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Novel developments for promoting health through microbiota modulation

Edited by
Carlos Gómez-Gallego and Hani El-Nezami

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Novel developments for promoting health through microbiota modulation

Topic editors

Carlos Gómez-Gallego — University of Eastern Finland, Finland

Hani El-Nezami — The University of Hong Kong, SAR China

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Table of contents

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EDITED AND REVIEWED BY
Christophe Lacroix,
ETH Zürich, Switzerland

*CORRESPONDENCE
Carlos Gómez-Gallego
✉ carlos.gomezgallego@uef.fi
Hani El-Nezami
✉ hani.el-nezami@uef.fi

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Editorial: Novel developments for promoting health through microbiota modulation

Carlos Gómez-Gallego[1]* and Hani El-Nezami[1,2]*

[1]Institute of Public Health and Clinical Nutrition, School of Medicine, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland, [2]School of Biological Science, Faculty of Science, The University of Hong Kong, Hong Kong, China

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Editorial on the Research Topic
Novel developments for promoting health through microbiota modulation

The human microbiota is a vast and complex ecosystem of microorganisms that play a pivotal role in health, influencing many important bodily functions, including digestion, immunity, and metabolism (1). Recent evidence has identified associations of differences in microbiota composition with certain conditions and diseases, for example, in late-onset breast milk jaundice (Guo Q. et al.), increasing the interest in microbiota modulation as a potential treatment of certain conditions and diseases. However, findings from microbiota-related studies are ambiguous and sometimes difficult to reproduce, while defining what constitutes a "healthy microbiota" still remains a challenge (2). Interventions to alter microbiota composition have been studied for decades, ranging from dietary changes to fecal transplants. However, continuing to explore novel and more effective interventions could help to combat the rise of non-communicable diseases worldwide.

Our Research Topic contributes to the advancement of the understanding of microbiota-related interventions and their impact on human health. The 15 publications collected explore the therapeutic potential of microbiota modulation using different interventions such as probiotics, synbiotics, dietary supplements and dietary interventions in improving immune system, obesity, type 2 diabetes (T2D) and gastrointestinal disorders. These studies highlight the emerging interest to develop strategies based on microbiota modulation in disease management and, due to the individual variation on human microbiota, underscore the importance of personalized approaches to promote overall health.

Fecal microbiota transplants have been studied since the 1980s with variying level of success. In the case of the irritable bowel syndrome (IBS), a meta-analysis conducted by Zhao et al. revealed that the success of these interventions is highly dependent on several factors such as dosage, frequency, delivery method and preparation method of donor stool, as well as differences in the microbiota of the selected donors. In particular, direct delivery to the gastrointestinal tract using more invasive routes like gastroscopy or colonoscopy is more likely to lead to significant improvements of IBS symptoms when compared with oral administration. This meta-analysis highlights the need for more standardized intervention processes for the use of fecal transplants when treating specific diseases like IBS.

Microbiota modulation represents a promising strategy for improving health outcomes and managing metabolic disorders, particularly T2D and obesity. In a systematic review from Paul et al., the authors delve into the potential role of microbiome therapies in ameliorating biomarkers of inflammation and oxidative stress in patients living with T2D. Compared with individual probiotics, synbiotics, and other prebiotics, multi-strain probiotic combinations and prebiotics like resistant dextrin and inulin seem to be more effective in reducing inflammation and oxidative stress associated with T2D and could contribute to their management. This reduction in oxidative stress and inflammation may also alleviate liver damage (Al-Najjar et al.).

Positive effects of a multi-strain probiotic blend tested on children living with obesity have also been demonstrated, emphasizing how probiotic combinations can be used to reshape the gut microbiota and improve lipid metabolism and reduce body weight (Chen et al.). An intervention conducted by Yildrim et al. in children living with obesity, which evaluated the use of multi-strain synbiotic supplementation in combination with a standard diet and increased physical activity, observed significant improvements in anthropometric measurements, including body weight, BMI, and waist circumference. Both studies highlight the synergy between dietary modifications and synbiotics during weight loss, and emphasize the potential role of multi-strain probiotics and synbiotics as part of an effective weight-loss strategy in pediatric obesity.

All together, these findings underscore the combined effect of multi-strain probiotics and synbiotics to modulate microbiota composition and activity in both children and adults, with the aim of improving health and enhancing disease management.

However, it is not only the combination of different strains that have potential applications for promoting health. The consumption of particular single probiotic strains may confer benefits for the management of specific conditions, as was recognized in guidelines recently published by the World Gastroenterology Organization.[1] In a study conducted by Guo Y. et al. in ICR mice, *Latilactobacillus sakei* Furu 2019, when administered alone or as a synbiotic with stachyose, significantly alleviates constipation, potentially through its regulation of inflammation, neurotransmitters, hormones and the gut microbiota. *Brevibacillus laterosporus* BL1 might be another promising probiotic for weight management. Prophylactic treatment of C57BL/6 mice with this strain led to reduced body weight gain, decreased fat mass, improved lipid profiles, and enhanced brown adipose tissue thermogenesis, while also positively modulating the gut microbiota (Weng et al.). *Lactiplantibacillus pentosus* P2020 was evaluated as potential preventive strategy for hyperuricemia (Wang et al.) using Kunming mice as an animal model. *Lactiplantibacillus pentosus* P2020 administration was able to reduce serum uric acid levels and mitigate renal inflammation, potentially by regulating inflammatory pathways, enhancing uric acid excretion, and improving intestinal barrier function. In germ-free Fischer 344 rats inoculated with infant fecal microbiota, Rocha Martin et al. studied how *Cutibacterium avidum* P279 can modulate microbiota composition and activity, reducing H_2-producing bacteria with a reduction in symptoms of infant colic. However, the efficacy of these probiotics must still be demonstrated in humans.

Various nutritional and dietary interventions have also been explored to understand their impact on microbiota modulation as an approach to manage different health conditions. Lakshmanan et al. studied the impact of a formulated fruit and vegetable supplement on gut microbiota composition and antioxidant capacity. Supplementation led to changes in microbiota composition and activity, with increased *Faecalibacterium* abundance and decreased *Ruminococcus*, along with higher production of short-chain fatty acids (SCFAs). These changes could potentially reduce pro-inflammatory responses and increase antioxidant capacity in healthy adults. In interleukin-10 knockout mice, dietary eggshell membrane supplementation demonstrated significant improvements in survival rates and mitigated gut dysbiosis, suggesting that this supplement might be explored in future studies as a dietary intervention for inflammatory bowel disease in humans (Yang et al.). Interestingly, Beaumont et al. proposed that specific amino acids my be used as a novel type of prebiotics due to their selective use by intestinal bacteria and the positive impact on host health, or as synbiotics when combined with specific probiotics. Intermittent fasting (IF) is a dietary approach that has gained popularity in recent years. Mohr et al. evaluated IF as an alternative to daily caloric restriction in adults with overweight and obesity. The study compared one-day and two-day IF protocols combined with protein pacing. While both IF groups exhibited differences in fecal microbiome and plasma metabolome, the two-day IF group displayed distinct metabolic changes in plasma, such as increased levels of specific metabolites, including trimethylamine oxide, levulinic acid, 3-aminobutyric acid, citrate, isocitrate, and glucuronic acid. This research highlights the potential of IF to beneficially modulate microbiota composition and activity and suggests that varying fasting durations may be of interest for specific dietary intervention strategies.

Changes in the microbiome can also be used as novel diagnostic tools of specific diseases, as discussed by Hardinsyah et al.. They hypothesized that salivary microbiome could be used as a diagnostic indicator for T2D. Although the relationship between the salivary microbiome and T2D is complex, with large individual variation in bacterial profiles, metabolic pathways, and oral health status, the authors highlight the potential of salivary microbiome profiling as a rapid, non-invasive test for detecting T2D. As microorganisms can respond quickly to changes in the human body, there is promise in the use of them as biological indicators, for both rapid diagnosis and monitoring the effectiveness of therapeutic interventions.

Microbiota modulation, as highlighted in our Research Topic, has great potential for improving health. It can positively impact conditions such as obesity, hyperuricemia, constipation, and T2D. The use of fecal microbiota transplants, probiotics, prebiotics, synbiotics, dietary supplements, and dietary interventions demonstrates the versatility of this approach. Future research should focus on refining specific microbial targets, personalized interventions, and addressing the interplay between diet, microbiota, and health. Additionally, exploringthe microbiome as

1 WGO. Available online at: https://www.worldgastroenterology.org (accessed October 31, 2023).

diagnostic tool, and harnessing microbiota modulation in other less explored areas like cardiovascular health and mental wellbeing, are exciting future possibilities. These studies contribute to set the basis for innovative applications and open the door to new frontiers in microbiota-related research.

Author contributions

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ORIGINAL RESEARCH
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Dietary Eggshell Membrane Powder Improves Survival Rate and Ameliorates Gut Dysbiosis in Interleukin-10 Knockout Mice

Yongshou Yang[1,2,3], Huijuan Jia[3], Weida Lyu[3], Kyohei Furukawa[3], Xuguang Li[3], Yukio Hasebe[4] and Hisanori Kato[3]**

[1] School of Life Sciences, Anhui University, Hefei, China, [2] Key Laboratory of Human Microenvironment and Precision Medicine of Anhui Higher Education Institutes, Anhui University, Hefei, China, [3] Health Nutrition, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan, [4] ALMADO Inc., Tokyo, Japan

Inflammatory bowel disease (IBD) is known to be associated with compositional and metabolic changes in the gut microbiota. The aim of this study was to investigate whether dietary eggshell membrane (ESM) improves survival rate or ameliorates gut dysbiosis in a spontaneous IBD model of interleukin-10 knockout (IL10$^{-/-}$) mice. Female C57BL/6J wild-type (WT) and IL10$^{-/-}$ mice (KO) were fed an AIN-93G basal diet or an ESM diet (KOE) for 19 weeks. Gut microbiota profiles were analyzed *via* 16S rRNA sequencing, and short-chain fatty acids in cecal content were analyzed with high-performance liquid chromatography. The results demonstrated that ESM supplementation significantly improved the survival rate and body composition in KO mice. Alpha diversity analysis of the microbiota revealed that ESM supplementation significantly increased gut microbial diversity, which was decreased in IL10$^{-/-}$ mice. The Firmicutes/Bacteroidetes ratio was recovered to a normal level by ESM supplementation, suggesting that ESM helps maintain the compositional balance of the gut microbiota. ESM increased relative abundance of commensal bacterial Ruminococcus and Bacteroidales S24-7 and reduced the abundance of the proinflammatory-related bacterium, Enterobacteriaceae. Additionally, ESM supplementation promoted the production of butyrate in cecal contents and downregulated the expression of proinflammatory genes, including interleukin-1β (*Il-1β*) and tumor necrosis factor-α (*Tnf-α*) in IL10$^{-/-}$ mice colon, indicating anti-inflammatory functions. These findings suggest that ESM may be used as a beneficial dietary intervention for IBD.

Keywords: inflammatory bowel disease, eggshell membrane, gut microbiota, IL10, colitis

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Carlos Gómez-Gallego, University of Eastern Finland, Finland

Reviewed by:
Jing Wang, Feed Research Institute (CAAS), China
Manuela M. Santos, Université de Montréal, Canada

***Correspondence:**
Huijuan Jia
akakeiken@g.ecc.u-tokyo.ac.jp
Hisanori Kato
akatoq@g.ecc.u-tokyo.ac.jp

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INTRODUCTION

Inflammatory bowel disease (IBD) is characterized as a cluster of chronic gut inflammation disorders, including Crohn's disease and ulcerative colitis (1). Despite extensive efforts, the exact underlying mechanisms of IBD have not been clearly elucidated. However, several risk factors, including genetics, immune system, and environmental factors, play important roles in the development of IBD (2). Recently, abnormal microbial colonization of the gastrointestinal tract

has been considered a possible pathogenesis of IBD in genetically susceptible individuals (3). Many previous studies have identified a perturbation of gut microbiome in both IBD patients and mouse colitis models (4–6). Compared to chemical-induced colitis models, interleukin-10 knockout (IL-$10^{-/-}$) mice are often applied in mechanistic studies to examine the pathogeny of spontaneous, immune-mediated, chronic gastrointestinal inflammation (7, 8). Germ-free IL-$10^{-/-}$ mice showed no proof of colitis or related immune system activation, suggesting the critical role of gut microbiota in IBD pathogenesis (9).

Current treatments for IBD mainly rely on anti-inflammatory drugs and surgery to relieve symptomatic complications, which have several side effects, including loss of immunotolerance and drug resistance (10). Administration of prebiotics, probiotics, or synbiotics has been demonstrated to ameliorate some colitis symptoms in both IBD patients and rodent colitis models (7, 11–13). These studies imply that targeting the gut microbiota is an effective strategy for IBD treatment (14).

