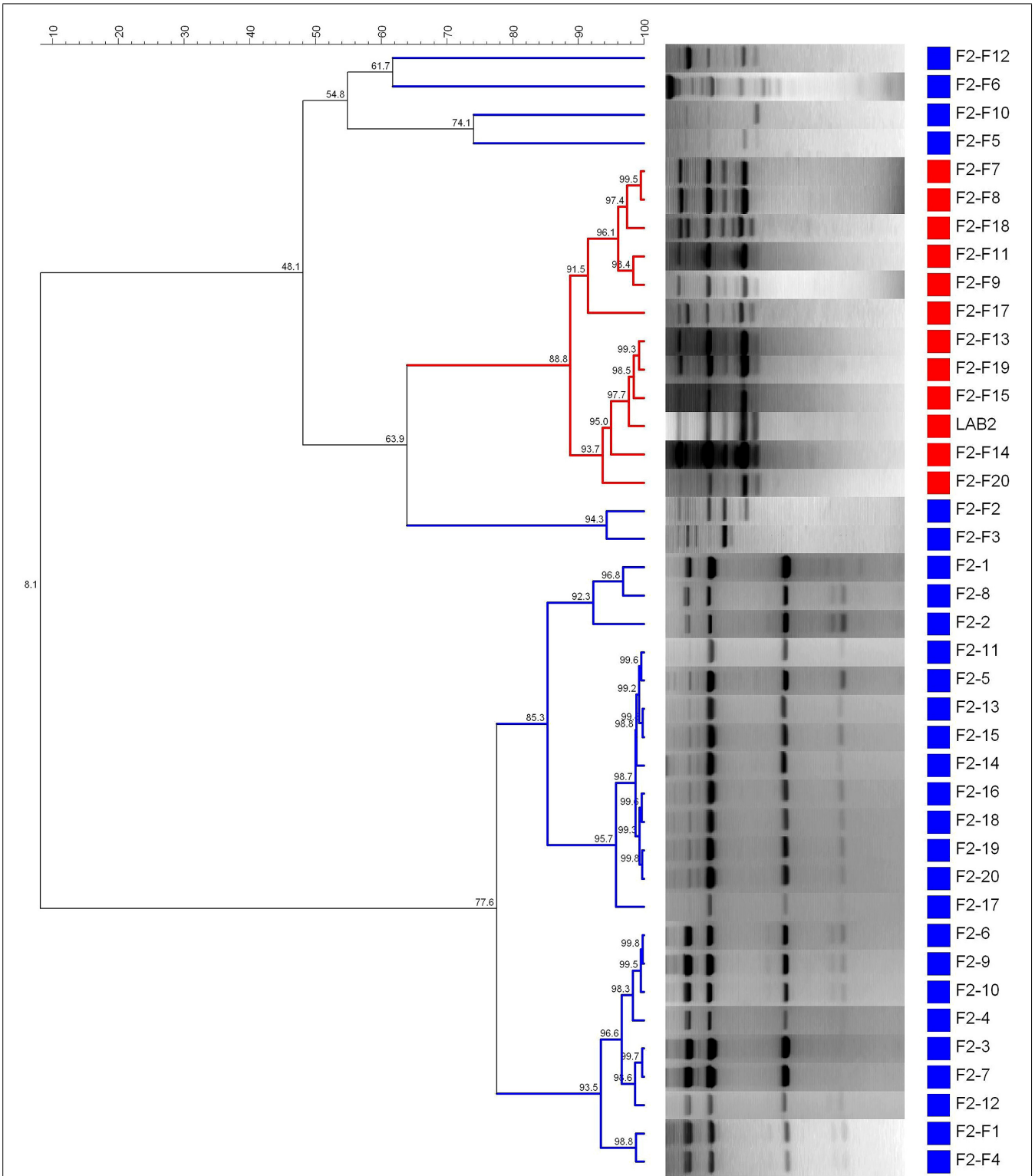


FIGURE 6 | Dendrogram generated after cluster analysis of the digitalized rep-PCR fingerprints with primer GTG_5 of 39 lactobacilli isolates obtained from fruits previously fermented using *L. pentosus* TOMC-LAB2 as starter culture. Part of fruits were just packaged (F2, control) or reinforced with the same strain at packing closing (F2-F), while 1–20 is the number for the isolates obtained within each treatment. Clustering parameters: 0.5% optimization and 5.0% curve smoothing.

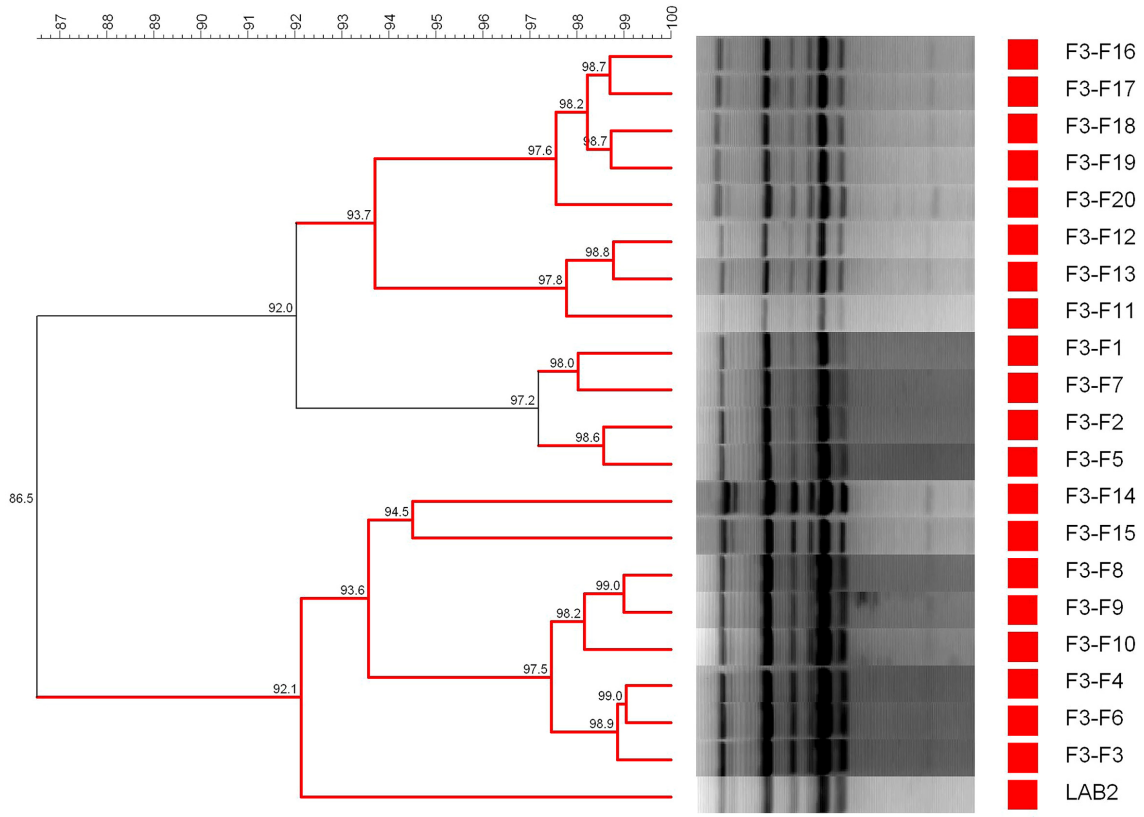


FIGURE 7 | Dendrogram generated after cluster analysis of the digitalized rep-PCR fingerprints with primer GTG_5 of 20 lactobacilli isolates obtained from fruits spontaneously fermented and then thermally treated (85°C for 5 min). Information corresponds to only F3-F treatment (fruits fortified with *L. pentosus* TOMC-LAB2 strain) since no growth was detected in F3, while 1–20 is the number for the isolates obtained in the treatment. Clustering parameters: 0.5% optimization and 0.0% curve smoothing.

the influence of the thermal treatment or bacteria growth on the sensory profile of packaged olives.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the EU's Seventh Framework Programme [FP7/2007-2013] under grant agreement no 243471 (PROBIOLIVES). We also thank the Spanish Government for financial support (projects AGL2009-07436/ALI, and AGL2010-15529/ALI partially financed by European regional development funds, ERDF), and the Junta de Andalucía (through financial support to group AGR-125). Thanks to JOLCA and ASEMESA for supplying the fruits and their own expertise for the development of this work. Francisco N. Arroyo-López wishes to express thanks for his Ramón y Cajal postdoctoral research contract (Spanish government).

REFERENCES

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A. G., Tsakalidou, E., Nychas, G. J. E., Panagou, E. Z., et al. (2013a). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol.* 33, 282–291. doi: 10.1016/j.fm.2012.10.005

Argyri, A. A., Lyra, E., Panagou, E. Z., and Tassou, C. C. (2013b). Fate of *Escherichia coli* O157:H7, *Salmonella enteritidis* and Listeria monocytogenes during storage of fermented green table olives in brine. *Food Microbiol.* 36, 1–6. doi: 10.1016/j.fm.2013.04.001

Argyri, A. A., Nisiotou, A. A., Malauchos, A., Panagou, E. Z., and Tassou, C. C. (2014). Performance of two potential probiotic *Lactobacillus* strains from the olive microbiota as starters in the fermentation of heat shocked green olives. *Int. J. Food Microbiol.* 171, 68–76. doi: 10.1016/j.ijfoodmicro.2013.11.003

Arroyo-López, F. N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., et al. (2012). Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style Manzanilla fermentations. *Food Microbiol.* 32, 295–301. doi: 10.1016/j.fm.2012.07.003

ASEMESA. (2014). Available at: http://www.asemesa.es/content/nutricion_y_salud [accessed February 19, 2014].

Bautista-Gallego, J., Arroyo-López, F. N., Kantsiou, K., Jiménez Días, R., Garrido Fernández, A., and Cocolin, L. (2013). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int.* 50, 135–142. doi: 10.1016/j.foodres.2012.10.004

Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Rodríguez-Gómez, F., and Garrido-Fernández, A. (2011). Evaluating the effects of Zinc Chloride as a preservative in cracked table olive packing. *J. Food Prot.* 74, 2169–2176. doi: 10.4315/0362-028X.JFP-11-201

Blana, V. A., Grounta, A., Tassou, C. C., Nychas, G. J., and Panagou, E. Z. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol.* 38, 208–218. doi: 10.1016/j.fm.2013.09.007

Böckelmann, U., Szewzyk, U., and Grohmann, E. (2003). A new enzymatic method for the detachment of particle associated soil bacteria. *J. Microbiol. Methods* 55, 201–211. doi: 10.1016/S0167-7012(03)00144-1

Champagne, C. P., and Gardner, N. J. (2005). Challenges in the addition of probiotic cultures to foods. *Crit. Rev. Food Sci. Nut.* 45, 61–84. doi: 10.1080/10408690590900144

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., and Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on the olive surface in fermentation sets inoculated with the probiotic strains *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int. J. Food Microbiol.* 140, 6–13. doi: 10.1016/j.ijfoodmicro.2010.02.024

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F. N., Garrido-Fernández, A., and Jiménez-Díaz, R. (2012). Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. *Int. J. Food Microbiol.* 157, 230–238. doi: 10.1016/j.ijfoodmicro.2012.05.011

Garrido-Fernández, A., Fernández Díez, M. J., and Adams, R. M. (1997). *Table olives: Production and Processing*. London: Chapman & Halls. doi: 10.1007/978-1-4899-4683-6

Garrido Fernández, A., Romero Barranco, C., García García, P., and Brenes Balbuena, M. (2001). "Aceitunas de mesa. composición y características nutricionales," in *Aceite de Oliva Virgen: Muestro Patrimonio Alimentario*, ed. J. Mataix Verdú (Granada: Fundación Puleva e Instituto Omega-3).

Gevers, D., Huys, G., and Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* 205, 31–36. doi: 10.1111/j.1574-6968.2001.tb10921.x

International Olive Council [IOC]. (2014). *Key Figures On The Work Marked For Table Olives*. Available at http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive- [accessed February 26, 2014].

Lavermicocca, P., Rossi, M., Russo, F., and Srirajaskanthan, R. (2010). "Table olives: a carrier for delivering probiotic bacteria to humans," in *Olives and Olive Oil in Health and Disease Prevention*, eds V. R. Preedy and R. R. Watson (Oxford: Academic Press), 735–743.

Lavermicocca, P., Valerio, F., Lisa Lonigro, S., De Angelis, M., Morelli, L., Callegari, M. L., et al. (2005). Study of adhesion and survival of *Lactobacilli* and *Bifidobacteria* on table olives with the aim of formulating a new probiotic food. *Appl. Environ. Microbiol.* 71, 4233–4240. doi: 10.1128/AEM.71.8.4233-4240.2005

Peres, C. M., Peres, C., Hernández-Mendoza, A., and Malcata, F. X. (2012). Review on fermented plant material as carriers and sources of potentially probiotic lactic acid bacteria – With an emphasis on table olives. *Trends Food Sci. Technol.* 26, 31–42. doi: 10.1016/j.tifs.2012.01.006

Ranadhera, R. D., Baines, C. S., 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

Reyes-Zurita, F. J., Rufino-Palomares, E. E., Lupiáñez, J. A., and Cascante, M. (2009). Maslinic acid, a natural triterpene from Olea europaea L., induces apoptosis in *HT29* human colon-cancer cells via the mitochondrial apoptotic pathway. *Cancer Lett.* 273, 44–54. doi: 10.1016/j.canlet.2008.07.033

Rodríguez-Gómez, F., Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Jiménez-Díaz, R., Garrido-Fernándeza, A., et al. (2013). Table olive fermentation with multifunctional *Lactobacillus pentosus* strains. *Food Control* 34, 96–105. doi: 10.1016/j.foodcont.2013.04.010

Weisstein, E. W. (2013). *"Prolate Spheroid." From MathWorld - A Wolfram Web Resource*. Available at: http://mathworld.wolfram.com/ProlateSpheroid.html [accessed June 11, 2013].

Conflict of Interest Statement: 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.

Received: 12 June 2014; paper pending published: 11 July 2014; accepted: 18 August 2014; published online: 03 September 2014.

Citation: Rodríguez-Gómez F, Romero-Gil V, García-García P, Garrido-Fernández A and Arroyo-López FN (2014) Fortification of table olive packing with the potential probiotic bacteria Lactobacillus pentosus TOMC-LAB2. Front. Microbiol. ***5****:467. doi: 10.3389/fmicb.2014.00467*

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

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 14 October 2014
doi: 10.3389/fmicb.2014.00540

Survival of pathogenic and lactobacilli species of fermented olives during simulated human digestion

Francisco N. Arroyo-López[1]*, Stéphanie Blanquet-Diot[2], Sylvain Denis[2], Jonathan Thévenot[2,3], Sandrine Chalancon[2], Monique Alric[2], Francisco Rodríguez-Gómez[1], Verónica Romero-Gil[1], Rufino Jiménez-Díaz[1] and Antonio Garrido-Fernández[1]

[1] Biotecnología de Alimentos, Instituto de la Grasa – Consejo Superior de Investigaciones Científicas, Seville, Spain
[2] Centre de Recherche en Nutrition Humaine Auvergne, EA 4678, Conception Ingénierie et Développement de l'Aliment et du Médicament, Clermont Université – Université d'Auvergne, Clermont-Ferrand, France
[3] Centre de Recherche en Nutrition Humaine Auvergne, M2iSH, UMR INSERM/Université d'Auvergne U1071 USC-INRA 2018, Clermont Université – Université d'Auvergne, Clermont-Ferrand, France

Edited by:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Chrysoula C. Tassou, Hellenic Agricultural Organization "DEMETER," Greece
Zhao Chen, Clemson University, USA

***Correspondence:**
Francisco N. Arroyo-López, Biotecnología de Alimentos, Instituto de la Grasa – Consejo Superior de Investigaciones Científicas, Avenida Padre García, Tejero nº 4, 41012 Seville, Spain
e-mail: fnarroyo@cica.es

The present survey uses a dynamic gastric and small intestinal model to assess the survival of one pathogenic (*Escherichia coli* O157:H7 EDL 933) and three lactobacilli bacteria with probiotic potential (*Lactobacillus rhamnosus* GG, *L. pentosus* TOMC-LAB2, and *L. pentosus* TOMC-LAB4) during their passage through the human gastrointestinal tract using fermented olives as the food matrix. The data showed that the survival of the *E. coli* strain in the stomach and duodenum was very low, while its transit through the distal parts (jejunum and ileum) resulted in an increase in the pathogen population. The production of Shiga toxins by this enterohemorrhagic microorganism in the ileal effluents of the *in vitro* system was too low to be detected by ELISA assays. On the contrary, the three lactobacilli species assayed showed a considerable resistance to the gastric digestion, but not to the intestinal one, which affected their survival, and was especially evident in the case of both *L. pentosus* strains. In spite of this, high population levels for all assayed microorganisms were recovered at the end of the gastrointestinal passage. The results obtained in the present study show the potential use of table olives as a vehicle of beneficial microorganisms to the human body, as well as the need for good hygienic practices on the part of olive manufacturers in order to avoid the possibility of contamination by food-borne pathogens.

Keywords: food carrier, lactobacilli, pathogen, probiotic, survival, Shiga toxins, table olives, TIM system

INTRODUCTION

Green Spanish-style table olives are considered ready-to-eat products which are directly consumed without any prior cooking, and often, without any pasteurization or sterilization treatment. This makes olives a splendid vehicle of microorganisms (beneficial or harmful) to the human body. Thereby, the analysis of the microbiota adhered to the fruits acquires a relevant importance, although, incomprehensibly, this point has received scarce attention by researchers. Fermented olives contain biofilm structures formed mainly by lactic acid bacteria (LAB) and yeasts, which can reach population levels up to 8 $\log_{10}$ CFU g^{-1} in the epidermis of the fruits (Nychas et al., 2002; Arroyo-López et al., 2012; Domínguez-Manzano et al., 2012). Native LAB or yeast strains isolated from olive microbiota which show potential probiotic characteristics could be used as starters either to initiate fermentation or to obtain a functional product after adhesion to the fruits (Rodríguez-Gómez et al., 2013; Blana et al., 2014). To exert their health effect, microorganisms to be evaluated as probiotics should be able to survive the harsh conditions found in the human digestive tract such as acidic pH or high concentrations of digestive enzymes and bile salts.

Regarding safety issues, it is necessary to provide more knowledge about the behavior of pathogens in ready-to-eat fermented olives for future risk assessments. Enterohemorrhagic *Escherichia coli* is a major food-borne pathogen that causes hemorrhagic colitis and a life-threatening sequelae, the hemolytic uremic syndrome (Pennington, 2010). The behavior of the enterohemorrhagic *E. coli* O157:H7 during olive processing (fermentation and packing) has been reported by several authors (Spyropoulou et al., 2001; Skandamis and Nychas, 2003; Argyri et al., 2013; Grounda et al., 2013) but there is hitherto no available information about the survival of this pathogen in the case of a hypothetical intake of contaminated olives. To cause human illness, *E. coli* O157:H7 should not only survive its passage through the gastrointestinal tract but also coordinate the expression of virulence genes especially that of encoding Shiga toxins (Foster, 2013).

Most of the available data on probiotic or pathogen survival in the human digestive tract has been obtained in static *in vitro* systems that are not representative of the continuously changing variable during the gastrointestinal transit. The TNO gastrointestinal tract model (TIM system, Zeist, Netherlands) is an alternative dynamic multi-compartmental *in vitro* system which currently allows the closest simulation of *in vivo* physiological processes

occurring within the stomach and small intestine of humans (Minekus et al., 1995; Guerra et al., 2012).

In this work, we used the TIM system to assess the survival and toxin production of an enterohemorrhagic *E. coli* strain inoculated in fermented olives during its transit through the upper human gastrointestinal tract. The survival of two potential probiotic strains isolated from table olives and belonging to *Lactobacillus pentosus* species (never studied before in dynamic digestive assays) was also evaluated and compared to that of a well-recognized probiotic microorganism (*L. rhamnosus* GG).

MATERIALS AND METHODS

OLIVES AND MICROORGANISMS

Fermented olives of the *Manzanilla* variety (*Olea europaea pomiformis*) were used in the present study. The fruits were previously fermented for two months according to the Spanish-style (Garrido-Fernández et al., 1997) and then pasteurized at 80°C for 15 min to avoid any microbial presence on the olive surface. Twenty-five grams of pasteurized olives were then homogenized with 250 mL of sterile water and independently inoculated with aerobic cultures of the reference strain *E. coli* O157:H7 EDL 933 (LB, 24 h, 37°C), the probiotic *L. rhamnosus* GG, and the olive isolates *L. pentosus* TOMC-LAB2 and *L. pentosus* TOMC-LAB4 (MRS broth, 24 h, 37°C) to reach a final population in the mix of 8.2, 9.1, 9.7, and 9.4 $\log_{10}$ CFU, respectively. Except for the pathogen strain, these inoculation levels are similar for the lactic acid population usually present in 25 g of fermented olives. The two *L. pentosus* strains, originally isolated from Spanish-style table olive fermentations and belonging to the table olive microorganisms collection (TOMC) from Food Biotechnology Department of Instituto de la Grasa (CSIC-Seville), were selected because of their previously described probiotic characteristics (Bautista-Gallego et al., 2013) and good performance as starter in previous fermentation trials carried out at laboratory scale (Rodríguez-Gómez et al., 2013). **Table 1** shows the main physicochemical conditions of the fermented olives at the moment of introduction into the TIM system.

SIMULATED HUMAN DIGESTIVE CONDITIONS

The TIM system, which simulates the physiological processes occurring in the stomach, duodenum, jejunum, and ileum, was programmed to reproduce the digestion of a solid food matrix in a healthy human adult using the protocol described by Etienne-Mesmin et al. (2011). The total duration of the digestions was 300 min with $n = 2$ digestions for each different microorganism. The parameters used for *in vitro* digestion are described in **Table 2**. Samples were taken in the test meal (initial intake) after inoculation and before its introduction into the artificial stomach, and regularly collected during digestion in the different compartments of the system (stomach, duodenum, jejunum, and ileum) as well as in the cumulative ileal effluents (CIE). Microbial counting was performed on LB agar (for *E. coli*) or on MRS agar (for the lactobacilli strains; Oxoid LTD, Basingstoke, Hampshire, England). Because food and microorganisms are in continuous transit from one compartment to the next, in order to assess microbial survival rates in the TIM system, control digestions ($n = 2$) were carried out with the same protocol conditions used for microorganisms, but with water containing only 0.8% (w/v) of blue dextran (Minekus et al., 1995). This compound is a non absorbable transit marker, which will represent a 100% survival percentage for bacteria. Thereby, curves below the transit marker will represent the mortality of the microorganism, while curves above the transit marker will be indicative of bacteria growth renewal.

DETERMINATION OF THE PRODUCTION OF SHIGA TOXINS BY THE *E. coli* STRAIN

Shiga toxins produced by *E. coli* O157:H7 EDL 933 in the ileal effluents of the TIM system were dosed by enzyme linked immunosorbent assay (ELISA) using the Ridascreen® Verotoxin kit (R-Biopharm, Darmstadt, Germany). Purified toxin Stx2 (Toxin Technology®, Sarasota, FL, USA) was used to establish standard calibration curves. Samples were diluted to half with the provided diluent and the dosage was made according to the manufacturer's instructions. Each digestive sample was analyzed in duplicate and serial dilutions of purified toxin were analyzed in triplicate.

STATISTICAL ANALYSIS

Significant differences among treatments ($p \leq 0.05$) were tested by analysis of variance (ANOVA) followed by a Fisher-LSD *post hoc* comparison test carried out with STATISTICA 7.0 software package (Statsoft Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

In the present study, fermented olives were independently inoculated with a food-borne pathogen and three potential beneficial microorganisms in order to determine their survival during their passage through the upper gastrointestinal tract of humans and the pathogen's ability to produce toxins. So far, there is no information available on the survival of microorganisms through the different compartments of the human gastrointestinal tract using table olives as the food matrix. The only related study was carried out by Lavermicocca et al. (2005), who fed volunteers with olives inoculated with the human origin strain *L. paracasei* IMPC2.1. These authors were able to recover the mentioned microorganism from fecal samples, but they did not obtain information on its resistance to specific gastric and intestinal conditions.

The behavior of the major food borne pathogen *E. coli* O157:H7 (strain EDL 933) during its transit through the different compartments of the TIM system is shown in **Figure 1**. In the stomach and duodenum, its population decreased considerably, probably due to the occurrence of stringent conditions such as gastric acidity, digestive enzymes, or bile salts (Smith, 2003). The highest cell mortality was observed in the artificial stomach from 90 min onward, when the pH fell below 3 units (see **Table 2**). At this time, the population decreased by more than 2 $\log_{10}$ CFU compared to the transit marker used as control, and no living cells were recovered from this compartment at 150 min. In the duodenum, at 180 min, around 3 $\log_{10}$ CFU were lost compared to the marker.

On the contrary, in the distal parts of the artificial gastrointestinal tract (jejunum and ileum), *E. coli* O157:H7 resumption was observed especially at the end of digestion. Thereby, at time 300 min, the counts for bacteria even exceeded that of

Table 1 | Experimental design and physicochemical conditions of the fermented olives used in the present work as food matrix.

Reference for treatment	Inoculated microorganism	Attributed effects on human health	Physicochemical conditions of fermented olives*
Ec	*Escherichia coli* O157:H7 EDL 933	Enterohemorrhagic pathogen	pH = 4.1 Salt = 50.0 g/l
Lr	*Lactobacillus rhamnosus* GG	Probiotic microorganism	Sugars ≤ 2.0 g/l
Lp2	*L. pentosus* LAB2	Potential probiotic strains	Phenolic compounds = 900 mg/kg
Lp4	*L. pentosus* LAB4	Isolated from table olives	

**Fruits had an average size of 4.06 ± 0.49 g and area surface of 10.93 ± 1.07 cm^2.*

Table 2 | Parameters of gastrointestinal digestion in the TIM system when simulating digestive conditions of a healthy adult after intake of a solid food matrix.

Compartment	Vol (ml)	pH/time (min)	Secretion	$t_{1/2}$ (min)	β coefficient
Stomach	300	2/0, 6/5, 5.7/15, 4.5/45,2.9/90, 2.3/120, 1.8/240, 1.6/300	0.25 ml/min of pepsin (2080 IU/ml), 0.25 ml/min of lipase (250.5 IU/ml), 0.25 ml/min of HCl (1.5 M) if necessary.	85	1.8
Duodenum	30	Maintained at 6.0	0.5 ml/min of bile salts (4% during the first 30 min of digestion and then 2%), 0.25 ml/min of pancreatic juice (10^3 USP/ml), 0.25 ml/min of intestinal electrolyte solution, 0.25 ml/min of $NaHCO_3$ (1 M) if necessary, 23,600 IU of trypsin (at the beginning of digestion).		
Jejunum	130	Maintained at 6.9	0.25 ml/min of $NaHCO_3$ (1 M) if necessary.		
Ileum	130	Maintained at 7.2	0.25 ml/min of $NaHCO_3$ (1 M) if necessary.	250	2.5

For each digestive compartment, the table gives the volume (initial volume for gastric compartment, maximal volume for intestinal compartments), the pH (depending on the time of digestion in the stomach), and the flow rates of digestive enzymes and bile salts. HCl and $NaHCO_3$ are added to the gastric and intestinal compartments, respectively, if the measured value of the pH deviates from the set point. The power exponential equation ($f = 1–2^{-(t/t1/2)\beta}$, where f represents the fraction of meal delivered, t the time of delivery, $t_{1/2}$ half the time of delivery, and β a coefficient describing the shape of the curve) was used for the computer control of gastric and ileal deliveries into the TIM system. Parameters used for $t_{1/2}$ and β for gastric and ileal deliveries are mentioned in the table.

the transit marker, chiefly in the jejunum (increase of 0.5 $\log_{10}$ CFU compared to the control marker). At 300 min, a high amount of viable cells were recovered from the ileal effluents, decreasing only by 0.2 $\log_{10}$ CFU with respect to the blue dextran. Bacterial growth renewal has been previously observed in the distal compartments of the TIM system for other *E. coli* strains (Ganzle et al., 1999; Etienne-Mesmin et al., 2011; Miszczycha et al., 2014). This growth resumption was probably linked to less stringent environmental conditions, such as a pH closer to neutrality, lower concentrations of bile salts (owing to their passive reabsorption in the TIM system), and/or an increase in the residence time of bacteria. This event led to an increase in the number of bacteria which could potentially enter into the colon and may potentiate the harmfulness of the food-borne pathogen in the hypothetical case of an intake of contaminated olives.

Large variations in survival rates have been obtained for *E. coli* O157:H7 in diverse *in vitro* digestion assays (Arnold and Kaspar, 1995; Takumi et al., 2000; Foster, 2004; Tamplin, 2005; Etienne-Mesmin et al., 2011). This wide range of response may be explained by differences between culture conditions, digestive systems (static or dynamic) and parameters, food matrices and also bacterial strains. Interestingly, the only studies that evaluated the behavior of *E. coli* O157:H7 in dynamic conditions gave survival rates after gastric digestion close to those obtained in the present work (Takumi et al., 2000; Etienne-Mesmin et al., 2011; Miszczycha et al., 2014). In table olive packing, this microorganism has shown also a considerable survival being present until 19th days of storage on olive fruits at low pH (4.2) and high salt concentration (60 g/l; Argyri et al., 2013).

Hitherto, there is no available data regarding the production (site and amount) of Shiga toxins by enterohemorrhagic *E. coli* strains during their transit through the human gastrointestinal tract. This study is the first to investigate toxin production by this food-borne pathogen in a human simulated digestive environment. Toxin levels in the ileal effluents of the TIM system were too low to be quantified by ELISA assays, which had a detection threshold value of 0.31 ng/ml (data not shown), indicating that under the tested conditions the pathogen was not able to produce Shiga toxins, its main virulence factor, at least at sufficient levels to be detected. It also suggests that toxin production, if it occurs, would rather

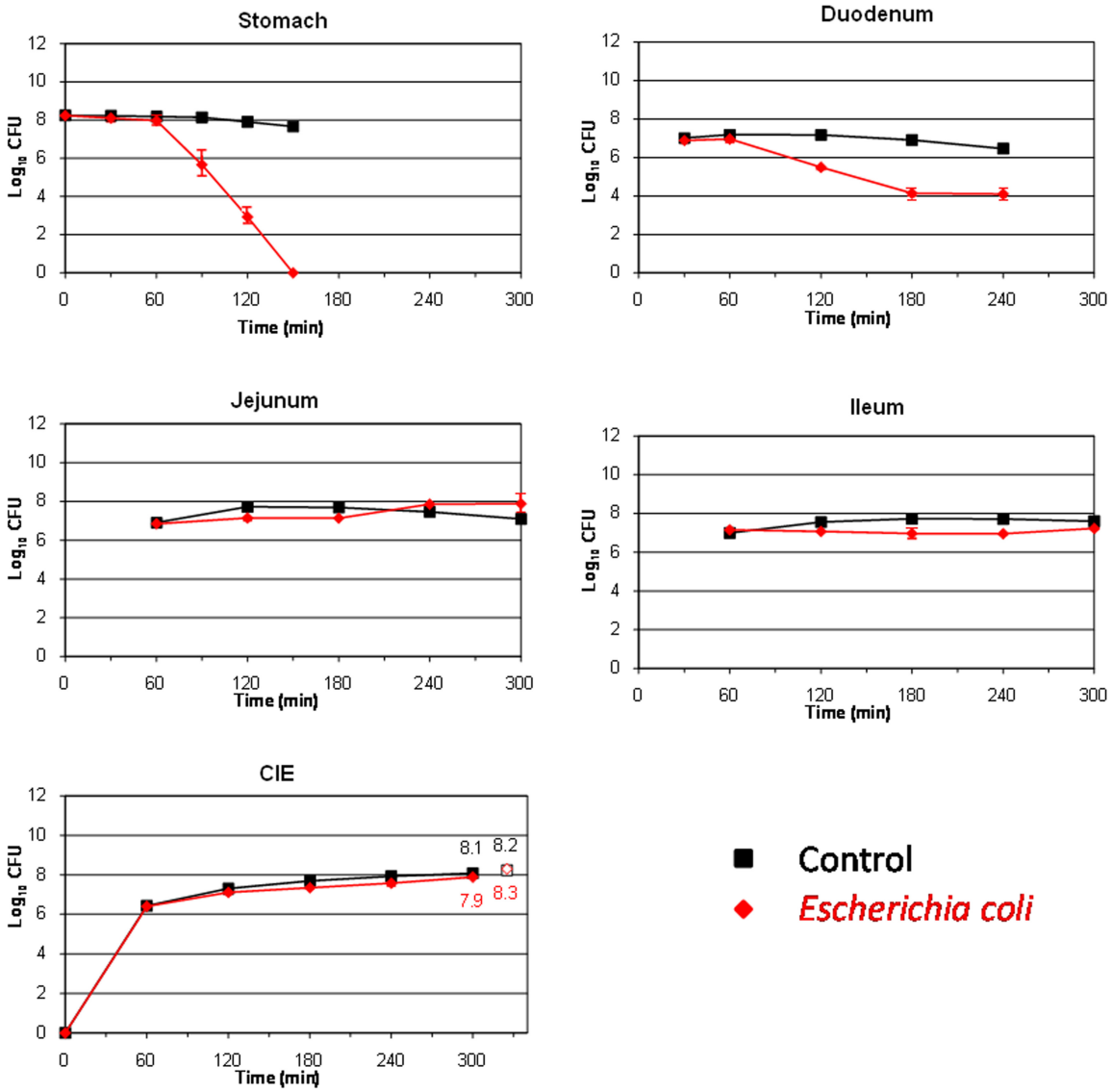


FIGURE 1 | Survival (plate counts versus time) of the food-borne pathogen *Escherichia coli* O157:H7 EDL 933 in the different compartments and ileal effluents (CIE) of the TIM system compared to a non absorbable transit marker, blue dextran, used as control. Results are expressed as $\log_{10}$ cfu ± SDs ($n = 2$).

take place in the colon, which is already described as the main site of pathogenicity for EHEC strains (Shigeno et al., 2002).

This study also aims to assess the survival of two *L. pentosus* strains isolated from table olives for their use as putative probiotics. LAB species are well known for their ability to resist and live in many different acidified fermented vegetables, among them table olives (Hurtado et al., 2012). The *L. pentosus* strains assayed in this work were originally isolated from table olive fermentations, so, presumably, they should be well adapted to the acidic environment which governs this type of product. **Figures 2** and **3** show the results obtained for the survival in the TIM system of *L. pentosus* TOMC-LAB2 and TOMC-LAB4, respectively. Both bacteria showed a considerable resistance to gastric digestion with a slight decrease at time 150 min of less than 0.1 $\log_{10}$ CFU with respect to the transit marker. However, their survival through the duodenum was considerably lower, especially evident in the case of the *L. pentosus* TOMC-LAB2 strain with a reduction of more than 3 $\log_{10}$ CFU at 240 min. On the contrary, the survival of neither microorganism was affected by the conditions encountered in the distal parts of the gastrointestinal tract (jejunum and ileum). At 300 min, compared to the transit marker, the amount of bacteria recovered from the ileal effluents was reduced by 2.1 and 1.3 $\log_{10}$ CFU for *L. pentosus* TOMC-LAB2 and TOMC-LAB4 strains, respectively.