Eggshell membrane (ESM) is a by-product of the egg process industry and is considered an environmental-burden waste. Our previous study found that dietary ESM exerts biochemical functions that can counter liver injury and fibrosis through modulating the PPARγ-endothelin 1 interaction signaling pathway (15). Moreover, dietary ESM was shown to ameliorate dextran sulfate sodium (DSS)-induced colitis through exerting anti-inflammatory effects and modulating the gut microbiota (16). The DSS-colitis model is widely used to mimic human IBD, mainly because of the massive epithelial injury and wholesale invasion of the lamina propria by gut microbiota, however, this is unlikely to be a crucial mechanism of human IBD (17). IL-$10^{-/-}$ mice display chronic intestinal inflammation through impaired immunoregulatory function of antigen-presenting cells as well as dysfunction of macrophages (17). The efficacy of ESM in IL-$10^{-/-}$ mice with spontaneous colitis has not yet been elucidated, thus we investigated the potential protective role of ESM on the effects of colitis and gut microbiota modulation in IL-$10^{-/-}$ female mice.

MATERIALS AND METHODS

Animal and Diets

The animal experiment was approved by the Animal Care and Use Committee of the University of Tokyo. Female wild-type (WT) mice and IL-$10^{-/-}$ C57BL/6J mice, aged 9 or 10 weeks, bred in our laboratory were used in this study. The mice were individually housed in cages under a controlled temperature (23 ± 2°C), 12 h light-dark cycle environment (lights on from 08:00 a.m. to 20:00 p.m.) with a relative humidity of 40–60%. The mice were acclimatized for 1 week and then randomly divided into three groups based on their genetic types and diets: WT (WT mice; control diet of AIN93G, $n = 10$), KO (IL-$10^{-/-}$ mice; control diet, $n = 16$), KOE (IL-$10^{-/-}$ mice; 8% ESM diet, $n = 16$) for 19 weeks. The dietary composition is shown in **Supplementary Table S1**. As the digestibility of ESM is ∼46%, the ESM diet was adjusted with cornstarch and casein to maintain the caloric and protein balance. Animals had free access to the experimental diet and tap water.

Sample Collection and Cecal Short-Chain Fatty Acid Analysis

At the end of experimental period, all 12 h-fasted mice were anesthetized using isoflurane before sacrificing. After sacrificing the mice, the tissues and cecal contents were collected, weighed, and stored at −80°C prior to analysis. The concentration of short-chain fatty acids (SCFAs) in cecal contents was measured using ion-exclusion high-performance liquid chromatography (HPLC), as described in a previous study (18). Briefly, 50 mg cecal contents were mixed with 100 μl distilled water and 15 μl 12% (v/v) perchloric acid. After centrifuging the mixture for 10 min at 13,000 × g, the collected supernatants were filtered with a 0.45 μm membrane filter (Cosmonice Filter W; Nacalai Tesque, Kyoto, Japan). The filtered samples were injected into an SIL-30AC autosampler (Shimadzu, Kyoto, Japan) for quantitative analysis.

Bacterial 16S rRNA Gene Sequencing

Bacterial genomic DNA from mouse cecal content was extracted with the QIAamp Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The V3–V4 of the 16S rRNA gene were amplified with the primer set, 341F: 5′-CCTACGGGNGGCWGCAG-3′; and 806R: 5′-GACTACHVGGGTATCTAATCC-3′, and then incorporated with Illumina adapters for subsequent sequencing. Thereafter, the libraries were conducted with a single Illumina MiSeq run (MiSeq Reagent Kit V3, 600 cycles; Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

Bioinformatic Analysis

The collected sequencing data was analyzed with QIIME (v. 1.8.0) software and the method is described in our previous study. First, fast length adjustment of short reads (FLASH) (v.1.2.11) was used to assemble the paired-end reads. Assembled reads with an average Q-value < 25 were filtered out using in-house script. The same number of filter-passed reads was randomly selected from each sample and used for further analysis. The selected reads were then processed using the QIIME pipeline. The high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and OTUs were assigned with the Greengenes database (v.13.8). The microbial functionality profiles were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v.2.1.4) to generate the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. The predicted metagenomic data were aligned to the KEGG database, and the differences between groups were compared using STAMP (Welch's t-test, two-sided).

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

The colonic mucosa was scraped off gently with a blunt-edged glass slide. Total RNA was extracted from colonic mucosa using TRIzol™ reagent (Ambion® Life Technologies™, Foster City, CA, USA) and RNA Isolation Kit (NucleoSpin RNA II; Macherey Nagel, Düren, German). cDNA was synthesized from extracted total RNA with the PrimeScript™ RT Master Mix (Takara Bio,

Tokyo, Japan) according to the manufacturer's instructions. The real-time quantitative polymerase chain reaction (qPCR) was performed using the Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc.). The primer sequences used in the study are described in **Supplementary Table S2**. The expression level of ribosomal protein lateral stalk subunit P1 (*Rplp1*) was used as the internal reference for normalization of the target gene mRNA expression.

Statistical Analysis

Data are expressed as mean ± standard error (SE) or boxplots with median, minimum, and maximum values. The normality and equal variances of the data were performed by Shapiro-wilk and Hartley's test firstly. Then, statistical analysis was performed using one-way ANOVA and Tukey-Kramer test. The survival analysis was performed using a Log-rank (Mantel-Cox) test in GraphPad Prism 9 (San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

ESM Administration Significantly Improved Survival Rate and Body Composition in IL10$^{-/-}$ Mice

During the 19-week experimental period, there was no significant difference in the total food intake among the experimental groups. After the 19-week treatment, the survival rate was significantly lower in the KO group than in the WT group ($P < 0.01$); however, ESM supplementation significantly improved the survival rate of IL10$^{-/-}$ mice (**Figure 1A**). From the 8- to 19-week treatment period, body weight was significantly lower in the KO group than in the WT group. ESM treatment largely reversed the body weight loss in IL10$^{-/-}$ mice (**Figure 1B**). At necropsy, KO mice showed reduced liver, visceral fat, and gastrocnemius muscle weights, which was mitigated by ESM supplementation (**Figures 1C–E**). The cecal content weight in KOE group mice was higher than that in WT mice (**Figure 1F**). The mice colon length was not significantly different among the experimental groups (**Figure 1G**). However, the mice colon weight was significantly elevated in the KO group compared to the WT group (**Figure 1H**). Accordingly, the ratio of the colon weight (mg) to length (cm), an indirect indicator of edema and inflammation, increased in the KO group compared to the WT group, which was slightly alleviated by ESM supplementation (**Figure 1I**).

ESM Administration Modulated the Composition of Gut Microbiota in IL10$^{-/-}$ Mice

To analyze the gut microbiota, a total of 28,313 filter-passed high-quality reads per sample were extracted for further QIIME pipeline analysis. Alpha diversity analysis showed that Shannon and Simpson indices were lower in the KO group than in the WT group (**Figures 2A,B**). However, Shannon index-based diversity was recovered by ESM supplementation. Principal component analysis of unweighted UniFrac analysis showed a distinct difference in the gut bacterial profiles between the WT and KO groups (**Figure 2C**). The KOE samples were clustered between the WT and KO groups.

Taxonomic analysis revealed that all treatment groups contained four dominant phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia (**Figures 3A,B**). At the phylum level, the abundance of Bacteroidetes was enriched in KO mice and reversed in KOE mice (**Figure 3B**). The abundance of Verrucomicrobia was decreased in KO mice but recovered by ESM supplementation. The ratio of Firmicutes to Bacteroidetes was significantly lowered in the KO group compared to the WT group, which was almost completely restored by ESM treatment (**Figure 3C**). At the genus level, commensal bacteria, such as *Ruminococcus*, were significantly reduced in the KO group but recovered in KOE, compared to the WT group. The family Bacteroidales S24-7 also followed this trend (**Figure 3D**). In contrast, *Bacteroides*, unclassified Enterobacteriaceae, and *Blautia* were significantly enriched in the KO group over the WT and KOE groups (**Figure 3D**). Collectively, these data indicated that ESM treatment markedly modulated the composition of gut bacteria in IL10$^{-/-}$ mice, resulting in a more similar bacterial structure to WT mice.

The concentrations of SCFAs in mice cecal contents were quantified using a HPLC system. The level of butyrate, which is considered a beneficial bacterial metabolite in the intestines, was significantly increased in the KOE group over the KO group (**Figure 4A**). The levels of other SCFAs, including acetate, propionate, isobutyrate, and valerate, were not altered by ESM treatment (**Figure 4A**). The concentration of isovalerate was significantly lower in the KOE group than the KO group. To understand the relationship between specific bacterial taxa and cecal SCFAs, a Pearson-correlation heatmap was generated by selecting taxa at the family level and the levels of cecal SCFAs (**Figure 4B**). Particularly, a strong positive correlation was observed between Ruminococcaceae, Oscillospira, Clostridiales, and Bacteroidales S24-7 and the level of cecal butyrate, respectively. *Bacteroides* showed a positive correlation with isovalerate levels and a negative correlation with acetate and butyrate levels.

To obtain a better understanding of the influence of ESM supplementation on gut bacteria, PICRUSt analysis was conducted to predict the bacterial gene functional profiles based on the 16S rRNA gene sequences. There are 28 KEGG pathways associated with gut microbiota that were significantly altered in the KO group compared to the WT group (**Supplementary Figure S1A**). ESM supplementation affected 21 KEGG pathways when compared to the KO group. Among them, 14 pathways (marked with *) that were altered in the KO group were recovered by ESM treatment (**Supplementary Figure S1B**).

ESM Inhibited the Augmented Proinflammatory Gene Expression in IL10$^{-/-}$ Mice

To further investigate the molecular mechanisms responsible for the observed beneficial effects of ESM on disease indices of IL10-KO mice, we analyzed relative gene expression in the

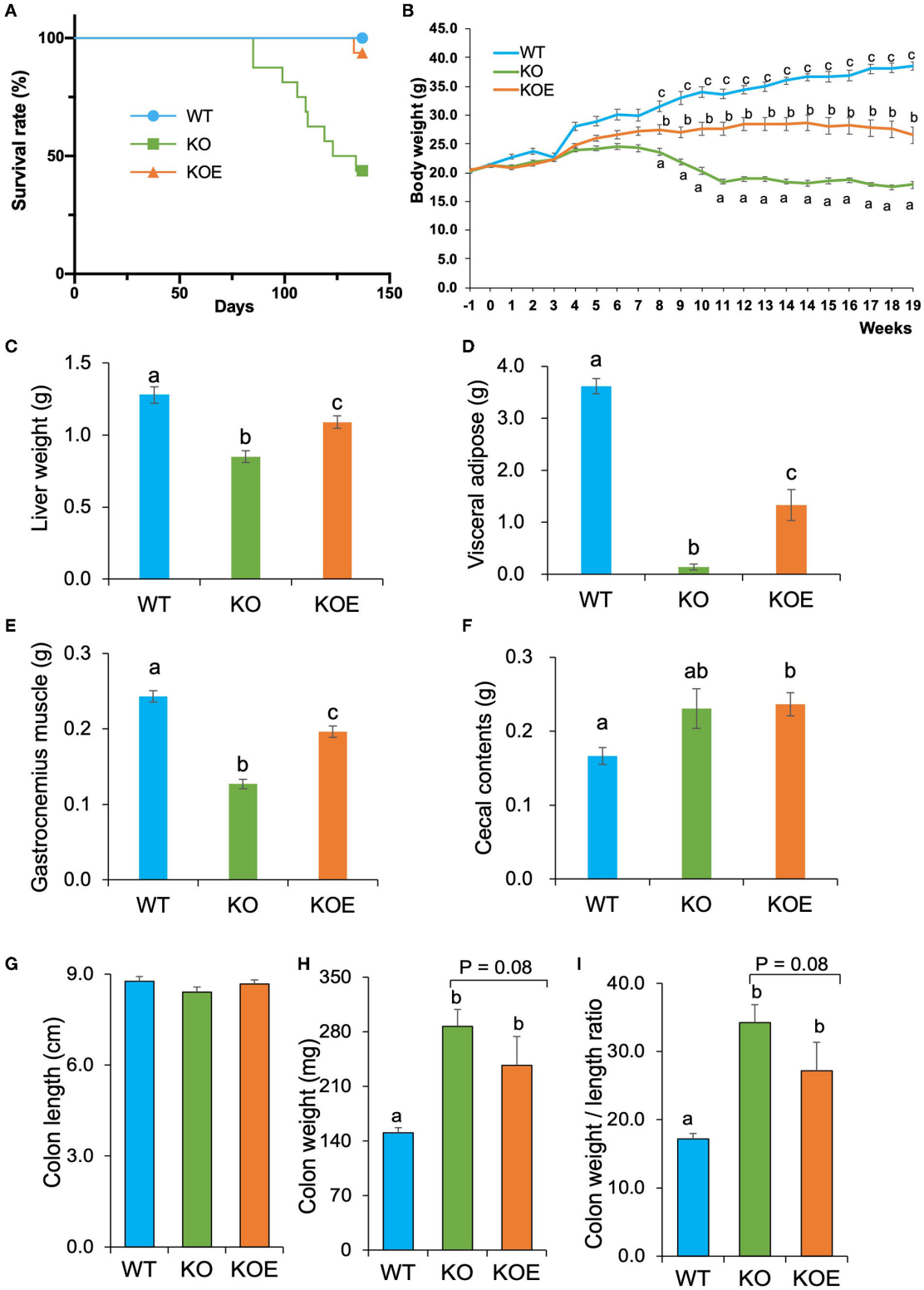


FIGURE 1 | Effect of eggshell membrane (ESM) supplementation on the survival rate and body composition in IL10$^{-/-}$ mice. **(A)** Survival rate in wild-type (WT) mice fed with control diet or IL10$^{-/-}$ mice fed with control diet (KO group) or ESM supplemental diet (KOE group) for 19 weeks. During the treatment period, body weight **(B)** was measured for each group. After 19-week treatment, liver weight **(C)**, visceral fat pad weight **(D)**, gastrocnemius muscle weight **(E)**, cecal content weight **(F)**, colon length **(G)**, colon weight **(H)**, and colon weight to length ratio **(I)** were calculated (WT: $n = 8$, KO and KOE: $n = 7$, respectively). Data are presented as mean ± standard error. Superscript with different letters indicate significant difference at $P < 0.05$.