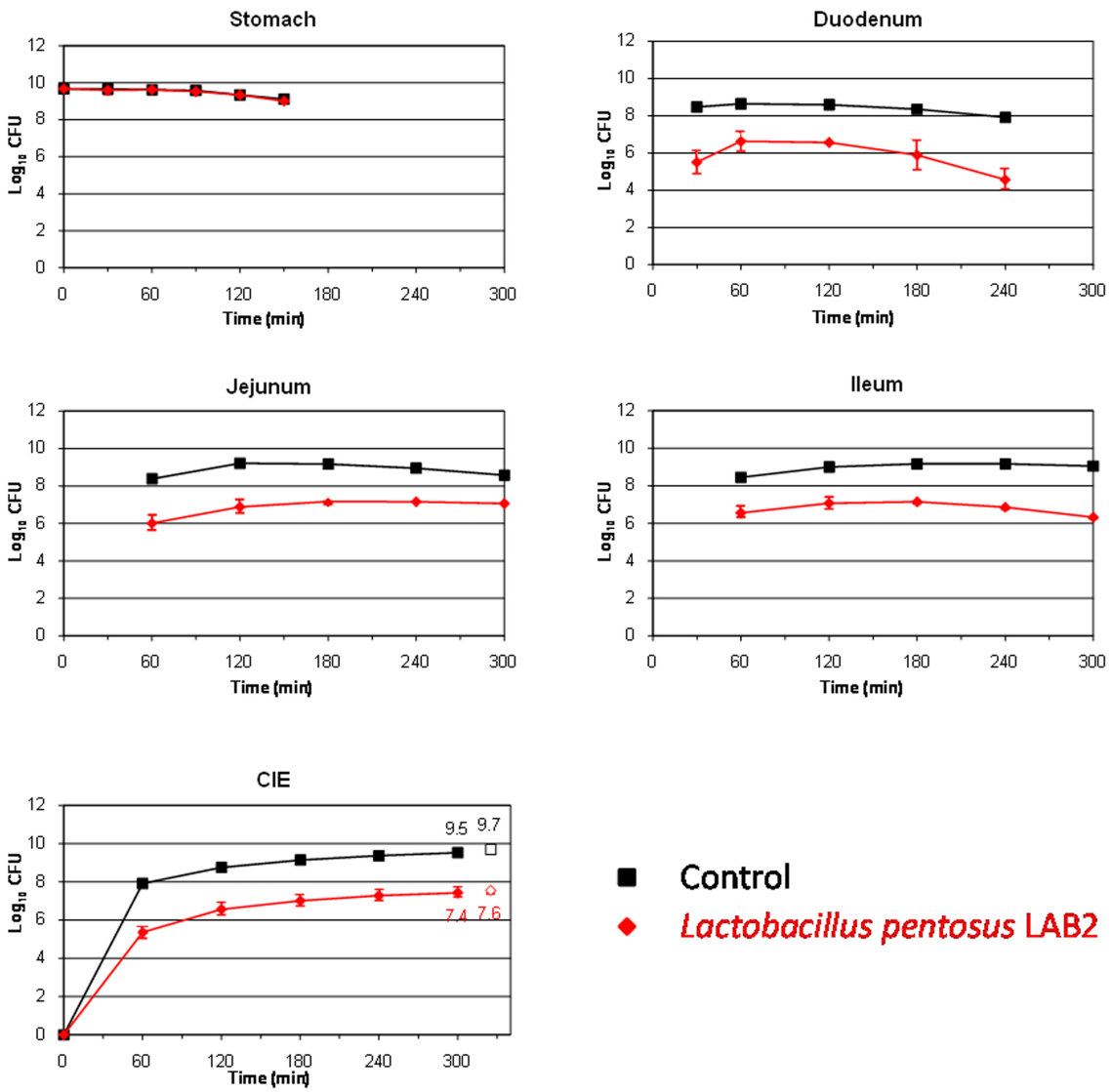


FIGURE 2 | Survival (plate counts versus time) of the potential probiotic strain *Lactobacillus pentosus* TOMC-LAB2, isolated originally from table olives, in the different CIE of the TIM system compared to a non absorbable transit marker, blue dextran, used as control. Results are expressed as $\log_{10}$ cfu ± SDs ($n = 2$).

To be effective and confer health benefits on the host, probiotics must be able to survive passage through the human stomach and small intestine and be present in sufficient number to colonize the colonic environment (Del Piano et al., 2006). The survival of the *L. pentosus* strains isolated from table olives was compared to that of the well-known probiotic *L. rhamnosus* GG (**Figure 4**). This microorganism was slightly affected during its transit through the stomach (only 0.1 $\log_{10}$ CFU reduction at time 150 min). Pitino et al. (2012) also found a high resistance of this species to stomach digestion in a dynamic gastric model. On the contrary, the population decreased in higher proportions at the end of its transit through the duodenum (~1 $\log_{10}$ CFU at time 240 min) and it seemed to be not affected by the conditions occurring in the jejunum and ileum (no additional mortality was observed in either compartment). At 300 min, high levels for this LAB species were recovered from the ileal effluents, decreasing by only 0.3 $\log_{10}$ CFU compared to the transit marker.

Table 3 shows the percentage of survival obtained for the four assayed bacteria after their transit through the different compartments of the TIM system as well as in the ileal effluents at the end of digestion. As can be clearly deduced even though *E. coli* O157:H7 is considered a highly acid resistant pathogen (Foster, 2004), this microorganism was the most affected bacteria by gastric digestion, with statistically significant differences ($p < 0.05$) compared to lactobacilli strains. *L. rhamnosus GG* was the most resistant bacteria during its transit through duodenum, although without

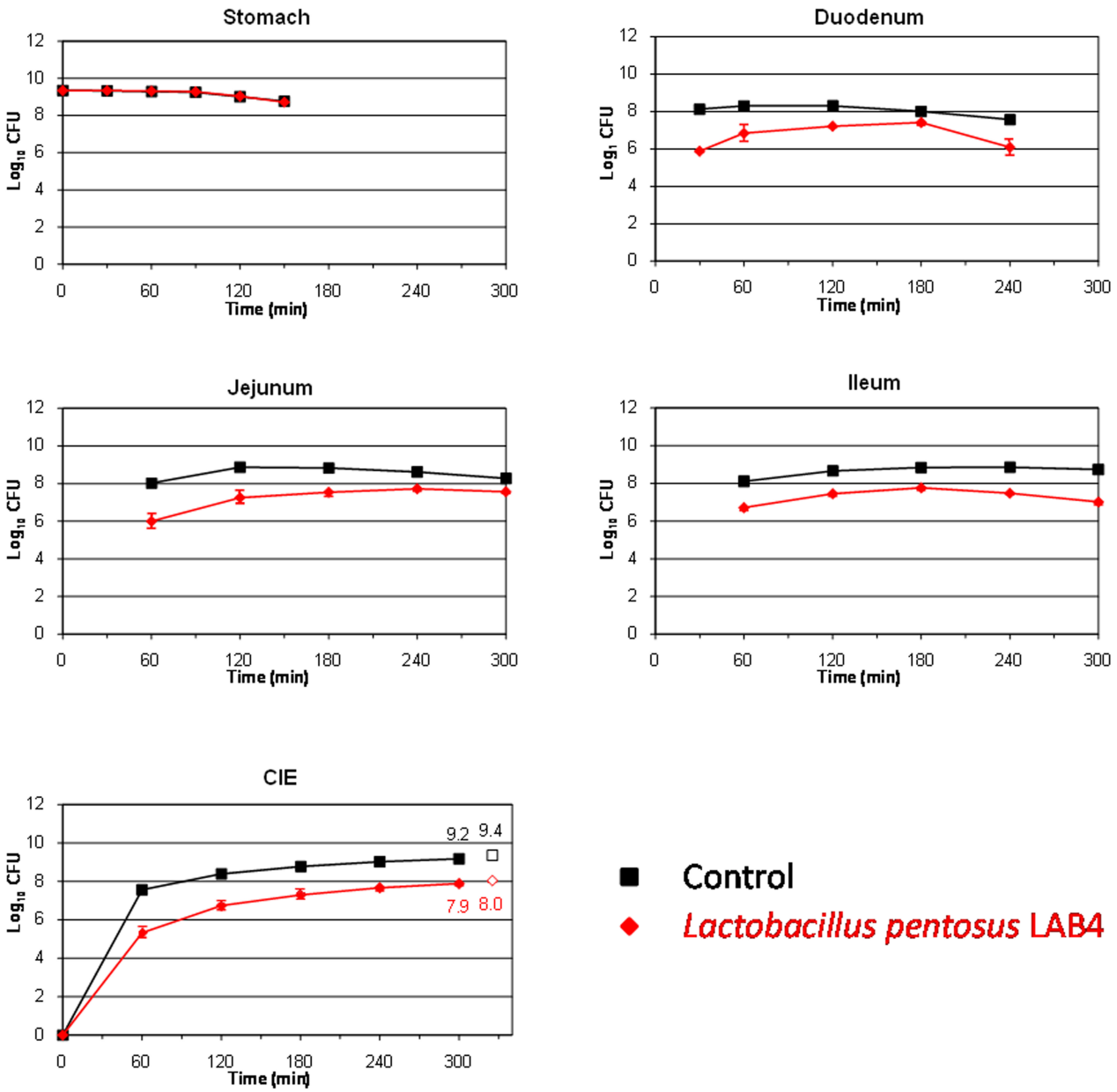


FIGURE 3 | Survival (plate counts versus time) of the potential probiotic strain *L. pentosus* TOMC-LAB4, isolated originally from table olives, in the different CIE of the TIM system compared to a non absorbable transit marker, blue dextran, used as control. Results are expressed as $\log_{10}$ cfu ± SDs ($n = 2$).

significant differences according to a Fisher-LSD *post hoc* comparison test. This is in agreement with previous results obtained by Silva et al. (1987) and Pitino et al. (2012) who cataloged *L. rhamnosus* as an acid and bile-resistant species. Bile salts are described as toxic at high concentrations for bacterial cells by disorganizing the lipid bi-layer structure of the cellular membranes (Thanassi et al., 1997). This is presumably the reason why all assayed microorganisms reduced their population during their transit through the duodenum. On the contrary, the food-borne pathogen was the most adapted microorganism in the distal parts of the gastrointestinal tract (jejunum and ileum), with significant differences ($p < 0.05$) compared to the lactobacilli species in the ileum. Thereby, after gastric and small intestinal transit, the survival rate was statistically higher for *E. coli* O157:H7 EDL 933 (117.5%) than for the rest of bacteria (from 0.7 to 40.5%), indicating that a higher percentage of the pathogen population could enter into the colon compared to the lactobacilli species in the case of a hypothetical intake of contaminated olives. *L. pentosus* TOMC-LAB2

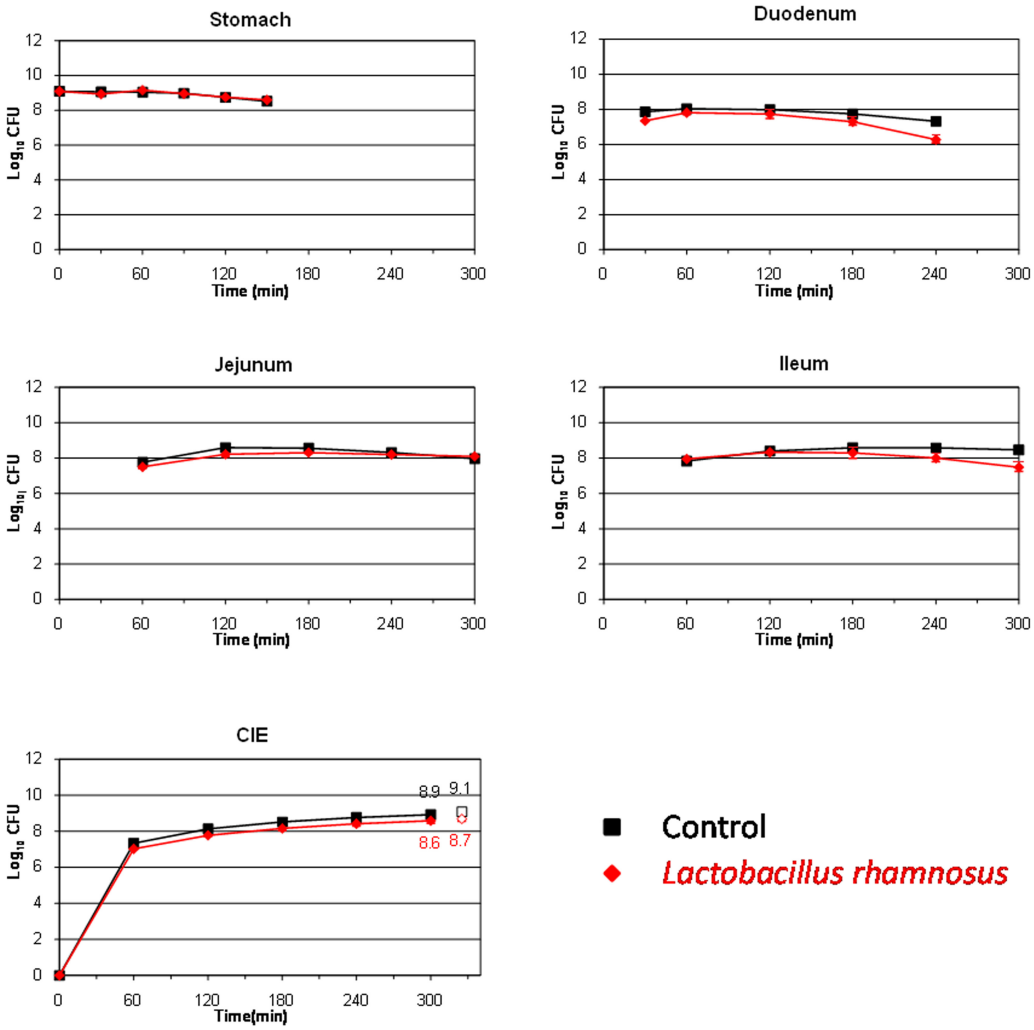


FIGURE 4 | Survival (plate counts versus time) of the probiotic strain *L. rhamnosus* GG in the different CIE of the TIM system compared to a non absorbable transit marker, blue dextran, used as control. Results are expressed as $\log_{10}$ cfu ± SDs ($n = 2$).

Table 3 | Survival (%) of the four tested strains at the end of *in vitro* digestions in the different compartments and ileal effluents (CIE) of the TIM system, using fermented *Manzanilla* olives as food matrix.

Microorganism	E_{150}	D_{240}	J_{300}	I_{300}	CIE_{300}	TF_{325}
E. coli O157:H7 EDL 933	0.00 (0.00)^{a}	0.01 (0.00)^{a}	11.55 (8.69)^{a}	45.27 (4.45)^{a}	45.45 (11.66)^{a}	117.47 (26.68)^{a}
L. rhamnosus GG	32.80 (3.47)^{c}	0.16 (0.05)^{a}	2.52 (0.01)^{a}	10.97 (4.55)^{b}	31.29 (5.91)^{a}	40.48 (7.37)^{c}
L. pentosus LAB2	18.13 (0.05)^{b}	0.001 (0.001)^{a}	0.05 (0.00)^{a}	0.24 (0.02)^{c}	0.55 (0.13)^{b}	0.74 (0.14)^{b}
L. pentosus LAB4	24.11 (5.22)^{b,c}	0.07 (0.05)^{a}	0.47 (0.06)^{a}	1.69 (0.66)^{c}	3.47 (1.03)^{b}	4.91 (1.04)^{b,c}

E_{150}, D_{240}, J_{300} and I_{300} stands for instantaneous percentages in the stomach (150 min), duodenum (240 min), jejunum (300 min), and ileum (300 min), respectively. CIE_{300} and TF_{325} stands for cumulative percentages from ileal effluents (0–300 min) and cumulative percentages from ileal effluents (0–300 min) plus final gastrointestinal residue, respectively. SD in parentheses. Values followed by different superscript letters, within the same column, are significantly different ($p < 0.05$) according to a Fisher-LSD post hoc comparison test.

and TOMC-LAB4 have already shown promising probiotic characteristics (Bautista-Gallego et al., 2013) as well as a considerable capacity for adhesion to the olive epidermis (Arroyo-López et al., 2012). Population levels higher than 7.5 $\log_{10}$ CFU were recovered for both strains after their transit through the artificial ileum and before entering into the colon (survival percentages of 0.6

and 3.5% for *L. pentosus* TOMC-LAB2 and TOMC-LAB4, respectively), compared to 8.5 $\log_{10}$ CFU obtained for *L. rhamnosus* GG (survival percentage of 31.3%). This makes them good candidates for use as probiotic agents, which could increase the functional value of table olives.

CONCLUSION

In summary, these results encourage further research on the development of table olives as a functional food, because, apparently, it is a good vehicle of microorganisms to the human body. Nevertheless, at the same time, they also show the necessity of good hygienic practices by olive manufacturers to avoid any possibility of intake of EHEC-contaminated olives, even though, apparently, the pathogen was not able to produce toxins during its transit through the upper human gastrointestinal tract following olive intake.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the EU's Seventh Framework Program (FP7/2007-2013) under grant agreement n° 243471 (PROBIOLIVES). Francisco N. Arroyo-López wish to express his gratitude to the Spanish Government for his Ramón y Cajal postdoctoral research contract, as well as CSIC for financial support of the research mobility program in foreign countries (A12012PM2-PA1003309). Verónica Romero-Gil thanks to the Agrifood Campus of International Excellence (ceiA3) for her predoctoral fellowship (program of formation of Ph.D. in companies).

REFERENCES

Argyri, A. A., Lyra, E., Panagou, E. Z., and Tassou, C. C. (2013). Fate of *Escherichia coli* O157:H7, *Salmonella Enteritidis* and *Listeria monocytogenes* during storage of fermented green table olives in brine. *Food Microbiol.* 36, 1–6. doi: 10.1016/j.fm.2013.04.001

Arnold, K. W., and Kaspar, C. W. (1995). Starvation and stationary-phase induced acid tolerance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 61, 2037–2039.