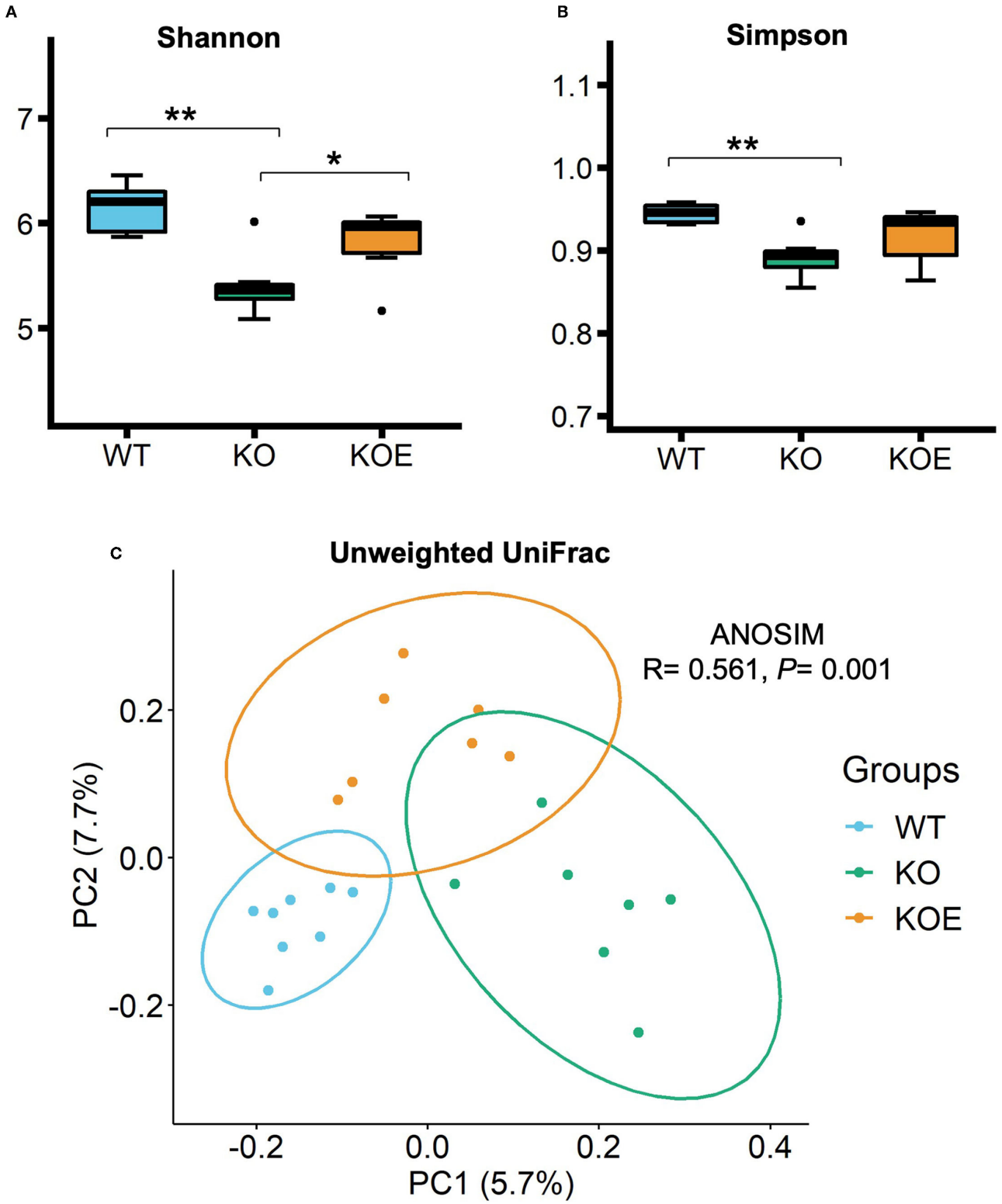
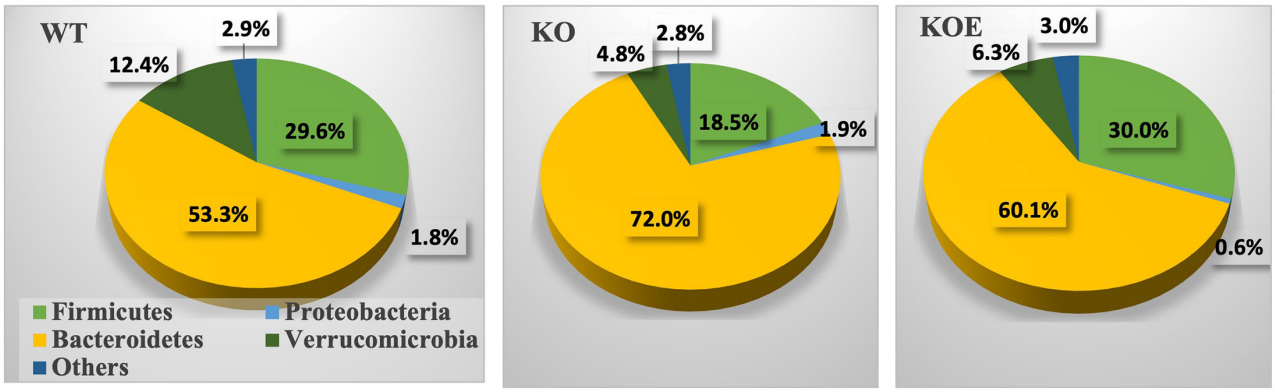


FIGURE 2 | The α- and β-diversities of gut bacteria in mice of treatment groups [wild-type (WT): $n = 8$, IL10$^{-/-}$ (KO) and KO with eggshell membrane supplementation (KOE): $n = 7$, respectively]. After the 19-week treatment, total bacterial DNA in cecal contents were extracted for 16S rRNA gene sequencing. Gut bacterial diversity and richness within samples were measured by **(A)** Shannon and **(B)** Simpson indices. The principal component analysis (PCoA) of Unweighted UniFrac **(C)** and ANOSIM analysis were conducted to compare the gut bacterial profiles among the three groups. Statistical analysis of α-diversity data was performed by ANOVA and the Tukey-Kramer test. $^{*}P < 0.05$, $^{**}P < 0.01$. The dots (•) in the boxplots indicate outliers.

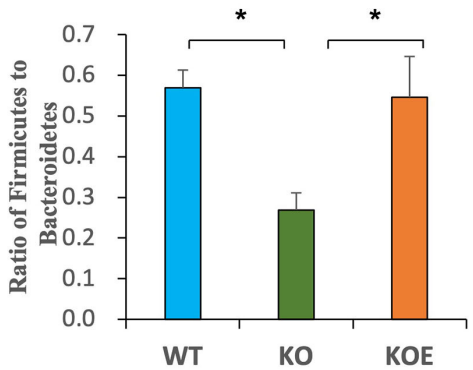
A



B

Taxa	WT	KO	KOE
Firmicutes	29.6±1.3	18.5±2.4	30.0±4.0
Bacteroidetes	53.3±2.5 [a]	72.0±3.0 [b]	60.1±4.2 [a]
Proteobacteria	1.8±0.3	1.9±0.4	0.6±0.1
Verrucomicrobia	12.4±1.9 [a]	4.8±1.5 [b]	6.3±1.2 [a]

C



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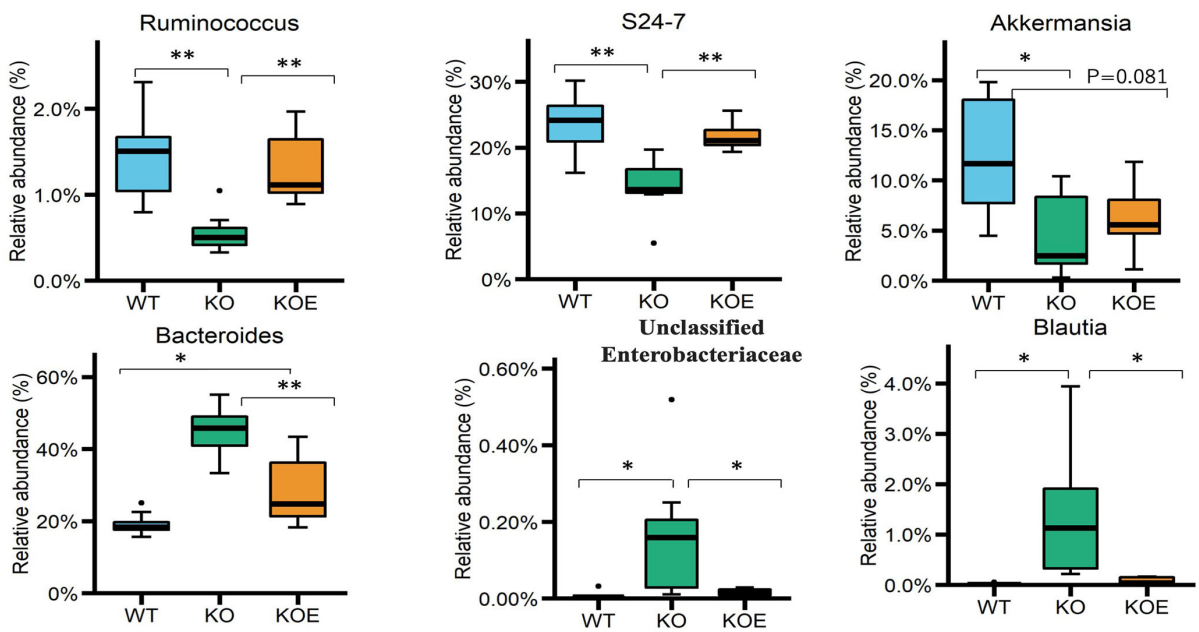

FIGURE 3 | Effect of eggshell membrane (ESM) on gut bacterial composition in IL10$^{-/-}$ (KO) mice [wild-type (WT): $n = 8$, KO and KO with ESM supplementation (KOE): $n = 7$, respectively]. Relative abundance of phyla is demonstrated in the pie graphs **(A)** and table **(B)**. **(C)** The ratio of Firmicutes to Bacteroidetes in the three groups. **(D)** The abundance of gut bacterial taxa at the genus level. Different superscript letters indicate significant differences at $P < 0.05$. *$P < 0.05$, **$P < 0.01$. The dots (•) in the boxplots indicate outliers.