Arroyo-López, F. N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., et al. (2012). Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style *Manzanilla fermentations. Food Microbiol.* 32, 295–301. doi: 10.1016/j.fm.2012.07.003

Bautista-Gallego, J., Arroyo-López, F. N., Rantsiou, K., Jiménez Díaz, R., Garrido Fernández, A., and Cocolin, L. (2013). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int.* 50, 135–142. doi: 10.1016/j.foodres.2012.10.004

Blana, V. A., Grounta, A., Tassou, C. C., Nychas, G. J., and Panagou, E. Z. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol.* 38, 208–218. doi: 10.1016/j.fm.2013.09.007

Del Piano, M., Morelli, L., Strozzi, G. P., Allesina, S., Barba, M., Deidda, F., et al. (2006). Probiotics: from research to consumer. *Dig. Liver Dis.* 38, 248–255. doi: 10.1016/S1590-8658(07)60004-8

Domínguez-Manzano, J., León-Romero, Á., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F. N., Garrido-Fernández, A., et al. (2012). Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. *Int. J. Food Microbiol.* 157, 230–238. doi: 10.1016/j.ijfoodmicro.2012.05.011

Etienne-Mesmin, L., Livrelli, V., Privat, M., Denis, S., Cardot, J. M., Alric, M., et al. (2011). Effect of a new probiotic *Saccharomyces cerevisiae* strain on survival of *Escherichia coli* O157:H7 in a dynamic gastrointestinal model. *Appl. Environ. Microbiol.* 77, 1127–1131. doi: 10.1128/AEM.02130-10

Foster, D. B. (2013). Modulation of the enterohemorrhagic *E. coli* virulence program through the human gastrointestinal tract. *Virulence* 4, 315–323. doi: 10.4161/viru.24318

Foster, J. W. (2004). *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat. Rev. Microbiol.* 2, 898–907. doi: 10.1038/nrmicro1021

Ganzle, M. G., Hertel, C., Van der Vossen, J. M., and Hammes, W. P. (1999). Effect of bacteriocin-producing *Lactobacilli* on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine. *Int. J. Food Microbiol.* 48, 21–35. doi: 10.1016/S0168-1605(99)00025-2

Garrido-Fernández, A., Fernández Díez, M. J., and Adams, R. M. (1997). *Table Olives. Production and Processing.* London: Chapman & Halls. doi: 10.1007/978-1-4899-4683-6

Grounda, A., Nychas, G. J. E., and Panagou, E. Z. (2013). Survival of foodborne pathogens on natural black table olives after post-processing contamination. *Int. J. Food Microbiol.* 161, 197–202. doi: 10.1016/j.ijfoodmicro.2012.12.017

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., and Alric, M. (2012). Relevance and challenges of in vitro gastrointestinal models in simulating human digestion. *Trends Biotechnol.* 30, 591–600. doi: 10.1016/j.tibtech.2012.08.001

Hurtado, A., Reguant, C., Bordons, A., and Rozès, N. (2012). Lactic acid bacteria from fermented table olives. *Food Microbiol.* 31, 1–8. doi: 10.1016/j.fm.2012.01.006

Lavermicocca, P., Valerio, F., Lisa Lonigro, S., De Angelis, M., Morelli, L., Callegari, M. L., et al. (2005). Study of adhesion and survival of *Lactobacilli* and *Bifidobacteria* on table olives with the aim of formulating a new probiotic food. *Appl. Environ. Microbiol.* 71, 4233–4240. doi: 10.1128/AEM.71.8.4233-4240.2005

Minekus, M., Marteau, P., Havenaar, R., and Huis in't Veild, J. H. (1995). A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *ATLA* 23, 197–209.

Miszczycha, S. D., Thévenot, J., Denis, S., Callon, C., Livrelli, V., Alric, M., et al. (2014). Survival of *Escherichia coli* O26:H11 exceeds that of *Escherichia coli* O157:H7 as assessed by simulated human digestion of contaminated raw milk cheeses. *Int. J. Food Microbiol.* 172, 40–48. doi: 10.1016/j.ijfoodmicro.2013.11.029

Nychas, G. J. E., Panagou, E. Z., Parker, M. L., Waldron, K. W., and Tassou, C. C. (2002). Microbial colonization of naturally black olives during fermentation and associated biochemical activities in the cover brine. *Lett. Appl. Microbiol.* 34, 173–177. doi: 10.1046/j.1472-765x.2002.01077.x

Pennington, H. (2010). *Escherichia coli* O157. *Lancet* 376, 1428–1435. doi: 10.1016/S0140-6736(10)60963-4

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., et al. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiol.* 31, 57–63. doi: 10.1016/j.fm.2012.02.013

Rodríguez-Gómez, F., Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Jiménez-Díaz, R., Garrido-Fernández, A., et al. (2013). Table olive fermentation with multifunctional *Lactobacillus pentosus* strains. *Food Control* 34, 96–105. doi: 10.1016/j.foodcont.2013.04.010

Shigeno, T., Akamatsu, T., Fujimori, K., Nakatsuji, Y., and Nagata, A. (2002). The clinical significance of colonoscopy in hemorrhagic colitis due to enterohemorrhagic *Escherichia coli* O157:H7 infection. *Endoscopy* 34, 311–314. doi: 10.1055/s-2002-23644

Silva, M., Jacobus, N. V., Deneke, C., and Gorbach, S. L. (1987). Antimicrobial substance from a human *Lactobacillus strain. Antimicrob. Agents Chemother.* 31, 1231–1233. doi: 10.1128/AAC.31.8.1231

Skandamis, P. N., and Nychas, G. J. E. (2003). Modeling the microbial interaction and the death of *Escherichia coli* O157:H7 during the fermentation of Spanish style green table olives. *J. Food Prot.* 66, 1166–1175.

Smith, J. L. (2003). The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions. *J. Food Prot.* 66, 1292–1303.

Spyropoulou, K. E., Chorianopoulos, N. G., Skandamis, P. N., and Nychas, G. J. E. (2001). Survival of *Escherichia coli* O157:H7 during the fermentation of Spanish-style green table olives (Conservolea variety) supplemented with different carbon sources. *Int. J. Food Microbiol.* 66, 3–11. doi: 10.1016/S0168-1605(00)00510-9

Takumi, K., De Jonge, R., and Havelaar, A. (2000). Modelling inactivation of *Escherichia coli* by low pH: application to passage through the stomach of

young and elderly people. *J. Appl. Microbiol.* 89, 935–943. doi: 10.1046/j.1365-2672.2000.01193.x

Tamplin, M. L. (2005). Inactivation of *Escherichia coli* O157:H7 in simulated human gastric fluid. *Appl. Environ. Microbiol.* 71, 320–325. doi: 10.1128/AEM.71.1.320-325.2005

Thanassi, D., Cheng, G. W. L., and Nikaido, H. (1997). Active efflux of bile salts by *Escherichia coli*. *J. Bacteriol.* 179, 2512–2518.

Conflict of Interest Statement: 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.

Received: 23 July 2014; paper pending published: 26 August 2014; accepted: 29 September 2014; published online: 14 October 2014.

Citation: Arroyo-López FN, Blanquet-Diot S, Denis S, Thévenot J, Chalancon S, Alric M, Rodríguez-Gómez F, Romero-Gil V, Jiménez-Díaz R and Garrido-Fernández A (2014) Survival of pathogenic and lactobacilli species of fermented olives during simulated human digestion. Front. Microbiol. ***5****:540. doi: 10.3389/fmicb.2014.00540*
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 17 February 2015
doi: 10.3389/fmicb.2015.00056

Chemicals to enhance microalgal growth and accumulation of high-value bioproducts

***Xinheng Yu*[1,2,3]*, Lei Chen*[1,2,3] *and Weiwen Zhang*[1,2,3] ***

[1] *Laboratory of Synthetic Microbiology, School of Chemical Engineering and Technology, Tianjin University, Tianjin, China*
[2] *Key Laboratory of Systems Bioengineering (Ministry of Education), Tianjin University, Tianjin, China*
[3] *SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin, China*

Edited by:
Maria De Angelis, University of Bari Aldo Moro, Italy

Reviewed by:
Ebrahim Hadavi, Islamic Azad University, Iran
Zhao Chen, Clemson University, USA

****Correspondence:***
Weiwen Zhang, Laboratory of Synthetic Microbiology, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
e-mail: wwzhang8@tju.edu.cn

Photosynthetic microalgae have attracted significant attention as they can serve as important sources for cosmetic, food and pharmaceutical products, industrial materials and even biofuel biodiesels. However, current productivity of microalga-based processes is still very low, which has restricted their scale-up application. In addition to various efforts in strain improvement and cultivation optimization, it was proposed that the productivity of microalga-based processes can also be increased using various chemicals to trigger or enhance cell growth and accumulation of bioproducts. Herein, we summarized recent progresses in applying chemical triggers or enhancers to improve cell growth and accumulation of bioproducts in algal cultures. Based on their enhancing mechanisms, these chemicals can be classified into four categories:chemicals regulating biosynthetic pathways, chemicals inducing oxidative stress responses, phytohormones and analogs regulating multiple aspects of microalgal metabolism, and chemicals directly as metabolic precursors. Taken together, the early researches demonstrated that the use of chemical stimulants could be a very effective and economical way to improve cell growth and accumulation of high-value bioproducts in large-scale cultivation of microalgae.

Keywords: chemicals, microalgae, growth, accumulation, bioproducts

INTRODUCTION

Microalgae are autotrophic organisms, which utilize light energy, and inorganic nutrients such as CO_2, nitrogen and phosphorus, to generate biomass and synthesize valuable metabolites. Some algal species cultivated under stress conditions accumulate specific secondary metabolites (i.e., pigments, vitamins, or lipids), which are high-value bioproducts that can be applied in the cosmetic, food, or pharmaceutical sectors (Skjanes et al., 2013). In contrast to higher plants that contain large amount of cellulose and hemicellulose, larger portion of algal biomass can be directly converted into biofuels or other high-value bioproducts *via* downstream processes (Wijffels et al., 2010; Vanthoor-Koopmans et al., 2013; Yen et al., 2013). One well-known area of such applications is microalgae-based biodiesel that has been proposed as good alternative to non-renewable fossil fuels (Sheehan et al., 1998), and another area of commercial exploitation of microalgae is the production of pharmaceutically and high-value industrial chemicals (Leu and Boussiba, 2014).

Although microalgae are known to synthesize a variety of bioproducts with potential commercial values, only a few of them, such as β-carotene and astaxanthin, have been produced at an industry-scale (Ben-Amotz, 1995; Sheehan et al., 1998; Borowitzka, 2013), which may be due to the low productivity of these products in the native microalgae and the difficulty in isolating them by economically feasible means (Clarens et al., 2010; Norsker et al., 2011; Razon and Tan, 2011; Soratana and Landis, 2011). While significant efforts have been undertaken to select high-yield strains, optimize cultivation and even modify the strains by genetic engineering in the past decades (Suen et al., 1987; Cerón Garcìa et al., 2005; Kilian et al., 2011), progress has yet to be fully satisfied.

As an alternative method to improve production or accumulation of bioproducts, chemicals as metabolic triggers or enhancers that are able to directly modulate cellular metabolism have been proposed and applied in various commercially viable microalgae. Unlike genetic modification, this approach relies on phenotypic screening and does not require specific knowledge of molecular targets in metabolic and catabolic pathways involved in synthesis of bioproducts. In a recent study, Franz et al. (2013) described a phenotypic screening of 42 chemicals for their roles on lipid metabolism in microalgae, and identified 12 chemicals that are capable of enhancing intracellular lipid levels by >100%, with three compounds (i.e., epigallocatechin gallate, CDK2 inhibitor 2 and cycloheximide) increasing intracellular lipids by 200–400% based on Nile Red fluorescence intensity. In addition, the researchers took a further step to verify these chemicals in large-scale cultures and concluded that propyl gallate and butylated hydroxyanisole could be used in large-scale applications considering the low cost of the chemicals and the lipid content increases (Franz et al., 2013), demonstrating that the application of chemical enhancer could be a valuable and practical approach in addressing the low productivity issue with microalgae-based processes. In this article, we review the recent progresses in applying chemicals to improve cell growth and accumulation of high-value bioproducts in microalgae (**Table 1**), with a focus on the molecular mechanisms of their stimulatory roles.

Table 1 | Summary of chemicals used in enhancing growth and product accumulation in microalgae.

Species	Products	Chemicals	Reference
Haematococcus pluvialis	Astaxanthin	2, 4-Epibrassinolide (EBR)	Gao et al. (2013b)
Chlorella vulgaris	Biomass	Brassinosteroids (BRs)	Bajguz and Piotrowska-Niczyporuk (2013)
Haematococcus pluvialis	Astaxanthin	Jasmonic acid (JA)	Gao et al. (2012b)
Haematococcus pluvialis	Astaxanthin	Salicylic acid (SA)	Gao et al. (2012a)
Haematococcus pluvialis	Astaxanthin	Methyl jsmonate (MJ), gibberellic acid (GA_3)	Lu et al. (2010)
Microcystis aeruginosa	Biomass	Polycyclic aromatic hydrocarbons	Zhu et al. (2012)
Chlorella zofingiensis	Astaxanthin	Pyruvate, citrate, and malic acid	Chen et al. (2009)
Haematococcus pluvialis	Astaxanthin	Gibberellic acid (GA_3)	Gao et al. (2013a)
Haematococcus pluvialis	Astaxanthin	Salicylic acid (SA), methyl jsmonate (MJ)	Vidhyavathi et al. (2008)
Schizochytrium sp. HX-308	DHA	Ethanol, sodium acetate, malic acid	Ren et al. (2009)
Chlorella vulgaris	Biomass	Indomethacin (IM)	Piotrowska et al. (2008)
Haematococcus pluvialis	Astaxanthin	Fe, sodium acetate	Kobayashi et al. (1993), Choi et al. (2002), Li et al. (2008), Wang et al. (2009), Su et al. (2014)
Synechocystis sp. PCC680, *Anabaena*. sp PCC7120, *Scenedesmus obliquus*	Biomass, lipid, and fatty acid composition	Ethanolamine	Cheng et al. (2012)
Haematococcus pluvialis	Astaxanthin	Methylene blue(MB), methyl viologen (MV), H_2O_2, 2,2′-azo-bis(2-amidinopropane)-dihydrochloride (AAPH)	Kobayashi et al. (1993), Rioboo et al. (2011)
Chlorococcum sp.	Astaxanthin	H_2O_2, methyl viologen (MV), Fe	Ma and Chen (2001a)
Chlorella zofingiensis	Astaxanthin	H_2O_2 and NaClO	Ip and Chen (2005)
Haematococcus pluvialis	Carotenoid	Sodium acetate, sodium chloride, Fe, methyl viologen (MV)	Steinbrenner and Linden (2001)
Dunaliella salina	β-carotene	Fe, cetate, malonate	Mojaat et al. (2008)
Chlorococcum sp.	Free trans-astaxanthin	H_2O_2	Ma and Chen (2001b)
Chlorella sorokiniana	Biomass and lipid	2-phenylacetic acid (PAA), Indole butyric acid (IBA), 1-naphthaleneacetic acid (NAA), Gibberellic acid (GA_3), Zeatin, thidiazuron, Humic acid, Kelp extrsct, Methanol, Fe, Putrescine, Supermidine	Hunt et al. (2010)

(Continued)

Table 1 | Continued

Species	Products	Chemicals	Reference
Chlorella pyrenoidosa	Biomass	Kinetin, gibberellic acid (GA_3), indole acetic acid (IAA)	Vance (1987)
Chlorella pyrenoidosa	Biomass and carotenoids	Indole butyric acid (IBA), indole acetic acid (IAA), indole-3-lactic acid, tryptamine, 2-(2,4-dichlorophenoxy) acetic acid (2,4-D), naphthaleneacetic acid (NAA), N-6-benzylaminopurine, N-6-furfu-rylamineopurine, allantoin (AT)	Czerpak and Bajguz (1997)
Chlorella vulgaris	Biomass	Brassinosteroids (BRs)	Bajguz and Czerpak (1996)
Chlorella vulgaris	Biomass	Salicylic acid (SA)	Czerpak et al. (2002)
Chlorella vulgaris	Biomass	Diamines, polyamines	Czerpak et al. (2003)
Monorapbidium convolutum and *Monorapbidium minutum*	Biomass	Humic substances	Karasyova et al. (2007)
Chlamydomonas reinhardtii and Chlorella vulgaris	Lipid	Brefeldin A	Kim et al. (2013)
Spirulina platensis	Total carotenoids and α-tocopherol, glutathione (GSH), and ascorbic acid (AsA)	H_20_2	Abd El-Baky et al. (2009)
Dunaliella salina	Biomass and glycerol	Copper	Lustigman et al. (1987)
Haematococcus pluvialis	Biomass and astaxanthin	Fe^{2+}-EDTA, Fe^{3+}-EDTA, $Fe(OH)_x^{32x}$, and $FeC_6H_5O_7$	Cai et al. (2009)
Spirulina platensis	Biomass and free proline concentration	2-(2,4-dichlorophenoxy) acetic acid (2,4-D)	Saygideger and Deniz (2008)
Chlorella vulgaris and Spirulina platensis	Biomass	2, 4-Epibrassinolide (EBR)	Saygideger and Okkay (2008)
Chlorella pyrenoidosa	Biomass	Anthranilic acid, tryptamine, 2-phenylacetic acid (PAA), 2-(2,4-dichlorophenoxy) acetic acid (2,4-D), naphthaleneacetic acid (NAA), naphthyl-3-sulphonic acid, indole acetic acid (IAA)	Czerpak et al. (1994)

(Continued)

Table 1 | Continued

Species	Products	Chemicals	Reference
Haematococcus pluvialis	Carotenoids	Abscisic acid (ABA) and its analogs	Kobayashi et al. (1997, 1998)
Selenastrum capricornutum	Biomass	Ethyl 2-methyl acetoacetate (EMA)	Hong et al. (2008)
Nannochloropsis salina, Nannoc hloropsis oculata, Nannochloris sp. and *Phaeodactylum tricornutum*	Lipid	Multiple chemical triggers (Forskolin, quinacrine, butyl hydroxy anisd (BHA), epigallocatechin gallate etc.)	Franz et al. (2013)
Chlamydomonas reinhardtii	Biomass and fatty acid	Indole acetic acid (IAA), gibberellic acid (GA_3), kinetin, 1-triacontanol, abscisic acid	Park et al. (2013)
Synechocystis PCC 6803	Biomass and lipid	Calliterpenone	Patel et al. (2013)
Nostoc muscorum and *Tolypothrix tenuis*	Biomass	2-phenylacetic acid (PAA)	Ahmad and Winter (1970)
Chlorella vulgaris	Biomass	Zeatin	Piotrowska and Czerpak (2009)
Scenedesmus obliquus	Biomass	Methanol	Theodoridou et al. (2002), Navakoudis et al. (2007)
Chlorella minutissima	Biomass	Methanol	Kotzabasis et al. (1999)
Chlorella vulgaris	Lipid	Fe	Liu et al. (2008)
Dunaliella primolecta	Biomass	Diamines and polyamines	Hourmant et al. (1994)

PHYTOHORMONES AND ANALOGS REGULATING MULTIPLE ASPECTS OF METABOLISM

TARGETING ON BIOSYNTHETIC PATHWAYS OF HIGH-VALUE PRODUCTS

It has been established that plants have developed a broad spectrum of molecular mechanisms to resist unfavorable environmental perturbations (Ren et al., 2009). Microalgae that share the evolutionary merits with plants also have mechanisms to deal with various environmental stress. One well-studied example is antioxidant pigment astaxanthin that plays a critical role in response to various stress conditions, such as high light, salinity, nutrient stress, and high carbon/nitrogen ratio, in chlorophyceae *Haematococcus pluvialis* (Tripathi et al., 1999; Sarada et al., 2002). The pathway of astaxanthin synthesis in *H. pluvialis* has been deciphered (Grünewald et al., 2000; Vidhyavathi et al., 2008) and several biosynthetic genes related to carotenoid have also been cloned and characterized (Lotan and Hirschberg, 1995; Sun et al., 1998; Linden, 1999; Steinbrenner and Linden, 2003; Huang et al., 2006). To increase the astaxanthin productivity, chemicals as metabolism enhancers were also evaluated recently. In one study, Lu et al. (2010) reported that gibberellic acid (GA_3) and methyl jsmonate (MJ) played roles in regulating gene expression of *bkts* that catalyzes β-carotene to canthaxanthin in the astaxanthin biosynthetic pathway (Lu et al., 2010). More recently, Gao et al. (2012a,b, 2013a,b) found that chemicals jasmonic acid (JA), salicylic acid (SA), GA_3, and 2, 4-epibrassinolide (EBR) can enhance astaxanthin production to 1.458 mg/L, 2.74 mg/L, 2.39 mg/L, 2.26 mg/L, respectively; and further analysis showed that the enhancing mechanisms of chemicals were concentration-dependent. For example, the results showed that 25 mg/L JA up-regulated the transcriptional expression of *pds*, *crt*R-B, and *lyc* of the astaxanthin biosynthetic pathway (>10-fold up-regulation) the most, while 50 mg/L JA impacted the transcriptional expression of *ipi-1*, *ipi-2*, *psy*, *crt*R-B, and *crt*O than on *pds*, *lyc*, and *bkt2* more significantly (Gao et al., 2012b). Based on a correlation analysis between their maximum mRNA transcripts of five carotenoid genes and astaxanthin production, Li et al. (2010) proposed that multiple regulatory mechanisms at transcriptional, translational, and post-translational levels of astaxanthin biosynthetic genes co-existed in controlling the overall carotenogenesis process in *H. pluvialis* (Li et al., 2010). Interestingly, different modes of regulation can be issued by the same chemical in *H. pluvialis*, such as JA that up-regulated *psy*, *pds*, *crt*R-B, *lyc*, *bkt*, and *crt*O genes at the transcriptional level, and up-regulated *ipi-1* and *ipi-2* genes at both transcriptional and post-transcriptional levels, respectively; and SA up-regulated *ipi-1*, *ipi-2*, *psy*, *crt*R-B, *bkt*, and *crt*O gene at the transcriptional level, and *lyc* at the post-transcriptional level and *pds* at both levels, respectively (Gao et al., 2012a,b).

INDUCING OXIDATIVE STRESS RESPONSES

Photosynthetic algae, like higher plants, generate reactive oxygen species (ROS) through chloroplast photosynthesis and mitochondrial respiration under stress condition, and ROS will then to be used as signal molecules to initiate production and accumulation of many bioproducts (Asada, 1994). The effects of SA and MJ on the antioxidant systems in *H. pluvialis* were investigated, and the results showed that at low concentrations, 100 μM SA increased astaxanthin content to 6.8-fold under low light (30 μmol m^{-2} s^{-1}), while 10 μM MJ showed marginal increase in astaxanthin. However, at high concentration of 500 μM, both SA and MJ reduced the growth of microalgae and inhibited astaxanthin accumulation. Further mechanism analysis showed that SA at high concentrations increased superoxide dismutase activity to 4.5- and 3.3-fold and ascorbate peroxidase (APX) activity to 15.5- and 7.1-fold under low and high light, respectively, while MJ increased catalase activity (1.4-fold) under high light and APX activity (5.4-fold) under low light, suggesting the low astaxanthin accumulation may be due to the free radicals being scavenged (Raman and Ravi, 2010).

REGULATING OTHER ASPECTS OF CELLULAR METABOLISM

Phytohormones are signal molecules synthetized by plants, and capable of efficiently regulating cellular metabolism at very low concentrations (Park et al., 2013). The application of phytohormones to improve growth and productivity has been reported, and the results with *Chlorella* species showed that use of natural and synthetic auxins, as well as their precursors, have considerable stimulating effects on algal growth and biomass composition (Czerpak et al., 1994, 1999; Czerpak and Bajguz, 1997; Hunt et al., 2010). In addition, a combination of chemicals from within the auxin family as well as with that of other families, such as 5 ppm 1-naphthaleneacetic acid (NAA) + 10 ppm GA_3 + 1 ppm zeatin (ZT), dramatically increased biomass productivity by 170% over the control in *Chlorella sorokiniana* (Hunt et al., 2010). Another study investigated the effects of phytohormones on microalgal growth and oil accumulation for biodiesel production in *Chlamydomonas reinhardtii*. The results indicated that all five of the tested phytohormones (i.e., indole-3-acetic acid, gibberellic acid, kinetin, 1-triacontanol, and abscisic acid) promoted cell growth. In particular, hormone treatment increased biomass production by 54–69% relative to the control growth medium, demonstrating their values in decreasing cost of commercial biodiesel production (Park et al., 2013).

Brassinosteroids (BRs) are hydroxylated derivatives of 5-cholestane and important plant growth regulators in multiple developmental processes, such as cell division and cell elongation (Bajguz and Czerpak, 1996; Bajguz and Tretyn, 2003). A recent study found that BRs cooperated synergistically with auxins in stimulating cell proliferation and endogenous accumulation of proteins, chlorophylls, and monosaccharides in *C. vulgaris* (Bajguz and Piotrowska-Niczyporuk, 2013).

In terms of the molecular mechanisms, auxins and their analogs have been found to affect photosynthetic efficiency and CO_2 fixation in microalgae. For example, a study showed that auxins had incentive effects on reactions of bonding CO_2 to 1, 5-biphosphoribulose and photosynthetic phosphorylation. As expected, the increase in intensity of photosynthesis reactions correlated well with higher contents of chlorophylls, pheophytins, and total carotenoids in cells treated with indomethacin that shares structural similarity with natural auxins (Piotrowska et al., 2008). Other studies also indicated that low concentrations of synthetic auxins, such as 2-(2,4-dichlorophenoxy) acetic acid (2,4-D), NAA and 2-phenylacetic acid (PAA), stimulated the photosynthetic rate and chlorophylls as well as carotenoids synthesis in green

algae *C. pyrenoidosa*, *Scenedesmus acuminatus*, and *S. qadricauda* (Czerpak et al., 1994, 1999, 2002; Wong, 2000).

Diamines and polyamines are polycation nitrogen compounds presented in almost all prokaryotic and eukaryotic microorganisms and belonged to specific cellular regulators of growth and metabolism (Rayle and Cleland, 1992). The study showed that in *C. vulgaris* treated with diamines and polyamines, the content of monosaccharides, primary products of Calvin cycle were intensively stimulated on 3 days of *C. vulgaris* culture, while chlorophyll content was enhanced on 9 days of *C. vulgaris* culture, indicating that the amines stimulated the dark phase of photosynthesis in the young cells, and the light synthesis phase in aging cells, respectively (Czerpak et al., 2003).

An acid growth theory has been proposed to explain the cell elongation triggered by auxins in plant cells, which refers to the auxin-induced acidification of free space in cell wall. The decrease of pH enhances the plasticity of cell wall thus contributes to the increased elongation rate of the plant tissues, and the phenomenon is presumably related to the activation of membrane-binding proton pumps by auxin (Rayle and Cleland, 1992; Hobbie et al., 1994). A study with algal *C. vulgaris* also showed that BR-stimulated cell growth depended at least partly on acid growth theory (Bajguz and Czerpak, 1996).

Cell phase and mitosis regulated by phytohormones was also reported in microalgae. A recent study showed that NAA (30 ppm) treatment stimulated higher biomass productivity between days 5 and 10 while PAA (5 ppm) treatment effected on the first 5 days in in *C. sorokiniana*, suggesting that NAA might prolong exponential phase and PAA might short initial lag phase before initiation of cell division. The combination of NAA (5 ppm) + PAA (30 ppm) showed 104% increase of biomass and demonstrated that auxins enhanced biomass growth by reducing generation time thus contributing to reducing generation time (Hunt et al., 2010). Another study on the synchronous culture of *C. pyrenoidosa* showed that the time to incipient cell division was reduced by GA and 6-furfurylaminopurine, suggesting these two phytohormones had played roles in eliminating the initial lag phase (Vance, 1987). Similarly, the cell number and dry weight of *C. vulgaris* was also significantly increased in response to optimal dose of IM (10^{-7} M) on a 5-day cultivation, suggesting that growth elicited by natural and synthetic auxins encompassed the stimulation of mitosis (Piotrowska et al., 2008).

Chlorophyll pigment presents challenges to lipid extraction and biodiesel conversion in downstream processing of algal biomass. Hence, chemicals led to higher biomass and lower pigment production will bring benefits. A study showed that the addition of NAA (30 ppm) and PAA (5 ppm) significantly increased biomass production, meanwhile decreased chlorophyll a synthesis in *C. sorokiniana* (Hunt et al., 2010). In addition, auxins at high concentrations can activate key regulatory enzyme in ethylene biosynthesis (Grossmann, 2000), and large amount of ethylene could then induce the degradation of photosynthetic pigments (Sunohara and Matsumoto, 1997).

As for other regulatory functions, an exposure of *C. vulgaris* cells to exogenous IM, synthetic analog of IAA, has been reported to increase cellular DNA level up to 48% and 20–43% more soluble proteins excreted to the environments (Piotrowska et al., 2008); and cytokinins and allantoin (AT) were found to stimulate carotenoids content by 185–190% and 124% in *C. pyrenoidosa*, possibly due to their inhibition of oxidases and dehydrogenases that are responsible for oxidation process and degradation of chlorophylls and carotenoids (Czerpak and Bajguz, 1997).

OTHER CHEMICALS INDUCING OXIDATIVE STRESS RESPONSES

Apart from phytohormones and analogs, other chemicals capable of inducing oxidative response for enhanceing microalgal growth and accumulation of high-value bioproducts were also investigated. An early study showed that Fe^{2+}, methylene blue (MB) for singlet oxygen ($^1O_2$), methyl viologen (MV) for superoxide anion radical (O_2^-), H_2O_2, and 2,2′-azo-*bis*(2-amidinopropane)-dihydrochloride (AAPH) for peroxy radical ($AO_2\cdot$), were capable of triggering astaxanthin biosynthesis in *H. pluvialis*, in which Fe^{2+} possibly served as an HO· generator *via* an iron-catalyzed Fenton reaction (Kobayashi et al., 1993). HO· or other active oxygen species ($^1O_2$, O_2^-, H_2O_2, and $AO_2\cdot$) might then enhance carotenoid formation in algal cyst cells by participating directly in the carotenogenic enzyme reactions as an oxidizer or an H acceptor (Beyer and Kleinig, 1989). In a recent study, Ip and Chen (2005) proposed sodium hypochlorite (NaClO) as another oxygen species to enhance astaxanthin production of *C. zofingiensis* in the heterotrophic cultivation medium.

CHEMICALS AS METABOLIC PRECURSORS

An early study showed that an addition of 100 mM pyruvate into the culture medium of *C. zofingiensis* enhanced the yield of astaxanthin from 8.36 to 10.72 mg/L. In addition, citrate and malic acid also had the similar stimulatory effects on the formation of astaxanthin. Pyruvate might serve as a precursor for isopentenyl pyrophosphate (IPP), the carotenoid precursor in *C. zofingiensis* and *H. pluvialis*, while the stimulatory effects of citrate and malic acid on astaxanthin biosynthesis in *C. zofingiensis* could be due to their conversions to pyruvate (Chen et al., 2009). For docosahexaenoic acid (DHA) accumulation in *Schizochytrium* sp. HX-308, an addition of 4 g/L malic acid to the culture medium at the rapid lipid accumulation stage can increase DHA content of total fatty acids from 35 to 60%. In addition to functioning as a possible carbon precursor, it was speculated that malic acid added at rapid lipid accumulation stage could activate malic enzyme activity and enhance NADPH generating reaction from malic acid to pyruvate (Ren et al., 2009). In addition, ethanol was also found to enhance lipid content by 35% in *Crypthecodinium cohnii*, in which ethanol can be converted to acetyl-CoA directly and in its metabolism might generate additional reducing power NADPH for lipogenesis (Lolke et al., 2005).

To aid in identifying metabolites associated with enhanced production of bioproducts, metabolomics, a measurement, and study of the small-molecule metabolites that constitute cellular metabolic networks, has been recently applied. In one study, Cheng et al. (2012) compared the metabolites between two cyanobacteria *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC 7120, and one microalga *S. obliquus* by gas chromatography coupled with time-of-flight mass spectrometry to

detect important metabolites intricately tied to the lipid content in cyanobacteria and microalgae. The results showed that nine metabolites including ethanolamine were associated with the different lipid accumulation, and further study confirmed that addition of exogenous ethanolamine (2 mmol/L) could increase the lipid content by 22% in *S. obliquus* (Cheng et al., 2012). In another study, Su et al. (2014) investigated mechanism of astaxanthin induction under various stress conditions using a metabolomics and network analysis, and found that several metabolites, such as D-(+) altrose, D-ribose 5-phosphate, L-glutamic acid, and α-ketoglutaric acid, were positively associated with the increased astaxanthin accumulation in *H. pluvialis*. Although further confirmation is still needed, it was speculated that the increased abundances of these metabolites might contribute to the enhanced carbon flow into the astaxanthin biosynthesis (Su et al., 2014). Taken together, these early studies demonstrated that metabolomics could be a valuable tool in identifying potential metabolites for enhancing target production in algae (Zhang et al., 2010). Effective mechanisms of the chemicals were schemed in **Figure 1**.

CONCLUSION

To produce bioproducts form microalgae in an economically feasible and sustainable way, one major hurdle that needs to be overcome is the low productivity. To address the issues, efforts have been undertaken to identify and apply chemical triggers or enhancers to enhance cell growth and accumulation of bioproducts in microalgae, and the studies have demonstrated that application of chemical triggers or enhancers could be a very practical method in large-scale fermentation of microalgae. In addition, the possible stimulatory mechanisms were also partially deciphered for some of the chemicals. However, to uncover new chemicals and expand the application, it is necessary to determine more accurately the metabolic mechanisms related to cell growth,

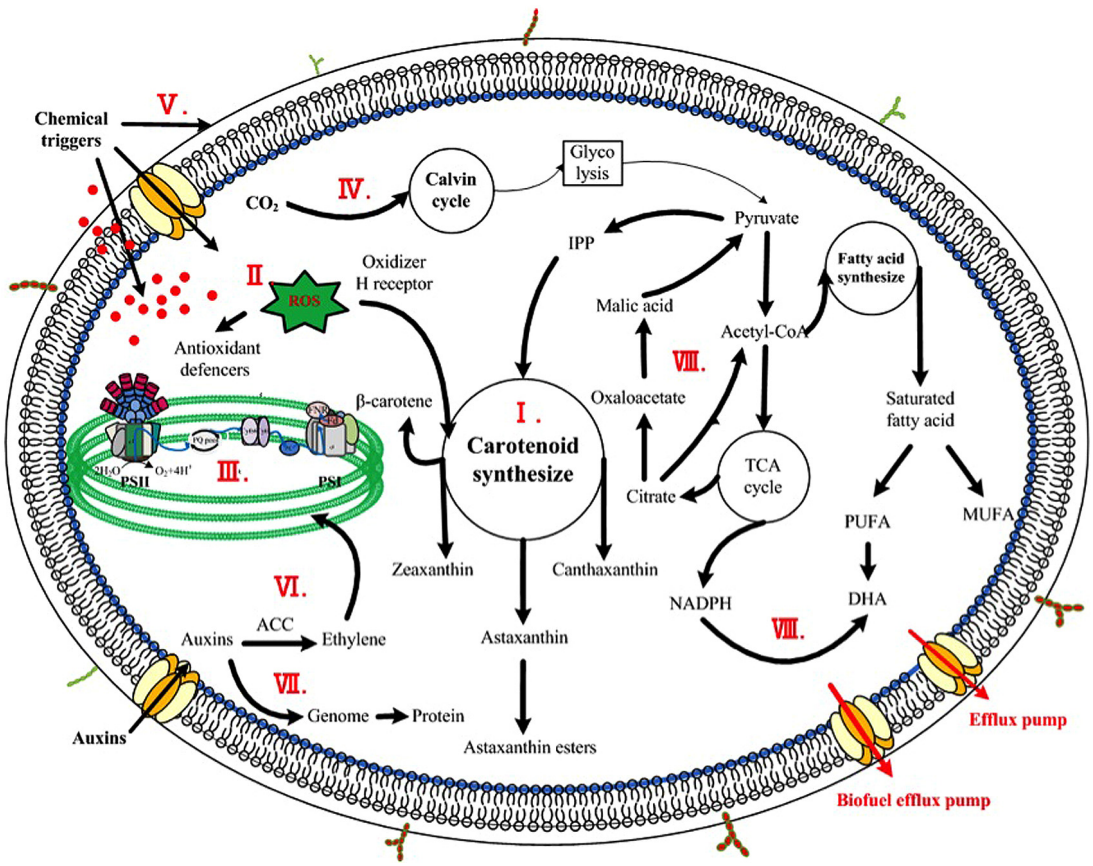


FIGURE 1 | Scheme of enhancing mechanisms of chemicals on microalgae. The major stimulatory mechanisms were indicted inside the cell. **(I)** Chemicals targeting on biosynthetic pathways of high-value product, such as JA, SA, GA, and EBR controlling the overall carotenogenesis process in *H. pluvialis;* **(II)** Chemicals inducing oxidative stress responses, including direct or indirect addition of active oxygen species and chemical triggers inducing antioxidant production; **(III)** Phytohormones and analogs effecting on photosynthetic efficiency, namely the light phase, including photosynthetic phosphorylation, photosynthetic rate, and chlorophylls synthesis; **(IV)** Phytohormones and analogs impacting CO_2 fixation, namely the dark phase of photosynthesis, such as diamines and polyamines stimulating production of Calvin cycle; **(V)** Phytohormones and analogs encompassed acid growth theory, alternating the plasticity of cell wall thus contributing to cell elongation; **(VI)** Degradation of photosynthetic pigments due to large amount of ethylene caused by high concentration of auxins; **(VII)** Phytohormones and analogs regulating genome and protein expression, such as IM modulating DNA and protein content in *C. vulgaris*; **(VIII)** Chemicals as metabolic precursors, such as pyruvate serving as a precursor of carotenoid synthesis thus stimulating the formation of astaxanthin and NADPH (led by malic acid) acting as a precursor of fatty acid synthesis increasing DHA content.

production and accumulation of bioproducts, and the modes of action (MOA) of chemicals in microalgae. For this regard, the application of various global-focused technologies, such as genomics, proteomics, and metabolomics, could be valuable tools in the future research.