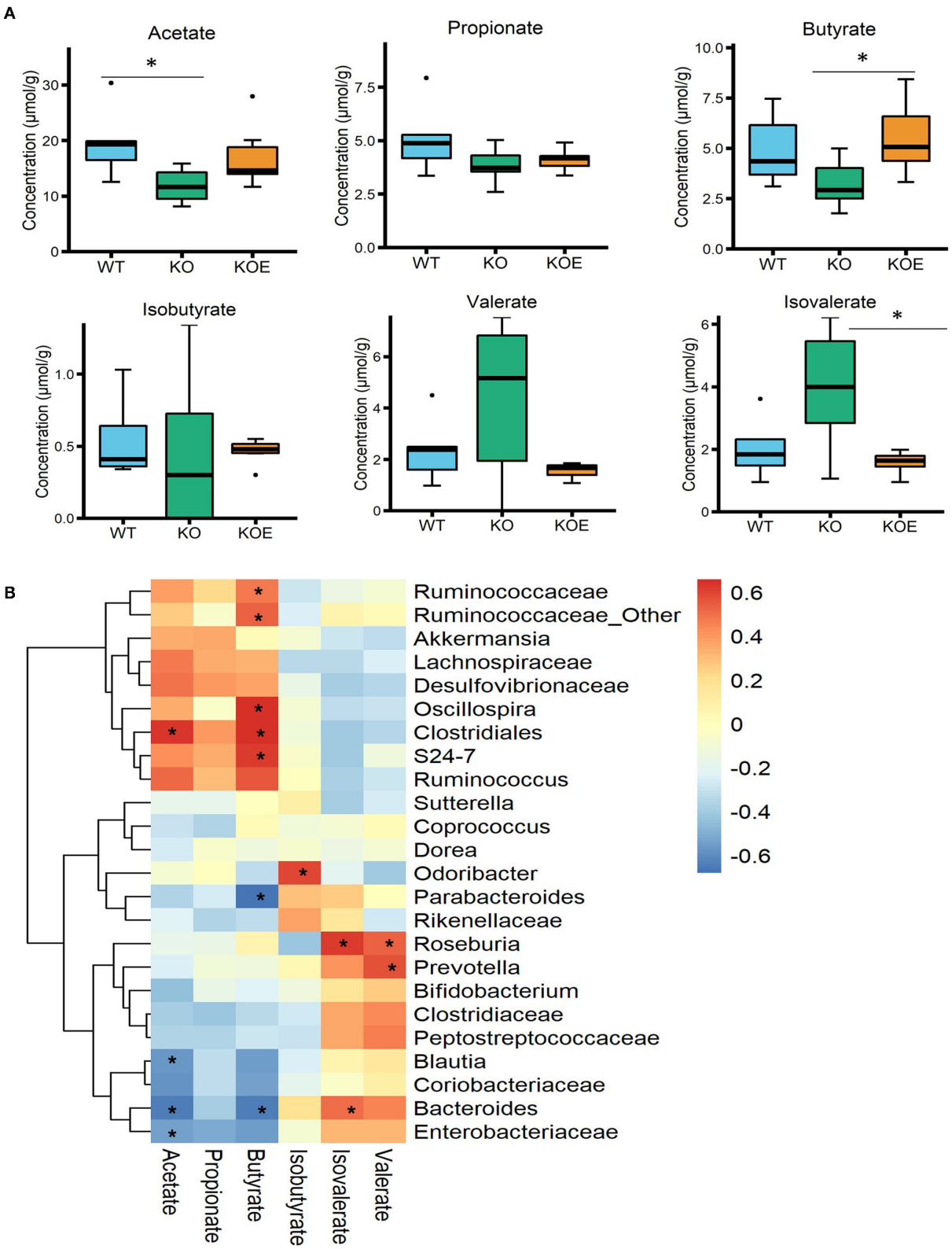

FIGURE 4 | Effects of eggshell membrane (ESM) on the **(A)** levels of short-chain fatty acids (SCFAs) in cecal contents [wild-type (WT): $n = 8$, IL10$^{-/-}$ (KO) and KO with ESM supplementation (KOE): $n = 7$, respectively]. **(B)** The heatmap showing the Pearson correlation between SCFAs and the relative abundance of the selected taxa at the family level. *$P < 0.05$. The dots (•) in the boxplots indicate outliers.

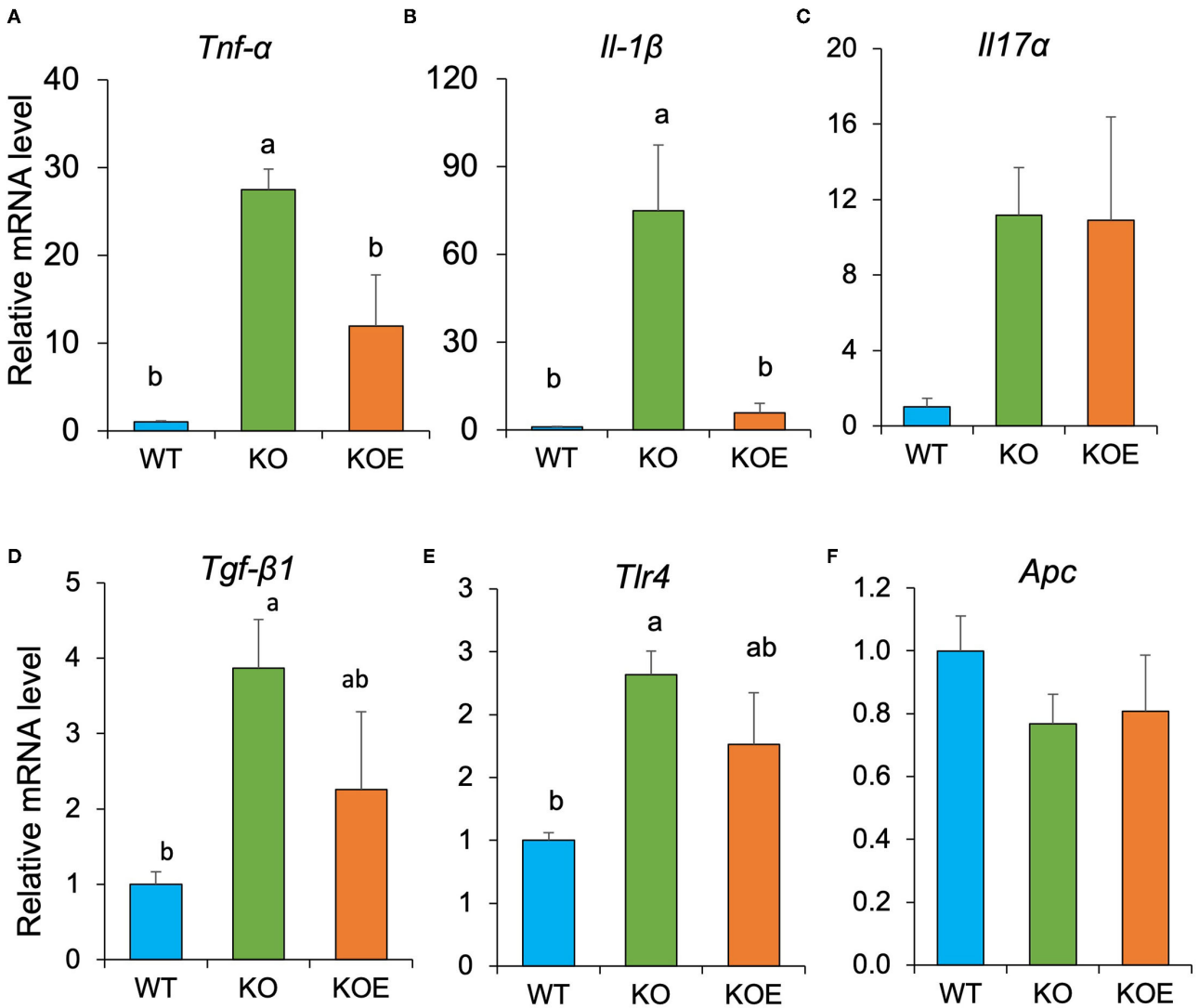

FIGURE 5 | Eggshell membrane (ESM) modulates relative gene expression of several proinflammatory cytokines relative to RPLP1 in the mouse colonic mucosa. After the 19-week treatment, relative mRNA expression of *Tnf-α* **(A)**, *Il1β* **(B)**, *Il17a* **(C)**, *Tgf-β1* **(D)**, *Tlr4* **(E)**, and *Apc* **(F)** in colonic mucosa were examined using quantitative real-time polymerase chain reaction (qPCR). Data are presented as mean ± standard error. Statistical analysis was performed by one-way ANOVA and the Tukey-Kramer test. Superscript with different letters indicate significant difference at $P < 0.05$.

mouse colonic mucosa with qPCR. The result showed the relative mRNA levels of *Tnf-α* and *Il1β* were dramatically upregulated in IL10$^{-/-}$ mice, however, the gene expression was significantly inhibited by ESM treatment (**Figure 5**). The expression of the other genes related to intestinal homeostatic functions, including *Il17, Tgf-β1, Tlr4,* and *Apc,* were not significantly altered by ESM supplementation.

DISCUSSION

Eggshell membrane (ESM), primarily composed of collagen-like proteins, is considered as a safety and novel functional dietary ingredient. In this study, IL-10$^{-/-}$ mice, as a spontaneous colitis model, were used to explore the effect of ESM on colitis. The survival rate and body weight loss were ameliorated following ESM treatment in the IL-10$^{-/-}$ mice. Coincidently, the reduced tissue weights in the liver, visceral adipose, and gastrocnemius muscles were reversed following ESM treatment by the end of experimental period. These findings suggested that the colitis mouse model was successfully constructed, and ESM supplementation was effective in ameliorating some symptoms of colitis.

The alteration of gut bacterial composition is considered to play a critical role in the onset and progression of IBD. Therefore, we examined the effects of ESM on the composition of gut bacteria via 16S rRNA gene sequencing. Our results indicated that bacterial diversity was reduced in IL-10$^{-/-}$ mice, but this was reversed by ESM supplementation. Moreover, the gut microbiota

of the three groups was clustered into different groups, where the KOE group was positioned between the WT and KO groups. These results suggest that the gut dysbiosis observed in colitis mouse model might be improved by ESM treatment. Firmicutes and Bacteroidetes were dominant in the mice intestine according to the taxonomic analysis. The Firmicutes / Bacteroidetes (F/B) ratio was reduced in IL-10$^{-/-}$ mice but was recovered to a normal level (similar to WT) following ESM treatment. The F/B ratio is generally considered to play an important role in maintaining regular gut homeostasis. The altered F/B ratio is a marker of dysbiosis, whereby an increase is normally observed with obesity, and a decrease is associated with IBD (19). However, an increased or unchanged F/B ratio was reported in other previous studies in IL-10$^{-/-}$ mice (7, 20). This disparity may be attributed to the difference in housing conditions, maturity or other environmental factors.

At the genus level, *Ruminococcus* was significantly decreased in the KO group, but recovered in KOE mice, compared to the WT group. The family Bacteroidales S24-7 followed the same trend. The symbiont of the genus *Ruminococcus* often inhabits both the animal and human gastrointestinal tract, and can use fermentable carbohydrates for growth. For instance, *Ruminococcus bromii* has the ability to ferment resistant starch and subsequently contribute dramatically to butyrate production in the gut (21). Consistently, our results showed that treatment with ESM enhanced the production of butyrate, which is positively associated with the elevated level of Ruminococcaceae. Meanwhile, members of the family S24-7, recently renamed as Muribaculaceae, are dominant in the murine gut microbiota and have been identified in the gastrointestinal tract of other animals (22). The abundance of S24-7 has been shown to decrease in mice fed with a high-fat diet but to increase in those fed with dietary fibers (23, 24). In addition, the abundance of S24-7 recovered to a higher level following treatment-induced colitis remission in a mouse model (25). In our study, S24-7 was found to have a positive correlation with cecal butyrate. These results imply that S24-7 may play a critical role in the amelioration of colitis by treatment with ESM.

In contrast, *Bacteroides*, unclassified Enterobacteriaceae, and *Blautia* were significantly higher in the KO group than in WT or KOE groups. The family Enterobacteriaceae contains symbiotic bacteria and several well-known pathogens, such as *Escherichia coli, Klebsiella*, and *Shigella*. An increased abundance of Enterobacteriaceae has been reported in both IBD patients and mouse colitis models (26, 27). Consistently, our previous study showed that ESM treatment decreased the abundance of Enterobacteriaceae in DSS-induced IBD model mice (16). The proinflammatory properties of Enterobacteriaceae have been well studied, but the mechanism by which Enterobacteriaceae is enriched in IBD is not fully clear. Meanwhile, the commensal bacterium *Blautia* is a dominant genus in the gut microbiota that plays a critical role in maintaining gut environmental balance and preventing gut inflammation through regulation of intestinal regulatory T cells and production of SCFAs (28). Decreased levels of *Blautia* have been found in patients with Crohn's disease or colorectal cancer (29, 30). However, several studies found higher levels of *Blautia* in the feces of IBD patients and ulcerative colitis than in healthy individuals (31, 32). Given these conflicting results, it will be interesting to explore the role of *Blautia* in the etiology of colitis. Due to the current shortcomings of 16S rRNA gene sequencing for gut microbiota, this study could not identify the gut bacterial taxa at the species or strain levels. Therefore, more in-depth studies should be performed to investigate the detailed roles of the gut microbiota, especially at the species or even strain levels.

Through the analysis of relative gene expression in the mouse colonic mucosa with qPCR, we confirmed aggravated colon inflammation through the increased expression of proinflammatory gene mRNA, such as *Tnf-α* and *Il1β*. However, ESM supplementation ameliorated the colon inflammatory state by downregulating these proinflammatory genes. Previous studies have showed that butyrate exerts anti-inflammatory effects by activating GPR41 and GPR43 and inhibiting NFκB activation (33, 34). Together, these results indicated the possibility that ESM ameliorates the colon inflammatory state through the modulation of gut microbiota and its metabolites. Further study is required to confirm this possibility.

CONCLUSIONS

In conclusion, the current study demonstrated the effectiveness of ESM supplementation in improving the survival rate and ameliorating the reduction of body weight, body fat, and muscle volume in IL10$^{-/-}$ mice. Furthermore, ESM supplementation improved the imbalanced gut microbiota composition, as indicated by the higher bacterial diversity and richness, the increased abundance of commensal gut bacteria (*Ruminococcus* and Bacteroidales S24-7), and the decreased level of potentially harmful bacteria (Enterobacteriaceae) in IL10$^{-/-}$ mice. Moreover, ESM supplementation promoted the production of butyrate in the gut and downregulated proinflammatory cytokine gene expression in the colon. These findings suggest that ESM could be used as a dietary intervention in colitis treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: DDBJ; DRA013317.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the University of Tokyo.