ACKNOWLEDGMENTS

The research was supported by grants from the National High-tech R&D Program (No. 2012AA02A707), and the Doctoral Program of Higher Education of China (No. 20120032110020 and 20130032120022).

REFERENCES

Abd El-Baky, H., El Baz, F., and El-Baroty, G. (2009). Enhancement of antioxidant production in *Spirulina platensis* under oxidative stress. *Acta physiol. plant.* 31, 623–631. doi: 10.1007/s11738-009-0273–278

Ahmad, M., and Winter, A. (1970). The effect of weak auxins on the growth of blue-green algae. *Hydrobiologia* 36, 305–316. doi: 10.1007/bf00035329

Asada, K. (1994). "Production and action of active oxygen species in photosynthetic tissues," in *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants*, eds C. H. Foyer and P. M. Mullineaux (Boca Raton, FL: CRC Press), 77–104.

Bajguz, A., and Czerpak, R. (1996). Effect of brassinosteroids on growth and proton extrusion in the alga *Chlorella vulgaris* Beijerinck (Chlorophyceae). *J. Plant Growth Regul.* 15, 153–156. doi: 10.1007/bf00198931

Bajguz, A., and Piotrowska-Niczyporuk, A. (2013). Synergistic effect of auxins and brassinosteroids on the growth and regulation of metabolite content in the green alga *Chlorella vulgaris* (Trebouxiophyceae). *Plant Physiol. Biochem.* 71, 290–297. doi: 10.1016/j.plaphy.2013.08.003

Bajguz, A., and Tretyn, A. (2003). The chemical characteristic and distribution of brassinosteroids in plants. *Phytochemistry* 62, 1027–1046. doi: 10.1016/S0031-9422(02)00656-8

Ben-Amotz, A. (1995). New mode of *Dunaliella* biotechnology: two-phase growth for β-carotene production. *J. Appl. Phycol.* 7, 65–68. doi: 10.1007/bf00003552

Beyer, P., and Kleinig, H. (1989). "On the desaturation and cyclization reactions of carotenes," in *Chromoplast Membranes, Carotenoids*, eds N. Krinsky, M. Mathews-Roth, and R. Taylor (New York, NY: Springer), 195–206.

Borowitzka, M. (2013). High-value products from microalgae-their development and commercialisation. *J. Appl. Phycol.* 25, 743–756. doi: 10.1007/s10811-013-9983–9989

Cai, M., Li, Z., and Qi, A. (2009). Effects of iron electrovalence and species on growth and astaxanthin production of *Haematococcus pluvialis*. *Chin. J. Oceanol. Limnol.* 27, 370–375. doi: 10.1007/s00343-009-9176–9171

Cerón Garcìa, M., Sánchez Mirón, A., Fernández Sevilla, J., Molina Grima, E., and Garcìa Camacho, F. (2005). Mixotrophic growth of the microalga *Phaeodactylum tricornutum*. *Process Biochem.* 40, 297–305. doi: 10.1016/j.procbio.2004.01.016

Chen, T., Wei, D., Chen, G., Wang, Y., and Chen, F. (2009). Employment of organic acids to enhance astaxanthin formation in heterotrophic *Chlorella zofingensis*. *J. Food Process. Preserv.* 33, 271–284. doi: 10.1111/j.1745-4549.2009.00391.x

Cheng, J., Niu, Y., Lu, S., and Yuan, Y. (2012). Metabolome analysis reveals ethanolamine as potential marker for improving lipid accumulation of model photosynthetic organisms. *J. Chem. Technol. Biotechnol.* 87, 1409–1418. doi: 10.1002/jctb.3759

Choi, Y., Yun, Y., and Park, J. (2002). Evaluation of factors promoting astaxanthin production by a unicellular green alga, *Haematococcus pluvialis*, with fractional factorial design. *Biotechnol. Prog.* 18, 1170–1175. doi: 10.1021/bp025549b

Clarens, A., Resurreccion, E., White, M., and Colosi, L. (2010). Environmental life cycle comparison of algae to other bioenergy feedstocks. *Environ. Sci. Technol.* 44, 1813–1819. doi: 10.1021/es902838n

Czerpak, R., and Bajguz, A. (1997). Stimulatory effect of auxins and cytokinins on carotenes, with differential effects on xanthophylls in the green alga *Chlorella pyrenoidosa* Chick. *Acta Soc. Bot. Pol.* 66, 41–46. doi: 10.5586/asbp.1997.006

Czerpak, R., Bajguz, A., Białecka, B., Wierzchołowska, L., and Wolańska, M. (1994). Effect of auxin precursors and chemical analogues on the growth and chemical composition in *Chlorella pyrenoidosa* Chick. *Acta Soc. Bot. Pol.* 63, 279–286. doi: 10.5586/asbp.1994.038

Czerpak, R., Dobrzyn, P., Krotke, A., and Kicinska, E. (2002). The effect of auxins and salicylic acid on chlorophyll and carotenoid contents in *Wolffia arrhiza* (L.) Wimm. (Lemnaceae) growing on media of various trophicities. *Pol. J. Environ. Stud.* 11, 231–235.

Czerpak, R., Krotke, A., and Mical, A. (1999). Comparison of stimulatory effect of auxins and cytokinins on protein, saccharides and chlorophylls content in *Chlorella pyrenoidosa* Chick. *Pol. Arch. Hydrobiol.* 46, 71–82.

Czerpak, R., Piotrowska, A., Dobrogowska, R., Matejczyk, M., and Wieslawski, W. (2003). Biochemical activity of di-and polyamines in the green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae). *Acta soc. bot. Pol.* 72, 19–24. doi: 10.5586/asbp.2003.003

Franz, A., Danielewicz, M., Wong, D., Anderson, L., and Boothe, J. (2013). Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity. *ACS Chem. Biol.* 8, 1053–1062. doi: 10.1021/cb300573r

Gao, Z., Meng, C., Gao, H., Li, Y., Zhang, X., Xu, D., et al. (2013a). Carotenoid genes transcriptional regulation for astaxanthin accumulation in fresh water unicellular alga *Haematococcus pluvialis* by gibberellin A3 (GA3). *Indian J. Biochem. Biophys.* 50, 548–553.

Gao, Z., Meng, C., Gao, H., Zhang, X., Xu, D., Su, Y., et al. (2013b). Analysis of mRNA expression profiles of carotenogenesis and astaxanthin production of *Haematococcus* pluvialis under exogenous 2, 4-epibrassinolide (EBR). *Biol. Res.* 46, 201–206. doi: 10.4067/S0716-97602013000200012

Gao, Z., Meng, C., Zhang, X., Xu, D., Miao, X., Wang, Y., et al. (2012a). Induction of salicylic acid (SA) on transcriptional expression of eight carotenoid genes and astaxanthin accumulation in *Haematococcus pluvialis*. *Enzyme Microb. Technol.* 51, 225–230. doi: 10.1016/j.enzmictec.2012.07.001

Gao, Z., Meng, C., Zhang, X., Xu, D., Zhao, Y., Wang, Y., et al. (2012b). Differential expression of carotenogenic genes, associated changes on astaxanthin production and photosynthesis features induced by JA in *H. pluvialis*. *PLoS ONE* 7:e42243. doi: 10.1371/journal.pone.0042243

Grossmann, K. (2000). Mode of action of auxin herbicides: a new ending to a long, drawn out story. *Trends Plant Sci.* 5, 506–508. doi: 10.1016/S1360-1385(00)01791-x

Grünewald, K., Eckert, M., Hirschberg, J., and Hagen, C. (2000). Phytoene desaturase is localized exclusively in the chloroplast and up-regulated at the mRNA level during accumulation of secondary carotenoids in *Haematococcus pluvialis* (Volvocales, Chlorophyceae). *Plant Physiol.* 122, 1261–1268. doi: 10.1104/pp.122.4.1261

Hobbie, L., Timpte, C., and Estelle, M. (1994). "Molecular genetics of auxin and cytokinin," in *Signals and Signal Transduction Pathways in Plants*, ed. K. Palme (Amsterdam: Springer), 263–283. doi: 10.1007/978-94-011-0239-1_15

Hong, Y., Hu, H., and Li, F. (2008). Growth and physiological responses of freshwater green alga *Selenastrum capricornutum* to allelochemical ethyl 2-methyl acetoacetate (EMA) under different initial algal densities. *Pestic. Biochem. Physiol.* 90, 203–121. doi: 10.1016/j.pestbp.2007.11.009

Hourmant, A., Mereau, N., Penot, M., Cann, C., and Caroff, J. (1994). Influence of polyamines on growth and metabolism of *Dunaliella primolecta*. *Acta Bot. Neerlandica.* 43, 129–136. doi: 10.1111/j.1438-8677.1994.tb00740.x

Huang, J., Chen, F., and Sandmann, G. (2006). Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. *J. Biotechnol.* 122, 176–185. doi: 10.1016/j.jbiotec.2005.09.002

Hunt, R., Chinnasamy, S., Bhatnagar, A., and Das, K. (2010). Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*. *Appl. Biochem. Biotechnol.* 162, 2400–2414. doi: 10.1007/s12010-010-9012–9012

Ip, P., and Chen, F. (2005). Employment of reactive oxygen species to enhance astaxanthin formation in *Chlorella zofingiensis* in heterotrophic culture. *Process Biochem.* 40, 3491–3496. doi: 10.1016/j.procbio.2005.02.014

Karasyova, T., Klose, E., Mensel, R., and Steinberg, C. (2007). Natural organic matter differently modulates growth of two closely related coccal green algal species. *Environ. Sci. Pollut. Res.* 14, 88–93. doi: 10.1065/espr2006.06.317

Kilian, O., Benemann, C., Niyogi, K., and Vick, B. (2011). High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proc. Natl. Acad. Sci. U.S.A.* 108, 21265–21269. doi: 10.1073/pnas.1105861108

Kim, S., Kim, H., Ko, D., Yamaoka, Y., Otsuru, M., Kawai-Yamada, M., et al. (2013). Rapid induction of lipid droplets in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* by Brefeldin A. *PLoS ONE* 8:e81978. doi: 10.1371/journal.pone.0081978

Kobayashi, M., Hirai, N., Kurimura, Y., Ohigashi, H., and Tsuji, Y. (1997). Abscisic acid-dependent algal morphogenesis in the unicellular green alga Haematococcus pluvialis. *Plant Growth Regul.* 22, 79–85. doi: 10.1023/A:1005862809711

Kobayashi, M., Kakizono, T., and Nagai, S. (1993). Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. *Appl. Environ. Microbiol.* 59, 867–873.

Kobayashi, M., Todoroki, Y., Hirai, N., Kurimura, Y., Ohigashi, H., and Tsuji, Y. (1998). Biological activities of abscisic acid analogs in the morphological change of the green alga *Haematococcus pluvialis*. *J. Ferment. Bioeng.* 85, 529–531. doi: 10.1016/S0922-338X(98)80076-7

Kotzabasis, K., Hatziathanasiou, A., Bengoa-Ruigomez, M., Kentouri, M., and Divanach, P. (1999). Methanol as alternative carbon source for quicker efficient production of the microalgae *Chlorella minutissima*: role of the concentration and frequence of administration. *J. Biotechnol.* 70, 357–362. doi: 10.1016/S0079-6352(99)80128-3

Leu, S., and Boussiba, S. (2014). Advances in the production of high-value products by microalgae. *Ind. Biotechnol.* 10, 169–183. doi: 10.1089/ind.2013.0039

Li, Y., Sommerfeld, M., Chen, F., and Hu, Q. (2008). Consumption of oxygen by astaxanthin biosynthesis: a protective mechanism against oxidative stress in *Haematococcus pluvialis* (Chlorophyceae). *J. Plant Physiol.* 165, 1783–1797. doi: 10.1016/j.jplph.2007.12.007

Li, Y., Sommerfeld, M., Chen, F., and Hu, Q. (2010). Effect of photon flux densities on regulation of carotenogenesis and cell viability of *Haematococcus pluvialis* (Chlorophyceae). *J. Appl. Phycol.* 22, 253–263. doi: 10.1007/s10811-009-9453-9456

Linden, H. (1999). Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. *Biochim. Biophys. Acta* 1446, 203–212. doi: 10.1016/S0167-4781(99)00088-3

Liu, Z., Wang, G., and Zhou, B. (2008). Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresour. Technol.* 99, 4717–4722. doi: 10.1016/j.biortech.2007.09.073

Lolke, S., Alistair, J., and Colin, R. (2005). "Alternative carbon sources for heterotrophic production of docosahexaenoic acid by the marine alga *Crypthecodinium cohnii*," in *Single Cell Oils*, eds C. Ratledge and Z. Cohen (Champaign, IL: AOCS Publishing), 131–149. doi: 10.1201/9781439822364.ch8

Lotan, T., and Hirschberg, J. (1995). Cloning and expression in *Escherichia coli* of the gene encoding β-C-4-oxygenase, that converts β-carotene to the keto-carotenoid canthaxanthin in *Haematococcus pluvialis*. *FEBS Lett.* 364, 125–128. doi: 10.1016/0014-5793(95)00368-J

Lu, Y., Jiang, P., Liu, S., Gan, Q., Cui, H., and Qin, S. (2010). Methyl jasmonate- or gibberellins A3-induced astaxanthin accumulation is associated with up-regulation of transcription of beta-carotene ketolase genes (bkts) in microalga *Haematococcus pluvialis*. *Bioresour. Technol.* 101, 6468–6474. doi: 10.1016/j.biortech.2010.03.072

Lustigman, B., McCormick, J., Dale, G., and McLaughlin, J. (1987). Effect of increasing copper and salinity on glycerol production by *Dunaliella salina*. *Bull. Environ. Contam. Toxicol.* 38, 359–362. doi: 10.1007/bf01606687

Ma, R., and Chen, F. (2001a). Induction of astaxanthin formation by reactive oxygen species in mixotrophic culture of *Chlorococcum* sp. *Biotechnol. Lett.* 23, 519–523. doi: 10.1023/A:1010370401235

Ma, R., and Chen, F. (2001b). Enhanced production of free trans-astaxanthin by oxidative stress in the cultures of the green microalga *Chlorococcum* sp. *Process Biochem.* 36, 1175–1179. doi: 10.1016/S0032-9592(01)00157-1

Mojaat, M., Pruvost, J., Foucault, A., and Legrand, J. (2008). Effect of organic carbon sources and Fe2+ ions on growth and β-carotene accumulation by *Dunaliella salina*. *Biochem. Eng. J.* 39, 177–184. doi: 10.1016/j.bej.2007.09.009

Navakoudis, E., Ioannidis, N., Dörnemann, D., and Kotzabasis, K. (2007). Changes in the LHCII-mediated energy utilization and dissipation adjust the methanol-induced biomass increase. *Biochim. Biophys. Acta* 1767, 948–955. doi: 10.1016/j.bbabio.2007.05.003

Norsker, N., Barbosa, M., Vermue, M., and Wijffels, R. (2011). Microalgal production-a close look at the economics. *Biotechnol. Adv.* 29, 24–27. doi: 10.1016/j.biotechadv.2010.08.005

Park, W., Yoo, G., Moon, M., Kim, C., Choi, Y., and Yang, J. (2013). Phytohormone supplementation significantly increases growth of *Chlamydomonas reinhardtii* cultivated for biodiesel production. *Appl. Biochem. Biotechnol.* 171, 1128–1142. doi: 10.1007/s12010-013-0386–389

Patel, V., Maji, D., Singh, A., Suseela, M., Sundaram, S., and Kalra, A. (2013). A natural plant growth promoter, calliterpenone, enhances growth and biomass, carbohydrate, and lipid production in cyanobacterium *Synechocystis* PCC 6803. *J. Appl. Phycol.* 26, 279–286. doi: 10.1007/s10811-013-0103–107

Piotrowska, A., and Czerpak, R. (2009). Cellular response of light/dark-grown green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae) to exogenous adenine- and phenylurea-type cytokinins. *Acta physiol. plant.* 31, 573–585. doi: 10.1007/s11738-008-0267-y

Piotrowska, A., Czerpak, R., Pietryczuk, A., Olesiewicz, A., and Wêdoowska, M. (2008). The effect of indomethacin on the growth and metabolism of green alga *Chlorella vulgaris* Beijerinck. *Plant Growth Regul.* 55, 125–136. doi: 10.1007/s10725-008-9267–9266

Raman, V., and Ravi, S. (2010). Effect of salicylic acid and methyl jasmonate on antioxidant systems of *Haematococcus pluvialis*. *Acta Physiol. Plant.* 33, 1043–1049. doi: 10.1007/s11738-010-0623–626

Rayle, D., and Cleland, R. (1992). The Acid Growth Theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* 99, 1271–1274. doi: 10.1104/pp.99.4.1271

Razon, L., and Tan, R. (2011). Net energy analysis of the production of biodiesel and biogas from the microalgae: *Haematococcus pluvialis* and *Nannochloropsis*. *Appl. Energy* 88, 3507–3514. doi: 10.1016/j.apenergy.2010.12.052

Ren, L., Huang, H., Xiao, A., Lian, M., Jin, L., and Ji, X. (2009). Enhanced docosahexaenoic acid production by reinforcing acetyl-CoA and NADPH supply in *Schizochytrium* sp. HX-308. *Bioprocess Biosyst. Eng.* 32, 837–843. doi: 10.1007/s00449-009-0310–314

Rioboo, C., González, Ó., Abalde, J., and Cid, Á. (2011). Flow cytometric analysis of the encystment process induced by paraquat exposure in *Haematococcus pluvialis* (Chlorophyceae). *Eur. J. Phycol.* 46, 89–97. doi: 10.1080/09670262.2011.561775

Sarada, R., Tripathi, U., and Ravishankar, G. (2002). Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. *Process Biochem.* 37, 623–627. doi: 10.1016/S0032-9592(01)00246-1

Saygideger, S., and Deniz, F. (2008). Effect of 24-epibrassinolide on biomass, growth and free proline concentration in *Spirulina platensis* (Cyanophyta) under NaCl stress. *Plant Growth Regul.* 56, 219–223. doi: 10.1007/s10725-008-9310–9317

Saygideger, S., and Okkay, O. (2008). Effect of 2,4-dichlorophenoxyacetic acid on growth, protein and chlorophyll-a content of *Chlorella vulgaris* and *Spirulina platensis* cells. *J. Environ. Biol.* 29, 175–178.