AUTHOR CONTRIBUTIONS

HJ, HK, and YH: conceptualization. HJ: methodology, supervision, and project administration. YY: formal analysis, writing original draft preparation, and visualization. WL, KF, and XL: data curation. HJ and HK: writing review and editing and funding acquisition. YH: resources. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.895665/full#supplementary-material

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Conflict of Interest: YH is working as a researcher in ALMADO Inc. in Tokyo.

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

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SYSTEMATIC REVIEW
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The Promising Role of Microbiome Therapy on Biomarkers of Inflammation and Oxidative Stress in Type 2 Diabetes: A Systematic and Narrative Review

*Pradipta Paul[1], Ridhima Kaul[1], Basma Abdellatif[1], Maryam Arabi[2], Rohit Upadhyay[3], Reya Saliba[4], Majda Sebah[2] and Ali Chaari[2]**

[1] Division of Medical Education, Weill Cornell Medicine-Qatar, Education City, Qatar Foundation, Doha, Qatar, [2] Division of Premedical Education, Weill Cornell Medicine-Qatar, Education City, Qatar Foundation, Doha, Qatar, [3] Department of Medicine—Nephrology and Hypertension, Tulane University, School of Medicine, New Orleans, LA, United States, [4] Distributed eLibrary, Weill Cornell Medicine-Qatar, Education City, Qatar Foundation, Doha, Qatar

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***Correspondence:**
Ali Chaari
alc2023@qatar-med.cornell.edu

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Background: One in 10 adults suffer from type 2 diabetes (T2D). The role of the gut microbiome, its homeostasis, and dysbiosis has been investigated with success in the pathogenesis as well as treatment of T2D. There is an increasing volume of literature reporting interventions of pro-, pre-, and synbiotics on T2D patients.

Methods: Studies investigating the effect of pro-, pre-, and synbiotics on biomarkers of inflammation and oxidative stress in T2D populations were extracted from databases such as PubMed, Scopus, Web of Science, Embase, and Cochrane from inception to January 2022.

Results: From an initial screening of 5,984 hits, 47 clinical studies were included. Both statistically significant and non-significant results have been compiled, analyzed, and discussed. We have found various promising pro-, pre-, and synbiotic formulations. Of these, multistrain/multispecies probiotics are found to be more effective than monostrain interventions. Additionally, our findings show resistant dextrin to be the most promising prebiotic, followed closely by inulin and oligosaccharides. Finally, we report that synbiotics have shown excellent effect on markers of oxidative stress and antioxidant enzymes. We further discuss the role of metabolites in the resulting effects in biomarkers and ultimately pathogenesis of T2D, bring attention toward the ability of such nutraceuticals to have significant role in COVID-19 therapy, and finally discuss few ongoing clinical trials and prospects.

Conclusion: Current literature of pro-, pre- and synbiotic administration for T2D therapy is promising and shows many significant results with respect to most markers of inflammation and oxidative stress.

Keywords: gut microbiome, gastrointestinal microbiota, clinical trial, inflammatory markers, dysbiosis, resistant dextrin, *Lactobacillus*, *Bifidobacterium*

INTRODUCTION

Type 2 Diabetes (T2D) is considered an ever-growing burden on public welfare, impacting both high- and low-income nations worldwide. Obesogenic lifestyles, environmental changes, genetic predispositions, and aging have been identified as contributing factors to these increasing trends (1). According to the International Diabetes Federation, as of 2021, an estimated 573 million individuals between 20 and 79 years of age were affected by T2D, representing ∼10% of the world's population; this figure is expected to cross 643 million by 2030 (2). T2D was responsible for more than 6.7 million deaths in the same year, making it one of the top 10 leading causes of death globally, costing almost 1 trillion USD in health expenditure. In the United States, approximately 21 million adults have been diagnosed with T2D, constituting 8.6% of the adult population (3). Generally, males have a slightly higher prevalence of T2D when compared to females, although this difference is insignificant (3). The onset of new diagnoses increases with increasing age, peaking at around the ages of 55–59 (3).

Type 2 Diabetes and Coronavirus Disease 2019

The high prevalence of T2D explains its significant role as commonly present comorbidity in patients of the severe acute respiratory syndrome coronavirus–2 (SARS-CoV-2) induced coronavirus disease (COVID-19) that has plagued the globe for two years and counting (4–8). Diabetics are not only increasingly susceptible to contracting the infection, but also have vastly higher mortality associated with the comorbidity, with rates ranging from 10.5% (China) to as high as 33.8% (NYC) and 35.5% (Italy) (9–11). This can be attributed to the compromised nature of the immune system in individuals with type 2 diabetes, where delayed and less effective immune reactions likely lead to longer recovery periods due to higher viral loads (12). The virus is also presumed to thrive in glucose-rich serum in conditions such as those in diabetic patients, since glycolysis induces viral proliferation due to the production of reactive oxygen species (ROS) in the mitochondria and the stimulation of hypoxia-inducible factor 1α (13). Diabetic patients who are infected with COVID-19 are more susceptible to uncontrolled inflammatory responses, thrombophilia, and morbidity (14, 15), although the most commonly presenting symptoms include diarrhea (up to 50%), nausea, vomiting and abdominal pain (16). Researchers elsewhere have recommended modulation of individual diet as a significant factor that should be considered during treatment of the disease given the strict relationship between diet and gut microbiota, and the latter to disease severity (12). It must be also noted, however, that a more pronounced and large-scale effect of the pandemic is the likely reduction of physical activity and healthy diet consumption with respect to not only the general population, but also diabetics, as a result of the lockdown that lags behind the shadow of the virus-mediated effects itself (17).

Microbiome Therapy in Type 2 Diabetes

The etiology of T2D is complex and is associated with both non-modifiable risk factors such as age, genetic predisposition, race, ethnicity, as well as modifiable factors such as diet, physical activity, apnea and the use of tobacco (18–20). Of these various factors, having poor dietary habits and following a sedentary lifestyle are the two major influences behind the rapidly rising incidence of the diabetes epidemic; corrective measures in one's lifestyle, including sleep, and diet can help to reduce the risk of onset and prevent or delay the progression of T2D (21–24). Experimental and clinical trials have shown that changes in gut microbiome composition can contribute to T2D pathogenesis; in this regard, pre- and probiotics have closely investigated for the potential to influence the microbiota and thereby promote anti-diabetic activity in a therapeutic manner (25). Probiotics refer to bioactive agents, naturally found in many foods, that administered in adequate amounts, whereas prebiotics refer to substrates utilized by these bioactive agents within the host to grow (26, 27). When provided in combination, they are referred to as synbiotics, and all three are traditionally aimed at improving the quality and quantity of the gastrointestinal microbiome of the host, leading to health benefits. Additionally, a novel category of biologically active substances known as postbiotics, defined as "probiotic-derived products obtained from food-grade microorganisms that confer health benefits when administered in adequate amounts," have also found to be promising in not only maintaining homeostasis of normal human health, but also for therapeutic purposes in diabetes mellitus (28). The concept of using natural dietary biomolecules, such as non-digestible fibers, flavonoids and other polyphenols, to serve as solutions to clinical challenges is not new, and their promise has been investigated extensively and verified across both literature and by legislative bodies of significance (29–34).

The Gut Microbiome and Its Dysbiosis

With an astounding composition of 10^{11-12} bacteria per gram in the large intestine, the gut microbiota is an active influence on the adiposity and fat storage capacity of the human body (25). In addition, the gut microbiota regulates the intestinal barrier along with the sensory, immune, neurological, and enteroendocrine systems (35–37). A dysbiosis in the microbiota alters the abundance of species and produces molecules like short chain fatty acids (SCFAs) and lipopolysaccharides (LPS) that affect these systems (26, 37–40). SCFAs, produced by the microbiota during the decomposition of indigestible polysaccharides, improve glucose tolerance by suppressing fat accumulation through binding a G-protein coupled receptor (GPRC) and ultimately increases insulin-sensitivity (38). It has been found that patients with T2D have lower levels of fecal SCFAs than control groups, which can be increased through a daily supplement of inulin-type fructans (41). Additionally, LPS has a high pro-inflammatory potential and drives endotoxemia low-grade inflammation which is suggested to be a potential cause for insulin resistance (38, 39). T2D patients are known to have an altered microbiota (42). Normally,

species of the Bacteroidetes and the Firmicutes phyla are the dominant groups of bacteria in the human gut. However, it was found that microbiota from the phylum Firmicutes and class Clostridia are significantly decreased in people with T2D (43). Moreover, the ratios of Bacteroidetes to Firmicutes, and the ratios of Bacteroides-Prevotella group to *Clostridium coccoides-Eubacterium rectale* group correlated positively with plasma glucose concentration (43). Decreased proportions of butyrate-producing bacteria and increased proportions of the previously *Lactobacillus* genus species has also been reported (39). Some of the differences observed between the typical microbiota and a T2D-deceased one supports the low-grade inflammation theory suggesting that increased proteobacteria-derived LPS and flagella can induce inflammation (43). A recent review concludes that gut microbiota dysbiosis that promotes inflammation is a general feature of T2D, but a specific signature for diabetes vs. other diseases in terms of biomarkers is not found (39). The review also suggests that the low decrease in α-diversity in T2D patients may explain the low-grade inflammation (39). Gut microbiota modulation in disease and due to biotics involves a multimodal approach; there is competition between various species facilitated by factors such as luminal pH, limited sources, bacterial toxins and SCFAs, in addition to modulation of gut barrier function and promotion of mucus secretion. Moreover, probiotics have been shown to be involved in the differentiation of regulatory T-cells, cytokine modulation and are also important factors in the communication through gut-brain axis level (44).

Inflammation and Oxidative Stress in Type 2 Diabetes

Chronic activation of the innate immunity, often characterized by cytokine-induced acute inflammation (also called low-grade-inflammation) has been shown to be associated with T2D pathogenesis, in addition to other linked metabolic complications (45). Inflammation has also repeatedly been linked to diabetes pathophysiology through its mediated effects on endothelial and cardiovascular pathology. Characterized by recruitment of leukocytes and their cytokine release, as well as various endothelial and tissue-specific crosstalk, inflammation has shown to play an acute role in metabolic cardiomyopathy (46). Hence, it is not surprising that the elevated presence of pro-inflammatory C-reactive protein (CRP), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and the reduction of anti-inflammatory cytokines such as IL-4 and IL-10 in the blood are predictive markers of T2D development and pathogenesis (45). Obesity and increased body weight, have been hypothesized to mediate the increased expression of inflammatory markers, leading to downregulation of the intracellular, downstream physiologic effects of insulin, and hence development of further insulin resistance (47). Increased oxidative stress arising from hyperglycemia also impairs glucose uptake in both muscle and fat cells, in addition to decreasing pancreatic β-cell mediated insulin secretion, further contributing to development of diabetes, and serving as an important marker for investigation of therapeutic intervention (48, 49). Whether as a cause or effect, dysbiosis of gut microbiome has been closely associated with T2DM presentation. Such an association could stem from the effect of microbiome dysbiosis on the levels of certain inflammatory markers such as CRP and TFN-α, further resulting in chronic low-grade inflammation following an atypical immune response (50, 51).

Pro-Inflammatory Markers in Type 2 Diabetes

Pro-inflammatory markers, such as acute-phase proteins, pro-inflammatory cytokines, and chemokines, are elevated in diabetic and obese patients, and are generally found to be at lower levels in individuals with a healthier lifestyle (52). Generally, there is a positive correlation between the levels of pro-inflammatory markers and insulin resistance (53). Several prospective studies have determined that these inflammatory markers may also be used to predict the onset of T2D (54).

C-reactive protein (CRP) is a plasma protein that serves as an early biomarker of inflammation, infection, or trauma (55). CRP is released through IL-6 induced hepatic synthesis and is the main mediator of the acute-phase response. Elevated levels of CRP has a significant association with the risk and onset of Type 2 diabetes, as well as insulin resistance syndrome (56). High concentrations of CRP in patients with T2D may be because of the stimulation of cells in the innate immune system due to increased amounts of nutrients (57). High-sensitivity CRP tests measure lower levels of the protein and is also positively correlated with the risk of type 2 diabetes (58).

Interleukin-6 (IL-6) secreted largely by Th cells, macrophages, fibroblasts, is a multifunctional cytokine involved in inflammatory responses as well as the regulation of cell growth and proliferation, activation, and differentiation of genes. IL-6 induces the production of several leukocytes and proteins such as CRP, and has been found to have parallel associations with T2D (56). The abnormal synthesis of IL-6 causes inflammation and instigates the formation of suppressor of cytokine signaling-3 (SOCS-3), which may serve as an inhibitor of insulin trans-signaling pathways (59). Thus, elevated amounts of this cytokine may serve as a predictor of T2D. Moreover, studies have also shown that IL-6 plays a role in anti-inflammatory processes and glucose metabolism (60).

Another pro-inflammatory cytokine that participates in the regulation of the body's inflammatory response is Interleukin-1β (IL-1β). IL-β has been associated with several autoimmune diseases, such as Type 1 Diabetes, as well as metabolic syndromes, including cardiovascular diseases and T2D. These disorders are characterized by elevation in IL-1β levels, which lead to weakened secretion of insulin in the pancreas's B cells (61). IL-1β also causes the breakdown of insulin receptor substrate (IRS) proteins by stimulating suppressors of cytokine signaling (SOCS) (62).

Interferon-gamma is also a pro-inflammatory cytokine that is involved in the preparation of macrophages for stimulation and start of an inflammatory response (63). IFN-γ is expressed by activated T cells and natural killer (NK) cells. In obese patients with T2D, an elevation in IFN-γ can be detected (64). This

cytokine has a key role in the maintenance of inflammatory responses in adipose tissues in T2D (65).

Tumor necrosis factor alpha (TNF-α) is a homotrimer that acts as a proinflammatory cytokine and is synthesized by the activation of natural killer (NK) cells, T lymphocytes, and macrophages. It plays critical roles in infection control, bone remodeling, and insulin resistance (66). Obesity and T2D have been associated with increased levels of TNF-α as TNF-alpha instigates insulin resistance in adipose and peripheral tissues through the phosphorylation of serine (67). Furthermore, TNF-α induces low-grade chronic inflammation through the production of ROS and stimulation of several pathways mediated by transcription (68).

Interleukin 17 (IL-17) is a pro-inflammatory T helper (Th) 17 cytokine. IL-17 induces inflammation by binding to a family of IL-17 receptors, which then initiate signaling that activates nuclear factor-Kb (69). This leads to the production of proinflammatory cytokines by monocytes, fibroblasts, and epithelial and endothelial cells (70). IL-17 also moves and employs granulocytes. Insulin resistance and inflammation in diabetes mellitus are linked to the expansion of both Th17 and Th1 (71). Patients with poor blood sugar regulation have elevated serum levels of Th17 cytokines such as IL-17 when compared with individuals with healthy glucose regulation (72).

Interleukin-8 is another proinflammatory chemokine secreted by cells such as adipocytes, macrophages, and endothelial and epithelial cells (73). It is a multifunctional interleukin involved in local and systemic inflammation and macrophage infiltration, and has also been implicated in the pathogenesis of T2D. Increased circulating levels of IL-8 has been reported in patients with T2D (74).

Interleukin-12 (IL-12) is a proinflammatory cytokine that is produced by antigen presenting cells from the innate immune system when in the presence of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (75). IL-12 is important for the programming of T cells into Th1 cells, thereby inducing an immune response (76). IL-12, like the other inflammatory cytokines, has been implicated in the pathogenesis of type 2 diabetes and insulin resistance. Levels of IL-12 were found to be the highest in T2D patients (77).

Lipopolysaccharide-binding protein (LBP) is a plasma protein that facilitates the interaction of lipopolysaccharides with other receptors, including toll-like receptor-4 (TLR4) (59). TLR4 activation initiates an intracellular signaling pathway leading to the production of inflammatory cytokines and the activation of the innate immune system (78). Higher concentrations of plasma LPS have been detected in individuals affected by T2D (79).

Anti-inflammatory Markers in Type 2 Diabetes

Anti-inflammatory markers, such as IL-10, are generally detectable in lower quantities in diabetic and obese patients when compared to controls, and these decreased levels could play a key role in the development of insulin resistance as well as other chronic diseases (80).

Interleukin-10 (IL-10), an anti-inflammatory cytokine, is considered to have a protective function in T2D as it prevents inflammation of immune cells (81). IL-10 is best known for its inhibitory effect on macrophage stimulation (82). Elevated glucose levels in diabetic patients have been found to minimize IL10-mediated activation of STAT3, a signaling protein of IL-10 (81). Thus high serum glucose and HbA1c are associated with low capacity of IL10 production (83).

Oxidative Stress Markers in Type 2 Diabetes

Oxidative stress is caused by an imbalance of free radicals and antioxidant defenses, and is suggested to have a potential impact on the pathogenesis of diabetes and the development of its complications (84). Values of oxidative stress markers are measured to be mostly higher in diabetic vs. non-diabetic subjects (85).

Superoxide dismutase (SOD) is an oxidative stress-related parameters linked closely to Type 2 diabetes (86). SOD catalyzes the breakdown of reactive oxygen species (ROS) in all tissues and cells (87). Oxidative stress and synthesis of ROS have been associated with the development and complications of diseases such as diabetes mellitus (88). The downregulation of SOD may be associated with the pathogenesis of diabetes mellitus (89). In diabetic patients that have an imbalance of oxidants to antioxidants, there are higher levels of ROS, which causes the total activity of SOD to be significantly higher than the antioxidant's activity in non-diabetic patients (90). However, the activity of SOD is also linked to the duration of the disease, with the level of antioxidant enzyme decreasing as the years of disease increase (91).

Another antioxidant enzyme is catalase (CAT), found in peroxisomes and the cytosol that mainly functions in the catalysis and disposal of hydrogen peroxide (H_2O_2) into O_2 and H_2O in erythrocytes (92). Catalase has significantly higher activities in patients T2D when compared with controls (93). Recent data suggests that the onset of diabetes in catalase-deficient patients happens more than 10 years earlier than subjects with normal levels of catalase (94).

Oxidative stress in diabetic patients leads to more severe diabetic complications. Lipid peroxidation results from the interaction of the lipid bilayer of a cell membrane with ROS and oxygen derived free radicals, producing malondialdehyde (MDA) (95, 96). Elevated levels of MDA lead in subjects with T2D to many physiological effects, such as influencing the structural integrity of the cell membrane, inactivating surface enzymes and receptors on the membrane, and causing errors in cell regulation (97).

Nitric oxide (NO) is a reactive nitrogen species (RNS) implicated in the pathogenesis of diabetes and complications. Nitric oxide is involved in impaired cellular function and increased expression of nitric oxide synthase (98). Since nitric oxide has a relatively short half-life, its metabolite nitrite, and nitrate are usually measured in blood and urine, and later used to calculate NO production. Diabetic patients have significantly higher basal levels of NO than non-diabetic individuals across

multiple individual studies (99–101), as well as a meta-analysis (102), and were found to have hypertension and microvascular complications. Interestingly, one study based in India showed a lower mean plasma NO level in diabetics compared to control group (103).

Glutathione is the most abundant, low molecular weight non-protein antioxidant produced by cells (104). It exists in the thiol-reduced (GSH) and the disulfide-oxidized (GSSG) forms (105). GSH has a key function in preserving redox homeostasis, transport of amino acids, preventing damage of tissue, and serving as a coenzyme for several reactions (106). As an antioxidant, GSH plays a major role in GSH peroxidase (GPx)—catalyzed reduction of H_2O_2, which can in turn be reduced into GSH again by GSH reductase (GR). Because of its function and omnipresence, GSH can also be employed as a biomarker of oxidative stress. More specifically, it has been found that diabetic patients have reduced GSH/GSSG ratios, causing inflammation, hyperlipidemia, and antioxidant imbalance (107, 108).

Oxidative stress exhibits itself in elevated ROS synthesis and oxidation of circulating low-density lipoprotein molecules. Oxidized low-density lipoprotein (oxLDL) activates the immune system and promotes inflammation by inducing dendritic cell maturation and T cell activation (109). The levels of oxLDL were found to be higher in patients with T2D and obesity-related traits relative to controls (110). Other studies have shown that oxLDL levels can predict the onset of the metabolic syndrome (111).

Damage due to oxidative stress can be measured by the amounts of primary or secondary products of peroxidation. One of the secondary byproducts of this reaction are F_2-isoprostanes (F_2-IsoP) (112). F_2-isoprostanes are a group of prostaglandins (PG). F_2-like products and are involved in many diseases including T2D. Diabetic patients have higher levels of F_2-IsoP than controls and can be used as a standard biomarker of oxidative stress in patients with T2D (113).

8-hydroxy-2'-deoxyguanosine (8-OHdG) is a modified guanine that is considered a sensitive indicator of oxidative DNA damage (114). A total of 8-OHdG can be used to measure the extent of oxidative stress in the human body as it relates to DNA oxidation ratio and repair of DNA (115, 116). The levels of urine 8-OHdG increases in diabetic patients, and the amount of 8-OHdG generally correlated with the severity of complications such as diabetic nephropathy (117).

Total antioxidant capacity (TAC) is a cumulative measure of small molecule antioxidants and proteins, or the amounts of small molecules alone (118). TAC is often employed to assess a biological sample's total antioxidant status (TAS). Since the synergistic impact of antioxidants is known to serve greater defense against free radicals than any antioxidant by itself, evaluating overall capacity can provide more insight to the body's collective mechanisms (119). A negative correlation was observed between HbA1c and plasma TAC and TAS among middle-aged diabetic patients in comparison to healthy controls of similar age (120, 121).

Various factors govern the potential development of T2D interventions using microorganisms to permeate gut microbiota in the host with the aim of targeting biomarkers of inflammation and oxidative stress. Therefore, there is a need in connecting and reporting of the complex data in the literature with aim of distilling T2D interventions using biotics as an effective treatment. **Table 1** provides the reference values of each of the above listed biomarkers of inflammation and oxidative stress as seen in controls and those with T2D. 2.

METHODS

Literature Sources and Searches

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol (122). Searches were conducted in PubMed, Scopus, Web of Science, Embase, and Cochrane. We also searched for gray literature through ClinicalTrials.org and ProQuest Dissertations and Theses. The initial search took place in June 2020 and we reran a final search in January 2022 to gather any new records that might have been produced or published.

The search strategy in PubMed included the following elements:

("Probiotics"[MeSH Terms] OR "probiotics"[Title/Abstract] OR "probiotic"[Title/Abstract] OR "Prebiotics"[MeSH Terms] OR "prebiotic"[Title/Abstract] OR "prebiotics"[Title/Abstract] OR "Synbiotics"[MeSH Terms] OR "synbiotics"[Title/Abstract] OR "synbiotic"[Title/Abstract] OR "symbiotic"[Title/Abstract] OR "symbiotics"[Title/Abstract] OR "gastrointestinal microbiome"[MeSH Terms] OR "gut microbiome"[Title/Abstract] OR "gut flora"[Title/Abstract]) AND ("diabetes mellitus, type 2"[MeSH Terms] OR "T2D"[Title/Abstract] OR "type 2 diabetes"[Title/Abstract]).

TABLE 1 | Reference levels of markers of inflammation and oxidative stress in patients with type 2 diabetes and controls.

Marker (units)	T2D	Controls	References
CRP (mg/mL)	3.05 ± 3.92	0.8 ± 1.63	(74)
TNF-a (pg/mL)	8.3 ± 49.9	2.7 ± 4.6	(74)
IL-6 (pg/mL)	9.2 ± 52.1	2.9 ± 4.3	(74)
IL-1β (pg/mL)	30.0 ± 1.2	11.3 ± 1.1	(240)
IL-8 (pg/mL)	69.27 ± 112.8	16.03 ± 24.2	(74)
IL-10 (pg/mL)	9.53 ± 2.27	16.11 ± 2.27	(241)
IL-12 (pg/mL)	147.1 ± 66.4	69.3 ± 41.6	(77)
IL-17 (pg/mL)	13.32 ± 2.87	5.23 ± 4.18	(242)
SOD (U/gm P/mL)	5.72 ± 0.98	6.67 ± 1.22	(243)
GSH (mg%)	12.20 ± 1.84	14.21 ± 2.55	(243)
GPx (U/gm Hb)	5.92 ± 0.64	8.44 ± 1.17	(243)
GR (U/gm P)	15.24 ± 0.73	16.53 ± 0.41	(243)
CAT (U/gm P/mL)	6.68 ± 0.97	5.79 ± 0.58	(243)
MDA (nmol/mL)	7.09 ± 1.15	4.69 ± 0.72	(243)
F_2-IsoP (pg/mL)	33.4 ± 4.8	22.2 ± 1.9	(113)
8-OhdG (nmol/mol crea)	2.93 ± 1.78	2.14 ± 0.94	(117)
TAS (mM)	2.19 ± 0.85	1.86 ± 0.65	(244)
Plasma NO_2^- (μ mol/L)	0.21 ± 0.22	0.16 ± 0.19	(101)
Plasma NO_3^- (μ mol/L)	58.5 ± 42.8	34.5 ± 15.6	(101)
oxLDL-Ab (EU/mL)	26.37 ± 12.86	18.31 ± 8.69	(245)

Inclusions

Studies had to be clinical trials and randomized studies relevant to the effect of diet using probiotics, prebiotics, and symbiotics on T2D to Prebiotics, Probiotics, and Synbiotics and T2D. Studies of adults of any age, sex, ethnicity, from any region worldwide, and published at any time were included. Further, only those studies reporting on markers of inflammation and/or oxidative stress were included among the final analysis. Reports available from inception of respective databases to accessed dates (final search: January 2022) were included. Covidence was utilized for importing and screening titles, abstracts and full-texts; extraction was performed using MS Excel.

Exclusions

The paper excluded studies on other type of diabetes, and any reviews, conferences, abstracts and proceedings, editorial and non-clinical papers, as well as animal studies. Studies in other language than English were excluded too. Studies not reporting on markers of inflammation and/or oxidative stress were excluded among the final analysis.