Sheehan, J., Dunahay, T., Benemann, J., and Roessler, P. (1998). *Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae*. Golden: National Renewable Energy Laboratory.

Skjanes, K., Rebours, C., and Lindblad, P. (2013). Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. *Crit. Rev. Biotechnol.* 33, 172–215. doi: 10.3109/07388551.2012.681625

Soratana, K., and Landis, A. (2011). Evaluating industrial symbiosis and algae cultivation from a life cycle perspective. *Bioresour. Technol.* 102, 6892–6901. doi: 10.1016/j.biortech.2011.04.018

Steinbrenner, J., and Linden, H. (2001). Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-Induced astaxanthinformation in the green alga *Haematococcus pluvialis*. *Plant Physiol.* 125, 810–817. doi: 10.1104/pp.125.2.810

Steinbrenner, J., and Linden, H. (2003). Light induction of carotenoid biosynthesis genes in the green alga *Haematococcus pluvialis*: regulation by photosynthetic redox control. *Plant Mol. Biol.* 52, 343–356. doi: 10.1023/a:1023948929665

Su, Y., Wang, J., Shi, M., Niu, X., Yu, X., Gao, L., et al. (2014). Metabolomic and network analysis of astaxanthin-producing *Haematococcus pluvialis* under various stress conditions. *Bioresour. Technol.* 170, 522–529. doi: 10.1016/j.biortech.2014.08.018.

Suen, Y., Hubbard, J., Holzer, G., and Tornabene, T. (1987). Total lipid production of the green alga *Nannochloropsis* sp. QII under different nitrogen regimes. *J. Phycol.* 23, 289–296. doi: 10.1111/j.1529-8817.1987.tb04137.x

Sun, Z., Cunningham, F., and Gantt, E. (1998). Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte. *Proc. Natl. Acad. Sci. U.S.A.* 95, 11482–11488. doi: 10.1073/pnas.95.19.11482

Sunohara, Y., and Matsumoto, H. (1997). Comparative physiological effects of quinclorac and auxins, and light involvement in quinclorac-induced chlorosis in corn leaves. *Pestic. Biochem. Physiol.* 58, 125–132. doi: 10.1006/pest.1997.2289

Theodoridou, A., Dörnemann, D., and Kotzabasis, K. (2002). Light-dependent induction of strongly increased microalgal growth by methanol. *Biochim. Biophys. Acta* 1573, 189–198. doi: 10.1016/S0304-4165(02)00438-5

Tripathi, U., Sarada, R., Rao, S., and Ravishankar, G. (1999). Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. *Bioresour. Technol.* 68, 197–199. doi: 10.1016/S0960-8524(98)00143-6

Vance, B. (1987). Phytohormone effects on cell division in *Chlorella pyrenoidosa* chick (TX-7-11-05) (chlorellaceae). *J. Plant Growth Regul.* 5, 169–173. doi: 10.1007/bf02087185

Vanthoor-Koopmans, M., Wijffels, R., Barbosa, M., and Eppink, M. (2013). Biorefinery of microalgae for food and fuel. *Bioresour. Technol.* 135, 142–149. doi: 10.1016/j.biortech.2012.10.135

Vidhyavathi, R., Venkatachalam, L., Sarada, R., and Ravishankar, G. (2008). Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. *J. Exp. Bot.* 59, 1409–1418. doi: 10.1093/jxb/ern048

Wang, J., Sommerfeld, M., and Hu, Q. (2009). Occurrence and environmental stress responses of two plastid terminal oxidases in *Haematococcus pluvialis* (Chlorophyceae). *Planta* 230, 191–203. doi: 10.1007/s00425-009-0932–934

Wijffels, R., Barbosa, M., and Eppink, M. (2010). Microalgae for the production of bulk chemicals and biofuels. *Biofuels Bioprod. Biorefin.* 4, 287–295. doi: 10.1002/bbb.215

Wong, P. (2000). Effects of 2, 4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauda* Berb 614. *Chemosphere* 41, 177–182. doi: 10.1016/S0045-6535(99)00408-7

Yen, H., Hu, I., Chen, C., Ho, S., Lee, D., and Chang, J. (2013). Microalgae-based biorefinery-from biofuels to natural products. *Bioresour. Technol.* 135, 166–174. doi: 10.1016/j.biortech.2012.10.099

Zhang, W., Li, F., and Nie, L. (2010). Integrating multiple 'omics' analysis for microbial biology: application and methodologies. *Microbiology* 156, 287–301. doi: 10.1099/mic.0.034793–34790

Zhu, X., Kong, H., Gao, Y., Wu, M., and Kong, F. (2012). Low concentrations of polycyclic aromatic hydrocarbons promote the growth of *Microcystis aeruginosa*. *J. Hazard. Mater.* 237–238, 371–375. doi: 10.1016/j.jhazmat.2012.08.029

Conflict of Interest Statement: 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.

Received: 14 November 2014; accepted: 16 January 2015; published online: 17 February 2015.

Citation: Yu X, Chen L and Zhang W (2015) Chemicals to enhance microalgal growth and accumulation of high-value bioproducts. Front. Microbiol. ***6****:56. doi: 10.3389/fmicb.2015.00056*

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

frontiers in Microbiology

PERSPECTIVE
published: 19 June 2015
doi: 10.3389/fmicb.2015.00629

Is lactate an undervalued functional component of fermented food products?

*Graciela L. Garrote[1], Analía G. Abraham[1,2] and Martín Rumbo[3]**

[1] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA - CONICET), Universidad Nacional de La Plata, La Plata, Argentina, [2] Área Bioquímica y Control de Alimentos, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina, [3] Instituto de Estudios Inmunológicos y Fisopatológicos (IIFP - CONICET), Universidad Nacional de La Plata, La Plata, Argentina

Although it has been traditionally regarded as an intermediate of carbon metabolism and major component of fermented dairy products contributing to organoleptic and antimicrobial properties of food, there is evidence gathered in recent years that lactate has bioactive properties that may be responsible of broader properties of functional foods. Lactate can regulate critical functions of several key players of the immune system such as macrophages and dendritic cells, being able to modulate inflammatory activation of epithelial cells as well. Intraluminal levels of lactate derived from fermentative metabolism of lactobacilli have been shown to modulate inflammatory environment in intestinal mucosa. The molecular mechanisms responsible to these functions, including histone deacetylase dependent-modulation of gene expression and signaling through G-protein coupled receptors have started to be described. Since lactate is a major fermentation product of several bacterial families with probiotic properties, we here propose that it may contribute to some of the properties attributed to these microorganisms and in a larger view, to the properties of food products fermented by lactic acid bacteria.

Keywords: fermentation, lactate, functional food, probiotics, bioactive properties

OPEN ACCESS

Edited by:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Giorgio Giraffa, Consiglio per la Ricerca e la Sperimentazione in Agricoltura – Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie, Italy
Carmen Wacher, Universidad Nacional Autónoma de México, Mexico

****Correspondence:***
Martín Rumbo, Instituto de Estudios Inmunológicos y Fisopatológicos (IIFP - CONICET), Universidad Nacional de La Plata, Calle 47 y 115, 1900 La Plata, Buenos Aires, Argentina
martinr@biol.unlp.edu.ar

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

Received: *30 April 2015*
Accepted: *09 June 2015*
Published: *19 June 2015*

Citation:
Garrote GL, Abraham AG and Rumbo M (2015) Is lactate an undervalued functional component of fermented food products? Front. Microbiol. 6:629. doi: 10.3389/fmicb.2015.00629

Lactate is a Major Component of Lactic-Acid Bacteria Fermented Foods

For about 40 centuries, without understanding the scientific basis, people have been using lactic acid bacteria to produce fermented products that were originally developed as a way to preserve food from microbial or physicochemical modification that may alter its sensory or nutritional value. By fermentation, it has been possible to develop a wide variety of products of different taste, texture, and function. Lactic acid bacteria are traditionally used in obtaining dairy products from all over the world, including yogurt, cheese, butter, buttermilk, kefir, and koumiss, among others. Lactic acid bacteria refer to a large group of bacteria that share genetic traits and produce lactic acid as main end product of fermentation. They are widespread in nature and are also found in the gastrointestinal tract.

Fermenting milk with lactic acid bacteria provide a final product that contains lactic acid as a hallmark among other metabolites that may contribute to product characteristics. Although they are best known for their role in the preparation of fermented dairy products, lactic acid bacteria are also used in non-dairy food processing such as pickling of vegetables, curing fish, meats, and

sausages as well as in traditional fermented products around the world such as pozole, pulque, chicha, gari, kimchi, among others (Nout, 2009). Furthermore, LAB is also a major contributor to fermentation process that takes place in ensilage (Jay et al., 2005). In the process of yogurt production, around 20% of lactose present in milk is transformed into lactic acid, and the content of lactic acid in yogurt is around 0.9% (Cheng, 2010). Other fermented milk product as kefir may reach to 2% of lactate (Garrote et al., 2010; Londero et al., 2012).

Lactic-Acid Bacteria-Fermented Products have Beneficial Health Properties

The popular belief that fermented products have beneficial health effects is probably very old, but only in the past decades these ideas have begun to find a scientific support. During the last 20 years, a major expansion of food with health-promoting properties has taken place leading to the so-called "functional foods." This type of food, consumed as part of normal daily diet, contains bioactive ingredients that offer health benefits.

Within functional foods, probiotics have acquired an important role, showing capacity to regulate metabolism and immunity of the consumer resulting in improvement of the quality of life (Tojo Sierra et al., 2003). Probiotics according to the World Health Organization (FAO/WHO Report, 2002) are live microorganisms which when administered in adequate doses confer beneficial effects on host health.

There have been proposed many mechanisms by which probiotics may contribute to consumer health, although for several of them the cellular and molecular bases are not completely elucidated. Probiotics may produce agents that suppress the growth of other microorganisms such as organic acid (Garrote et al., 2000) or other inhibitory compounds (Holzapfel et al., 1995; Beshkova and Frengova, 2012); furthermore, they can compete for receptors and binding sites with other intestinal microbes on the intestinal mucosa exerting a protective affect against pathogen infection (Golowczyc et al., 2007; Kakisu et al., 2013). Probiotics can modulate the intestinal immunity and alter the responsiveness of the intestinal epithelia and immune cells to microbes in the intestinal lumen (Thomas and Versalovic, 2010). In this regard, numerous studies have shown that lactic acid bacteria in fermented milk improve different parameters of immune function (Matar et al., 2001; Isolauri et al., 2004; Tsai et al., 2012).

The consumption of fermented food and/or probiotics also modifies the intestinal microbiota which plays an important role in the function and integrity of the gastrointestinal tract, maintenance of immune homeostasis and host energy metabolism (Hemarajata and Versalovic, 2013; Flint et al., 2015). Microbes in the gastrointestinal tract can exert numerous effects on different cells of the mucosal immune system and, in turn, induce the production of cytokines, which prime the innate immune response (O'Flaherty et al., 2010). Recent studies revealed that microbiota, including their metabolites, modulate key signaling pathways involved in the inflammation of the mucosa. The underlying molecular mechanisms of host–microbiota interactions are still not fully elucidated; however, manipulation of microbiota by probiotics or prebiotics is becoming increasingly recognized as an important therapeutic option, especially for the treatment of the dysfunction or inflammation of the intestinal tract (Kanauchi et al., 2013). The metabolic output of the modification of gut microbiota is the production of different profile of short chain fatty acids (SCFA) such as butyrate, propionate, and acetate. It has been reported that SCFA show anti-inflammatory properties (Maslowski and Mackay, 2011). Furthermore, it has been shown that metabolites present in the supernatants of fermented dairy products can exert a protective effect *ex vivo* on intestinal mucosa exposed to inflammatory insults (Tsilingiri et al., 2012). Based in these results, Rescigno and coworkers has recently proposed the concept of postbiotics, meaning metabolites produced upon microbial fermentation that may have bioactive capacity and that could be useful for modulation of host response in cases of inflammatory diseases (Tsilingiri and Rescigno, 2013).

Many factors can be involved in the health promoting properties of a fermented food, such as the presence of probiotic microorganisms themselves, the metabolites produced during fermentation, products coming from the hydrolysis of the components of the food matrix, or changes in the microbiota induced by any of these factors. Taking into account that lactate is the main metabolite of many fermented products, it is conceivable to ask if lactate plays a role in the health promoting properties of fermented food.

Lactate has Bioactive Capacities Acting through Different Mechanisms

Lactate has been considered as a mere carbon metabolite with specific organoleptic/antimicrobial properties; however, different bioactive capacities of lactate have been recently shown (**Figure 1**). The lactic acid produced by the probiotic lactobacilli has been shown to be critical in modulating inflammation in a model of small intestine injury caused by indomethacin (Watanabe et al., 2009). We have recently shown that lactate abrogates TLR and IL1b dependent activation of intestinal epithelial cells (Iraporda et al., 2014). Moreover, besides immunomodulation, Okada et al. (2013) showed that luminal lactate stimulated enterocyte proliferation in a murine model of hunger-feedback, contributing to maintain intestinal barrier function. Beyond intestinal epithelial cells, lactate could have bioactive effects on myeloid cells. Lactate in the 10–20 mM range has been shown to modulate LPS-dependent monocyte activation (Dietl et al., 2010), whereas this activity is enhanced at pH 6.6 (Peter et al., 2015). In this case inhibition of NF-κB activation was also evidenced. Watanabe et al. (2009) also showed that lactate can modulate NF-κB signaling in myeloid cells. Furthermore, modulation of DC activation by lactate has also been described (Gottfried et al., 2006; Nasi et al., 2010; Nasi and Rethi, 2013; Iraporda et al., 2015).

Although there is evidence that lactate modulates key functions of main players of innate response such as myeloid and epithelial cells, the mechanisms responsible for these activities are still not yet fully elucidated but several options are possible (**Figure 1**). In recent years, several G protein-coupled receptors (GPCRs) have been characterized as sensors of small molecules such as fatty acids, sugars, or endogenous intermediate metabolites from microbial or food sources, having a profound impact on various biological processes (Blad et al., 2012). Among these receptors, GPR81 (or HCA1 or HCAR1) is specific for lactate (Offermanns, 2013), constituting an interesting candidate to mediate lactate bioactive effects. GPR81 is expressed primarily in adipocytes and have an antilipolytic effect (Liu et al., 2009). However, it has been shown that this receptor is also expressed in intestinal tissue (Iraporda et al., 2014) and it mediates macrophage dependent anti-inflammatory effects in mouse models of hepatitis and pancreatitis (Hoque et al., 2014). GPR81 dependent anti-inflammatory effects of lactate on macrophages are independent on G_i proteins and dependent on β-arrestin2 mediated signaling (Hoque et al., 2014; Liu et al., 2014). It still has to be confirmed if GPR81 may contribute to lactate bioactive properties observed in intestinal models of inflammation.

Beyond the signaling capacity through GPR81, lactate can also modulate histone deacetylase activity, showing specific patterns of gene expression regulation (Latham et al., 2012). Several modulatory effects on macrophages and epithelial cells were also associated with histone deacetylase capacity (Latham et al., 2012; Schilderink et al., 2013; Chang et al., 2014). Furthermore, high concentrations of lactate in extracellular milieu have also effects on modulation of cell metabolism, specially affecting glycolysis rate, which has been correlated with modulation of the production of proinflammatory mediators, such as TNFα by macrophages (Dietl et al., 2010). It has been recently shown that MCT4, a lactate membrane transporter, is induced in macrophages upon TLR activation and is critical for the management of lactate produced upon cell activation (Tan et al., 2015). Blocking the capacity of the macrophages to export lactate (Tan et al., 2015) or high concentrations of lactate in extracellular milieu (Dietl et al., 2010) have similar effects on cell metabolism that can contribute to modulation of proinflammatory mediator production. Furthermore, at systemic level lactate has other important regulatory actions on energy metabolism (Sola-Penna, 2008) that could also be triggered by enhanced intestinal lactate absorption. Changes in systemic levels of SCFA were reported by enhanced intestinal production and absorption (Macia et al., 2015), and this also could be the case for lactate.