After removing duplicates, the authors independently scanned the title and abstract of all articles referring to the inclusion and exclusion criteria. The same process was also used for full-text screening and any conflicts were resolved by consensus. The included articles were processed for qualitative analysis and the relevant information were grouped by themes and expanded through the discussion.

RESULTS

Review Process for Data Extraction

According to the PRISMA flowchart (see **Figure 1**), a total of 5,985 studies were imported from all databases, from which 3,250 duplicates were excluded, while 2,377 articles were found irrelevant through title and abstract screening and 20 others could not be retrieved. Therefore, a total of 338 articles were assessed for eligibility out of which only 47 were included in the final review. The reasons for excluding 311 full-text articles from the study can be summarized as follows: 68 studies did not have the adequate study protocol; 52 studies were non-clinical; 56 studies were not relevant; 35 did not report on the levels of inflammation or oxidative stress; 24 studies were systematic reviews and meta-analysis; 20 studies were not available in full-text; 19 studies had incorrect intervention; 17 studies were reviews, abstract, or proceedings; 17 studies were duplicates; and 3 studies were not in English language.

Effect of Probiotics on Inflammatory and Oxidative Stress Markers in Type 2 Diabetes

Pro-inflammatory Markers in Type 2 Diabetes

Effect on C-Reactive Protein and High-Sensitivity C-Reactive Protein

Sixteen studies have investigated and reported the effect of probiotics on either CRP or High-Sensitivity C-Reactive Protein (hs-CRP). While not all studies have reported significant ($p \leq 0.05$) effects, most have shown that administration of probiotics leads to a general decreasing trend in these markers of inflammation (**Table 2**).

Sabico et al. (123) reported a significant decrease of 2.9 mg/L (-52.7%) in CRP following supplementation of a multi-species probiotic in a cohort of 31 T2D patients when compared to baseline values; however, these results were not found to be significant compared to control despite an increase of 0.4 mg/L (+ 13.3%) in CRP markers in this group. Another group of researchers from Iran reported a significant decrease of 0.777 ± 0.441 mg/L of hs-CRP following an 8-week intervention of probiotics compared to a control group (124). Similar significant results have been reported following 12-week multi-species probiotic supplementation in three cohorts of T2D patients with coronary heart disease (CHD), with adjusted mean differences of –1.04, –0.88, and –0.95 mg/L in hs-CRP compared to control (125–127). Three other studies investigating the effects of multi-species produced insignificant results, with two reporting slight increases and another reporting a decrease in hs-CRP compared to baseline values or that of control (128–130). Of interest, Bayat et al. (131) have shown that an 8-week probiotic of an unknown number of species administration with and without *Cucurbita ficifolia* resulted in significant reductions in serum hs-CRP compared to baseline, control, and another receiving only *C. ficifolia*.

The results of single-species probiotic supplementation on CRP and hs-CRP appear to be non-conclusive, with independent studies reporting largely non-significant ($p > 0.05$) mean increase and decrease in these markers in the intervention groups compared to control or baseline. Andreasen et al. (132) and Feizollahzadeh et al. (133) have reported mean decreases of 0.3 and 0.2 mg/L in CRP compared to baseline following supplementation with soymilk containing *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum)* and freeze-dried *Lactobacillus acidophilus* in T2D-associated nephropathic patients and a mixed group of diabetic or those with impaired or normal glucose tolerance, respectively. Hsieh et al. (134) have reported that consuming live and heat-killed *Limosilactobacillus reuteri* (previously *Lactobacillus reuteri*) resulted in an increase of 0.3 ± 1.9 and 0.5 ± 2.4 mg/L from baseline(s), respectively, in two cohorts of T2D patients without any specified/reported comorbidities; although, since these results were all non-significant, no solid conclusion can be made regarding the effect of the difference in probiotic species administered or the presence of diagnosed comorbidities. Similar conflicting and non-significant results have been shown for changes in hs-CRP following single-probiotic supplementation, while one study by Sato et al. (135) have shown that Shirota-fermented milk with *Lacticaseibacillus casei* (previously *Lactobacillus casei*) significantly increased detectable hs-CRP levels by 92.0 mg/dL compared to an increase of 32.5 mg/dL in the control group over a period of 16 weeks.

Effect on Tumor Necrosis Factor-α

Eleven studies have investigated and reported the effect of probiotics either single or multi-species, on TNF-α. While

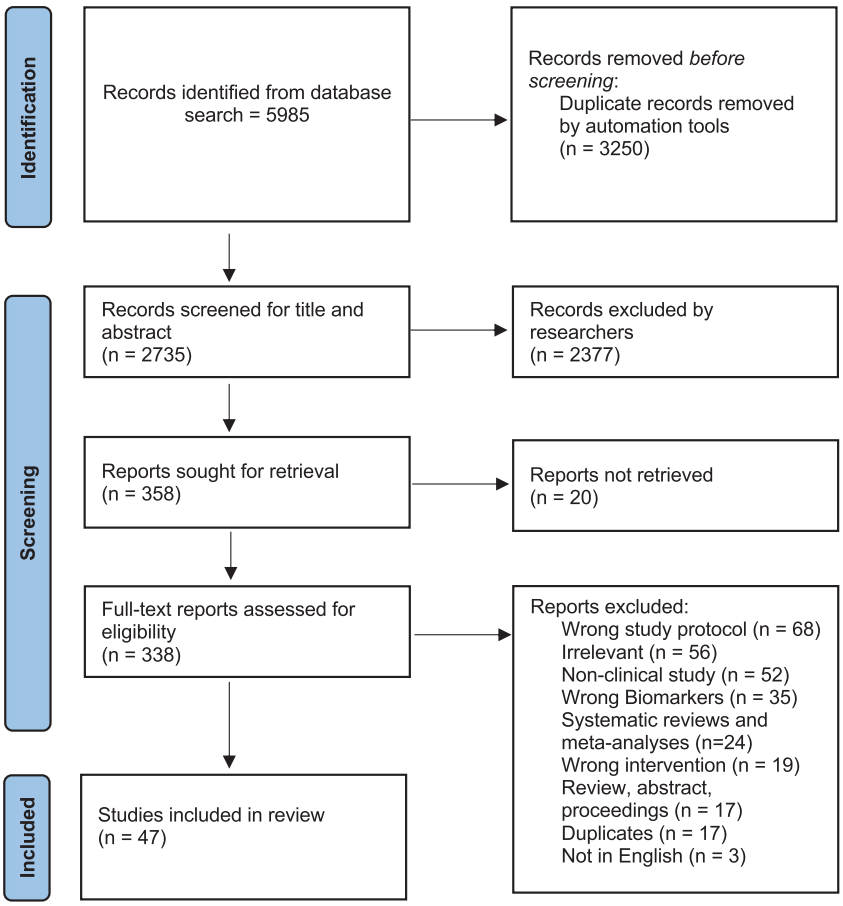

FIGURE 1 | Flowchart of search strategy, inclusion, and exclusion criteria.

some studies have reported non-significant ($p > 0.05$) changes post probiotic supplementation, most trials have successfully concluded that administration of probiotics lead to significant overall decreases in levels of this pro-inflammatory marker (**Table 2**).

Sabico et al. (123) reported that a multi-species probiotic supplementation led to a 0.6 pg/mL or 66.7% decrease from baseline in a cohort of diabetic patients from Saudi Arabia. Similarly, another group reported a near 33% reduction in the marker from baseline, a change that was also found to be significant compared to control (129). Through a series of four studies, Kobyliak et al. (136–139) have reported significant changes of −5.81 ± 9.13, −7.95 ± 1.27, −5.02 ± 9.33, and −6.75 ± 7.73 pg/mL in TNF- α from baseline compared to control following multi-species probiotic supplementation.

On the other hand, there is no consensual trend among studies investigating the effect of single-species probiotics due to the lack of statistically significant observations. However, interestingly, *L. reuteri* supplementation produced a directionally different change depending on whether the bacteria was provided in heat-killed or live form (134). Other studies have reported little to no change in TNF-α following single-species probiotic administration (132, 133, 135).

*Effect on Interleukin-1*β

Information for the effect of single or multi species probiotics on IL-1β is available from five clinical studies, each reporting significant results of decrease in marker compared to either baseline or control in the intervention groups (**Table 2**).

Hsieh et al. (134) have demonstrated that supplementation with heat-killed *L. reuteri* over a course of 6 months lead to a significant decrease of 1.43 ± 2.70 pg/mL in IL-1β compared to the change in control group over the same period. Interestingly, supplementation of the live form of the same bacteria in a lower dose resulted in a 50% reduction in this change from baseline and was not significant compared to control in the same study.

The effect of multi-species probiotic supplementation has been more consistently reproduced across multiple studies by Kobyliak et al. (136–139). Based on multispecies probiotic "Symbiter Forte" supplementation across four cohorts of diabetics, the group has reported significant mean differences of −4.1 ± 8.36, −4.91 ± 8.23, −6.74 ± 15.59, and −5.44 ± 1.51 pg/mL in serum IL-1β from baseline, with the latter two results also reported to be statistically significant compared to effects of placebos with organoleptically similar formulation as the probiotic media.

TABLE 2 | Studies investigating the effect of probiotics on markers of inflammation and oxidative stress in T2D.

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Probiotic (Single Sp.)	DB, PC, R (Denmark)	Patients with T2D, IGT or NGT $n = 24$ (24M) 60 (55-66) 28.7 (26.1-31.3)	Patients with T2D, IGT or NGT $n = 21$ (21M) 55 (48-61) 28.1 (25.1-31.1)	1:1 ratio of silicium dioxide and lactose	Freeze-dried *Lactobacillus acidophilus* NCFM capsules (1x10^{10} CFU/g)	1 × 1g/d	4 weeks	TNF-α (§)	0 pg/ml vs +0.1 pg/ml (§)	(132)
								↑IL-6 (§)	+0.1 pg/ml vs +0.2 pg/ml (§)	
								↑IL-1ra (§)	+2 pg/ml vs +31 pg/ml (§)	
								↓CRP (§)	−0.2 mg/l vs +0.4 mg/l (§)	
Probiotic (Single Sp.)	DB, R, PC (Taiwan)	$n = 22$ (13M/9F) 55.77 ± 8.55 27.53 ± 3.15	ADR1 group $n = 22$ (12M/10F) 52.32 ± 10.20 28.04 ± 4.29	NS	Live *Limosilactobacillus reuteri*, ADR-1 (2 × 10^9 CFU/capsule)	2 × 1 capsules/d	6 months + 3 months follow-up	↓IL-1β (§)	−0.72 ± 1.94 pg/ml vs 0.21 ± 1.52 pg/ml (§)	(134)
								↑CRP (§)	+0.03 ± 0.19 mg/dl vs +0.08 ± 0.28 mg/dl (§)	
								↑IL-6 (§)	+0.95 ± 2.65 ng/ml vs +0.90 ± 1.80 ng/ml (§)	
								↑IL-10 (§)	+1.48 ± 3.09 ng/ml vs +1.04 ± 2.41 ng/ml (§)	
								↑IL-17 (§)	+0.66 ± 3.24 ng/ml vs −0.35 ± 3.63 ng/ml (§)	
								↓TNF-α (§)	−32.00 ± 81.24 pg/ml vs −3.07 ± 72.22 pg/ml (§)	
								↑SOD (§)	+0.93 ± 0.95 U/ml vs +0.39 ± 0.82 U/ml (§)	
								↓GPX (§)	−0.34 ± 3.24 U/ml vs −0.40 ± 3.79 U/ml (§)	
			ADR3 group $n = 24$ (13M/11F) 53.88 ± 7.78 28.03 ± 3.88	NS	Heat-killed *Limosilactobacillus reuteri*, ADR-3 (1 × 10^{10} cells/capsule)	2 × 1 capsules/d	6 months + 3 months follow-up	↓IL-1β	−1.43 ± 2.70 pg/ml vs 0.21 ± 1.52 pg/ml	
								↑CRP (§)	+0.05 ± 0.24 mg/dl vs +0.08 ± 0.28 mg/dl (§)	
								↑IL-6 (§)	+1.55 ± 2.41 pg/ml vs +0.90 ± 1.80 pg/ml (§)	
								↑IL-10 (§)	+2.05 ± 3.25 ng/ml vs +1.04 ± 2.41 ng/ml (§)	
								↑IL-17 (§)	+0.47 ± 2.91 ng/ml vs −0.35 ± 3.63 ng/ml (§)	
								↑TNF-α (§)	+12.81 ± 86.00 pg/ml vs −3.07 ± 72.22 pg/ml (§)	
								↑SOD (§)	+0.04 ± 1.31 U/ml vs +0.39 ± 0.82 U/ml (§)	
								↓GPX (§)	−0.27 ± 4.36 U/ml vs −0.40 ± 3.79 U/ml (§)	

(Continued)

TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Probiotic (Single Sp.)	R, DB, C, CT (Iran)	Control Group *n* = 27 (12M/15F) 58.2 ± 11.8 BMI NR	Probiotic Group *n* = 30 (10M/20F) 59.7 ± 12.2 29.8 ± 5.7 BMI NR	Capsule containing 0.5 g of rice flour powder	Capsule containing *Lactobacillus acidophilus* (10^8 CFU)	1 capsule/d	3 months	↑CAT	2.44 ± 0.50 U/ml (I, 3m) vs 1.95 ± 0.34 U/ml (C, 3m)	(144)
								↑GPX	92.15 ± 8.41 U/mL (I, 3m) vs 84.89 ± 6.52 U/mL (C, 3m)	
								↑SOD	4.58 ± 0.42 U/mL (I, 3m) vs 3.99 ± 0.27 U/mL (C, 3m)	
								↓OxLDL (§)	16.85 ± 1.53 mU/L (I, 3m) vs 17.07 ± 1.01 mU/L (C, 3m) (§)	
Probiotic (Single Sp.)	R, DB, C, CT (Iran)	Control Bread (CB) *n* = 27 (5M/22F) 53.4 ± 7.5 30.5 ± 4.1	Probiotic Bread *n* = 27 (5M/22F) 52.0 ± 7.2 29.8 ± 5.7	Control bread	Bread containing Bacillus coagulans (1×10^8 CFU/g)	40 × 3 g/d	8 weeks	↓hs-CRP (§)	−1,330.2 ± 2,924.1 ng/ml vs −586.9 ± 2,009.2 ng/ml (§)	(160)
Probiotic (Single sp.)	DB, R, PG, PC (Sweden)	T2D and obese patients* *n* = 15 (11M/4F) 65 ± 5 30.7 ± 4.0	T2D and obese patients*; Low Dose group *n* = 15 (12M/3F) 66 ± 6 30.6 ± 4.5	Capsule with mildly sweet tasting powder in an aluminum laminate stick pack	Capsule containing low-dose *Limosilactobacillus reuteri* DSM 17938 (10^8 CFU/capsule)	1 capsule/d	12 weeks	ůhs-CRP (§)	2.3 ± 2.8 mg/L (I, 12w) vs 2.3 ± 2.8 mg/L (I, B) (§)	(246)
			T2D and obese patients*; High dose group *n* = 14 (11M/3F) 64 ± 6 32.3 ± 3.4	Capsule with mildly sweet tasting powder in an aluminum laminate stick pack	Capsule containing high-dose *Limosilactobacillus reuteri* DSM 17938 (10^{10} CFU/capsule)	1 capsule/d	12 weeks	↑hs-CRP (§)	2.4 ± 2.1 mg/L (I, 12w) vs 2.0 ± 1.4 mg/L (I, B) (§)	
Probiotic (Single Sp.)	PG, DB, RCT (Iran)	T2D patients with nephropathy* *n* = 20 (10M/10F) 53.6 ± 1.6 26.58 ± 0.73	T2D patients with nephropathy* *n* = 20 (9M/11F) 56.90 ± 1.81 26.68 ± 0.71	Conventional soy milk	Probiotic soy milk containing Lactiplantibacillus plantarum A7 (2×10^7 CFU/mL)	200 mL/d	8 weeks	↑(x)TNF-α (§)	172.83 ± 7.6 pg/ml (I, 8w) vs 172.44 ± 5.7 pg/ml (I, B) (§)	(133)
								↓CRP (§)	4.2 ± 1.4 mg/L (I, 8w) vs 4.5 ± 1.9 mg/L (I, B) (§)	
Probiotic (Single Sp.)	PG, DB, RCT (Iran)	T2D patients with nephropathy* *n* = 20 (10M/10F) 53.6 ± 1.6 26.58 ± 0.73	T2D patients with nephropathy* *n* = 20 (9M/11F) 56.90 ± 1.81 26.68 ± 0.71	Conventional soy milk	Probiotic soy milk containing Lactiplantibacillus plantarum A7 (2×10^7 CFU/mL)	200 mL/d	8 weeks	↓MDA (§)	1.28 ± 0.11 μmol/L (I, 8w) vs. 1.35 ± 0.05 μmol/L (I, B) (§)	(141)

(Continued)

TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
								↓MDA	Significant reduction vs control, markers NS	
								↑TAC (§)	989.06 ± 30.25 mmol/L (I, 8w) vs. 960.06 ± 35.20 mmol/L (I, B) (§)	
								↑GSH	732.96 ± 61.95 μmol/L (I, 8w) vs. 600.66 ± 69.61 μmol/L (I, B); also significant MD vs control, markers NS	
								↓GSSG	19.00 ± 0.70 μmol/L (I, 8w) vs. 30.37 ± 0.20 μmol/L (I, B); also significant MD vs control, markers NS	
								↑GPX	1.30 ± 1.25 U/g Hb (I, 8w) vs. 0.87 ± 1.00 U/g Hb (I, B); also significant MD vs control, markers NS	
								↑GR	1.35 ± 0.05 U/g Hb (I, 8w) vs. 0.97 ± 1.02 U/g Hb (I, B); also significant MD vs control, markers NS	
Probiotic (Single Sp.)	R, DB, PC (Denmark)	*n* = 18 (18M) 60.6 ± 5.2 27.7 ± 3.3	*n* = 23 (23M) 58.5 ± 7.7 29.2 ± 3.8	Artificially acidified milk	"Cardi04" yogurt containing *Lactobacillus helveticus*	300 × 1 mL/d	3 months	↑(x) hsCRP (§)	0.6 (0.4; 1.6) mg/L (I, 3m) vs 0.7 (0.4; 2.1) mg/L (I, B) (§)	(247)
								↑TNF-α (§)	1.2 ± 0.4 pg/ml (I, 3m) vs 1.1 ± 0.3 pg/ml (I, B) (§)	
Probiotic (Single Sp.)	R, DB, PC, CT (Iran)	Control Bread (CB) *n* = 27 (5M/22F) 53.4 ± 7.5 30.5 ± 4.1	Probiotic Bread *n* = 27 (5M/22F) 52.0 ± 7.2 29.8 ± 5.7	Control bread	Bread containing Bacillus coagulans (1 × 10^8 CFU/g)	40 × 3 g/d	8 weeks	↑NO (§)	+18.5 ± 36.2 μmol/L vs −0.8 ± 24.5 μmol/L (§)	(142)
								↑TAC (§)	+78.6 ± 218.4 mmol/L vs. −45.7 ± 240.3 mmol/L (§)	
								↑GSH (§)	+6.2 ± 347.2 μmol/L vs +18.8 ± 417.8 μmol/L	
								↑CAT (§)	+4.1 ± 20.2 U/mL vs +2.7 ± 14.9 U/mL	
								↑MDA (§)	+0.6 ± 1.7 μmol/L vs +0.5 ± 1.5 μmol/L (§)	
Probiotic (Single sp.)	PG, DB, RCT (Iran)	T2D patients with nephropathy* *n* = 20 (10M/10F) 53.6 ± 1.6 26.58 ± 0.73	T2D patients with nephropathy* *n* = 20 (9M/11F) 56.90 ± 1.81 26.68 ± 0.71	Conventional soy milk	Probiotic soy milk containing Lactiplantibacillus plantarum A7 (2 × 10^7 CFU/mL)	200 mL/d	8 weeks	↑SOD	Markers NS	(147)
								↓8-OHdG	Markers NS	

(Continued)

TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Probiotic (Single sp.)	R, PC (Japan)	*n* = 34 (20M/14F) 65.0 ± 8.3 24.6 ± 2.6	*n* = 34 (29M/5F) 64.0 ± 9.2 24.2 ± 2.6	Fermented milk without probiotics	Lacticaseibacillus casei strain Shirota-fermented milk (> 4 × 10^{10} cells per bottle)	80 mL/d	16 weeks	↑hs-CRP	+92.0 mg/dL vs +32.5 mg/dL	(135)
								·TNF-α (§)	+0.0 ± 0.5 pg/ml vs +0.0 ± 0.3 pg/ml (§)	
								↑IL-6 (§)	+0.4 ± 2.8 pg/ml vs +0.2 ± 1.0 pg/ml (§)	
								·LBP (§)	+0.0 ± 3.8 μg/mL vs −0.5 ± 4.0 μg/mL (§)	
Probiotic (Multi sp.)	DB, PC, RCT (Saudi Arabia)	*n* = 30 (NS) 46.6 ± 5.9 30.1 ± 5.0	*n* = 31 (NS) 48.0 ± 8.3 29.4 ± 5.2	Freeze-dried maize starch and maltodextrins	EcologicBarrier (2.5 × 10^9 CFU/g; Bifidobacterium bifidum W23, *Bifidobacterium animalis* subsp. lactisW52, *Lactobacillus acidophilus* W37, *Levilactobacillus brevis*W63, *Lacticaseibacillus casei* W56, *Ligilactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58) with maize starch and maltodextrins	2 × 2 g/d	6 months	↓IL-6	−3.9 pg/ml (−76.5%)	(123)
								↓IL-6 (§)	−3.9 pg/ml (−76.5%) vs −2.8 pg/ml (−77.8%) (§)	
								↓TNF-α	−0.6 pg/ml or −66.7%	
								↓TNF-α (§)	−0.6 pg/ml (−66.7%) vs −0.2 (−40.0%) (§)	
								↓CRP	−2.9 μg/ml or −52.7%	
								↓CRP (§)	−2.9 μg/ml (−52.7%) vs +0.4 μg/ml (+13.3%) (§)	
								↓Endotoxin	−3.2 IU/ml or −69.6%	
								↓Endotoxin (§)	−3.2 IU/ml (−69.6%) vs +0.8 IU/ml (+38.1%) (§)	
Probiotic (Multi sp.)	DB, PC, RCT (Saudi Arabia)	*n* = 39 (21M/18F) 46.6 ± 5.9 30.1 ± 5.0	*n* = 39 (19M/20F) 48.0 ± 8.3 29.4 ± 5.2	Maize starch and maltodextrins	EcologicBarrier (2.5 × 10^9 CFU/g) containing *Bifidobacterium bifidum* W23, *Bifidobacterium animalis* subsp. *lactis*W52, *Lactobacillus acidophilus* W37, *Levilactobacillus brevis*W63, *Lacticaseibacillus casei* W56, *Ligilactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58 with maize starch and maltodextrins	2 × 2 g/d	3 months	↓Endotoxin	−2.40 IU/mL (−52.2%) vs −0.20 (−9.5%) IU/mL	(140)

(Continued)

TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
								↑Endotoxin (§)	+0.15 IU/mL (§)	
Probiotic (Multi sp.)	DB, R, C, CT (Iran)	*n* = 30 (12M/18F) 51.00 ± 7.32 29.14 ± 4.30	*n* = 30 (11M/19F) 50.87 ± 7.68 28.95 ± 3.65	Conventional yoghurt containing Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus	Probiotic yoghurt containing *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium animalis* subsp. *lactis* Bb12 (1.79–6.04 × 10^6 CFU/g) and *Lactobacillus acidophilus* La5 (1.85–7.23 × 10^6 CFU/g)	300 g/d	6 weeks	↑Erythrocyte SOD	1113.69 ± 177.77 U/g Hb (I, 6w) vs 975.80 ± 238.34 U/g Hb (I, B); significant MD vs control, markers NS	(143)
								↑GPX	29.81 ± 4.58 U/g Hb (I, 6w) vs 29.03 ± 4.29 U/g Hb (I, B); significant MD vs control, markers NS	
								↓CAT (§)	146.57 ± 34.05 K/g Hb (I, 6w) vs 148.81 ± 34.56 K/g Hb (I, B) (§)	
								↑TAS	0.96 ± 0.18 mmol/L (I, 6w) vs 0.90 ± 0.18 mmol/L (I, B); significant MD vs control, markers NS	
								↓MDA	2.53 ± 0.65 μmol/L (I, 6w) vs 2.79 ± 0.62 μmol/L (I, B)	
Probiotic (Multi sp.)	DB, R, C, CT (Iran)	T2D and overweight patients* *n* (I+C) = 42 (10M/32F) 49.00 ± 7.08 29.22 ± 3.20	T2D and overweight patients* *n* (I+C) = 42 (10M/32F) 53.00 ± 5.9 28.36 ± 4.14	Conventional yoghurt containing Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus	Probiotic yoghurt containing *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium animalis* subsp. *lactis* Bb12 (~3.7 × 10^6 CFU/g) and *Lactobacillus acidophilus* La5 (~3.7 × 10^6 CFU/g)	300 g/d	8 weeks	↓hs-CRP (§)	2.80 ± 1.48 mg/l (I, 8w) vs 3.26 ± 1.36 mg/l (I, B) (§)	(129)
								↓IL-6 (§)	22.18 ± 2.56 pg/ml (I, 8w) vs 22.60 ± 2.81 (I, B) (§)	
								↓TNF-α	2.92 ± 1.16 pg/ml (I, 8w) vs 4.36 ± 1.9 pg/ml (I, B); also significant MD vs control, markers NS	

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TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Probiotic (Multi sp.)	SB, CT (Iran)	n = 18 (I+C = 8M/26F) 51.8 ± 10.2 27.