Considering Probiotic Properties from a Different Perspective

Taking into consideration the different bioactive properties of lactate mentioned above, a novel framework to interpret evidence on probiotic activity could be considered. So far, viability has been a major characteristic that has been the quintessence of probiotic action. Metabolic capacity, including fermentation is always dependent on microbial viability. As shown by Watanabe et al. (2009) and Flint et al. (2015), lactic acid production *in situ* is a key aspect for intestinal inflammation modulation by lactobacilli. Capacity to generate terminal fermentation metabolites has been shown to be critical for *in vivo* action of other microbial species with probiotic properties such as *Bifidobacterium* (Fukuda et al., 2011). Furthermore, adhesion to the intestinal mucosa is a desirable property for probiotic microorganisms and has been related to many of their health benefits (Servin and Coconnier, 2003). Autoaggregation and surface hydrophobicity of probiotic strains have been also correlated to adhesive capacity and have been also considered as positive traits in potentially probiotic

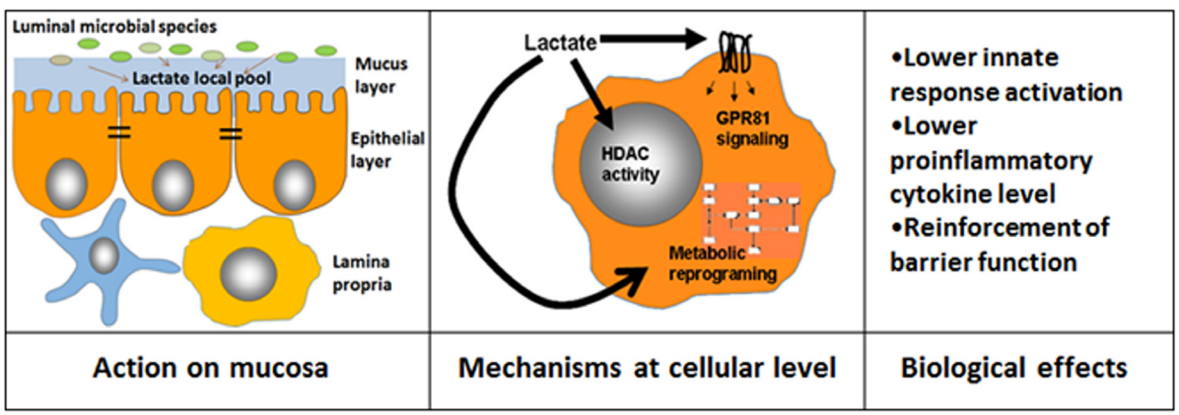


FIGURE 1 | Different mechanisms that mediate lactate bioactive effects. Lactate luminal intestinal levels are contributed by lactate present in ingested food and also by that produced by intestinal microorganisms. The local lactate pool in the mucosal cellular environment is contributed by microbial species able to adhere to mucus/cell surface and may target epithelial cells as well as immune cells present in the lamina propria **(Left)**. Lactate may influence cellular activities by at least three independent ways: (i) by modulating gene expression through modification of histone deacetylase activity (HDAC), (ii) by triggering different signaling pathways by GPR81, (iii) by inducing changes in metabolic pathways such as reducing glycolysis rate **(Center)**. As a consequence of these cellular processes, different functional effects are achieved **(Right)**.

strain selection studies (Kos et al., 2003; Collado et al., 2008; Botta et al., 2014; Papadimitriou et al., 2015). While it has been shown that adhesion to mucus/epithelial surface can be beneficial by blocking adhesion sites for potentially pathogens (Candela et al., 2008), the mechanistic basis of the immunomodulatory capacity associated to adhesion has been elusive. Without excluding other possible mechanisms that mediate this action, it is reasonable to assume that adhesion to epithelium increases the exposition of epithelial cells and intraepithelial leukocytes to bacterial fermentation products. Consequently, although luminal concentration of lactate may be high enough -at least in colon- to exert a modulatory activity on mucosa, the presence of adhesive bacteria with high metabolic capacity to produce lactate may even increase local concentration of this metabolite and consequently enhance immunomodulation, even with modest changes in luminal lactate concentration (Watanabe et al., 2009; Flint et al., 2015). Taking this into consideration, the evaluation of the capacity to use lactic acid fermentative pathway with high rate may be also considered as a desirable feature for probiotic candidate selection.

Beyond the capacity of probiotics to conduce lactic fermentation *in situ*, the health promoting effects of consumption of food containing relatively high amount of lactate such as yogurt, may be of considerable interest. In this case, the proximal mucosal sites, such as stomach will be exposed to highest concentration of lactate. In this case, the acid pH may also potentiate lactic acid modulatory effects (Dietl et al., 2010; Peter et al., 2015). Benefits of consumption of yogurt in different gastric inflammatory models have been reported, without establishing the mechanisms beyond this action (Uchida and Kurakazu, 2004; Uchida et al., 2010). On the other hand, lactate-rich food consumption may also impact in the microbiota composition independently of incorporation of viable bacteria, as has been shown by Garcia-Albiach et al. (2008) who compared gene structure of intestinal microbiota in groups receiving yogurt or heat-treated yogurt, finding comparable effects in both groups. Although in these cases many components of ingested food may contribute to microbiota shift, the presence of lactate, which is also an energy source for several intestinal microbial populations would possibly play a role.

So far, lactate bioactive properties have been disregarded. In the light of the accumulated evidence on lactate bioactivity and its mechanistic basis, it may be reasonable to reconsider the attribution of different properties of functional foods to different food components. Lactate as food component itself or as a bioactive metabolite generated *in situ* on the intestinal mucosa may contribute to health promoting-properties and should be valorised.

Acknowledgments

GG, AA, and MR are members of National Research Council (CONICET). This work is supported by grants from CONICET and National Agency for Promotion of Science and Technology (ANPCYT).

References

Beshkova, D., and Frengova, G. (2012). Bacteriocins from lactic acid bacteria: microorganisms of potential biotechnological importance for the dairy industry. *Eng. Life Sci.* 12, 419–432. doi: 10.1002/elsc.201100127

Blad, C. C., Tang, C., and Offermanns, S. (2012). G protein-coupled receptors for energy metabolites as new therapeutic targets. *Nat. Rev. Drug Discov.* 11, 603–619. doi: 10.1038/nrd3777

Botta, C., Langerholc, T., Cencic, A., and Cocolin, L. (2014). In vitro selection and characterization of new probiotic candidates from table olive microbiota. *PLoS ONE* 9:e94457. doi: 10.1371/journal.pone.0094457

Candela, M., Perna, F., Carnevali, P., Vitali, B., Ciati, R., Gionchetti, P., et al. (2008). Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. *Int. J. Food Microbiol.* 125, 286–292. doi: 10.1016/j.ijfoodmicro.2008.04.012

Chang, P. V., Hao, L., Offermanns, S., and Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2247–2252. doi: 10.1073/pnas.1322269111

Cheng, H. (2010). Volatile flavor compounds in yogurt: a review. *Crit. Rev. Food Sci. Nutr* 50, 938–950. doi: 10.1080/10408390903044081

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

Dietl, K., Renner, K., Dettmer, K., Timischl, B., Eberhart, K., Dorn, C., et al. (2010). Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes. *J. Immunol.* 184, 1200–1209. doi: 10.4049/jimmunol.0902584

FAO/WHO Report. (2002). *Guidelines for the Evaluation of Probiotics in Food*. Geneva: FAO/WHO.

Flint, H. J., Duncan, S. H., Scott, K. P., and Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. *Proc. Nutr. Soc.* 74, 13–22. doi: 10.1017/S0029665114001463

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469, 543–547. doi: 10.1038/nature09646

Garcia-Albiach, R., Pozuelo de Felipe, M. J., Angulo, S., Morosini, M. I., Bravo, D., Baquero, F., et al. (2008). Molecular analysis of yogurt containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in human intestinal microbiota. *Am. J. Clin. Nutr.* 87, 91–96.

Garrote, G. L., Abraham, A. G., and De Antoni, G. L. (2000). Inhibitory power of kefir: the role of organic acids. *J. Food Prot.* 63, 364–369.

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: Novel Applications*, Chap. 18, eds F. Mozzi, R. R. Raya, and G. M. Vignolo (Ames, IO: Wiley-Blackwell), 327–340. doi: 10.1002/9780813820866.ch18

Golowczyc, M. A., Mobili, P., Garrote, G. L., Abraham, A. G., and De Antoni, G. L. (2007). Protective action of *Lactobacillus* kefir carrying S-layer protein against *Salmonella enterica* serovar Enteritidis. *Int. J. Food Microbiol.* 118, 264–273. doi: 10.1016/j.ijfoodmicro.2007.07.042

Gottfried, E., Kunz-Schughart, L. A., Ebner, S., Mueller-Klieser, W., Hoves, S., Andreesen, R., et al. (2006). Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* 107, 2013–2021. doi: 10.1182/blood-2005-05-1795

Hemarajata, P., and Versalovic, J. (2013). Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therap. Adv. Gastroenterol.* 6, 39–51. doi: 10.1177/1756283X12459294

Holzapfel, W. H., Geisen, R., and Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int. J. Food Microbiol.* 24, 343–362. doi: 10.1016/0168-1605(94)00036-6

Hoque, R., Farooq, A., Ghani, A., Gorelick, F., and Mehal, W. Z. (2014). Lactate reduces liver and pancreatic injury in Toll-like receptor- and inflammasome-mediated inflammation via GPR81-mediated suppression of innate immunity. *Gastroenterology* 146, 1763–1774. doi: 10.1053/j.gastro.2014.03.014

Iraporda, C., Errea, A., Romanin, D. E., Cayet, D., Pereyra, E., Pignataro, O., et al. (2015). Lactate and short chain fatty acids produced by microbial fermentation downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells. *Immunobiology* (in press). doi: 10.1016/j.imbio.2015.06.004

Iraporda, C., Romanin, D. E., Rumbo, M., Garrote, G., and Abraham, A. (2014). The role of lactate in the immunomodulatory properties of kefir non bacterial fraction. *Food Res. Int.* 62, 247–253. doi: 10.1016/j.foodres.2014.03.003

Isolauri, E., Salminen, S., and Ouwehand, A. C. (2004). Microbial-gut interactions in health and disease. Probiotics. *Best Pract. Res. Clin. Gastroenterol.* 18, 299–313. doi: 10.1016/j.bpg.2003.10.006

Jay, J. M., Loessner, M. J., and Golden, D. A. (2005). Nondairy fermented foods and products in modern food microbiology. *Food Sci. Text Series Springer* 175–195. doi: 10.1007/0-387-234133-6_8

Kakisu, E., Abraham, A. G., Farinati, C. T., Ibarra, C., and De Antoni, G. L. (2013). *Lactobacillus plantarum* isolated from kefir protects vero cells from cytotoxicity by type-II shiga toxin from *Escherichia coli* O157:H7. *J. Dairy Res.* 80, 64–71. doi: 10.1017/S0022029912000659

Kanauchi, O., Andoh, A., and Mitsuyama, K. (2013). Effects of the modulation of microbiota on the gastrointestinal immune system and bowel function. *J. Agric. Food Chem.* 61, 9977–9983. doi: 10.1021/jf402441f

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J., and Matosic, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* 94, 981–987. doi: 10.1046/j.1365-2672.2003.01915.x

Latham, T., Mackay, L., Sproul, D., Karim, M., Culley, J., Harrison, D. J., et al. (2012). Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. *Nucleic Acids Res.* 40, 4794–4803. doi: 10.1093/nar/gks066

Liu, C., Wu, J., Zhu, J., Kuei, C., Yu, J., Shelton, J., et al. (2009). Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. *J. Biol. Chem.* 284, 2811–2822. doi: 10.1074/jbc.M806409200

Liu, Y., Chen, L. Y., Sokolowska, M., Eberlein, M., Alsaaty, S., Martinez-Anton, A., et al. (2014). The fish oil ingredient, docosahexaenoic acid, activates cytosolic phospholipase A(2) via GPR120 receptor to produce prostaglandin E(2) and plays an anti-inflammatory role in macrophages. *Immunology* 143, 81–95. doi: 10.1111/imm.12296

Londero, A., Hamet, M. F., De Antoni, G. L., Garrote, G. L., and Abraham, A. G. (2012). Kefir grains as a starter for whey fermentation at different temperatures: chemical and microbiological characterisation. *J. Dairy Res.* 79, 262–271. doi: 10.1017/S0022029912000179

Macia, L., Tan, J., Vieira, A. T., Leach, K., Stanley, D., Luong, S., et al. (2015). Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat. Commun.* 6:6734. doi: 10.1038/ncomms7734

Maslowski, K. M., and Mackay, C. R. (2011). Diet, gut microbiota and immune responses. *Nat. Immunol.* 12, 5–9. doi: 10.1038/ni0111-5

Matar, C., Valdez, J. C., Medina, M., Rachid, M., and Perdigon, G. (2001). Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. *J. Dairy Res.* 68, 601–609. doi: 10.1017/S0022029901005143

Nasi, A., Fekete, T., Krishnamurthy, A., Snowden, S., Rajnavolgyi, E., Catrina, A. I., et al. (2010). Dendritic cell reprogramming by endogenously produced lactic acid. *J. Immunol.* 191, 3090–3099. doi: 10.4049/jimmunol.1300772

Nasi, A., and Rethi, B. (2013). Disarmed by density: a glycolytic break for immunostimulatory dendritic cells? *Oncoimmunology* 2:e26744. doi: 10.4161/onci.26744

Nout, M. J. (2009). Rich nutrition from the poorest – cereal fermentations in Africa and Asia. *Food Microbiol.* 26, 685–692. doi: 10.1016/j.fm.2009.07.002

Offermanns, S. (2013). Free fatty acid (FFA) and hydroxy carboxylic acid (HCA) receptors. *Annu. Rev. Pharmacol. Toxicol.* 54, 407–434. doi: 10.1146/annurev-pharmtox-011613-135945

O'Flaherty, S., Saulnier, D. M., Pot, B., Versalovic, J. (2010). How can probiotics and prebiotics impact mucosal immunity? *Gut Microbes* 1, 293–300. doi: 10.4161/gmic.1.5.12924

Okada, T., Fukuda, S., Hase, K., Nishiumi, S., Izumi, Y., Yoshida, M., et al. (2013). Microbiota-derived lactate accelerates colon epithelial cell turnover in starvation-refed mice. *Nat. Commun.* 4:1654. doi: 10.1038/ncomms2668

Papadimitriou, K., Zoumpopoulou, G., Foligne, B., Alexandraki, V., Kazou, M., Pot, B., et al. (2015). Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. *Front. Microbiol.* 6:58. doi: 10.3389/fmicb.2015.00058

Peter, K., Rehli, M., Singer, K., Renner-Sattler, K., and Kreutz, M. (2015). Lactic acid delays the inflammatory response of human monocytes. *Biochem. Biophys. Res. Commun.* 457, 412–418. doi: 10.1016/j.bbrc.2015.01.005

Schilderink, R., Verseijden, C., and de Jonge, W. J. (2013). Dietary inhibitors of histone deacetylases in intestinal immunity and homeostasis. *Front. Immunol.* 4:226. doi: 10.3389/fimmu.2013.00226

Servin, A. L., and Coconnier, M. H. (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Pract. Res. Clin. Gastroenterol.* 17, 741–754. doi: 10.1016/S1521-6918(03)00052-0

Sola-Penna, M. (2008). Metabolic regulation by lactate. *IUBMB Life* 60, 605–608. doi: 10.1002/iub.97

Tan, Z., Xie, N., Banerjee, S., Cui, H., Fu, M., Thannickal, V. J., et al. (2015). The monocarboxylate transporter 4 is required for glycolytic reprogramming and inflammatory response in macrophages. *J. Biol. Chem.* 290, 46–55. doi: 10.1074/jbc.M114.603589

Thomas, C. M., and Versalovic, J. (2010). Probiotics-host communication: modulation of signaling pathways in the intestine. *Gut Microbes* 1, 148–163. doi: 10.4161/gmic.1.3.11712

Tojo Sierra, L., Leis, T. R., and Tojo González, R. (2003). Prebiotics and probiotics in childhood health and disease. *Gastroenterol. Hepatol.* 26(Suppl. 1), 37–49.

Tsai, Y. T., Cheng, P. C., and Pan, T. M. (2012). The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. *Appl. Microbiol. Biotechnol.* 96, 853–862. doi: 10.1007/s00253-012-4407-3

Tsilingiri, K., Barbosa, T., Penna, G., Caprioli, F., Sonzogni, A., Viale, G., et al. (2012). Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model. *Gut* 61, 1007–1015. doi: 10.1136/gutjnl-2011-300971

Tsilingiri, K., and Rescigno, M. (2013). Postbiotics: what else? *Benef. Microbes* 4, 101–107. doi: 10.3920/BM2012.0046

Uchida, M., and Kurakazu, K. (2004). Yogurt containing *Lactobacillus gasseri* OLL2716 exerts gastroprotective action against [correction of agaisnt] acute gastric lesion and antral ulcer in rats. *J. Pharmacol. Sci.* 96, 84–90. doi: 10.1254/jphs.FPJ04027X

Uchida, M., Shimizu, K., and Kurakazu, K. (2010). Yogurt containing *Lactobacillus gasseri* OLL 2716 (LG21 yogurt) accelerated the healing of acetic acid-induced gastric ulcer in rats. *Biosci. Biotechnol. Biochem.* 74, 1891–1894. doi: 10.1271/bbb.100287

Watanabe, T., Nishio, H., Tanigawa, T., Yamagami, H., Okazaki, H., Watanabe, K., et al. (2009). Probiotic *Lactobacillus casei* strain Shirota prevents indomethacin-induced small intestinal injury: involvement of lactic acid. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297, G506–G513. doi: 10.1152/ajpgi.90553.2008

Conflict of Interest Statement: 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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership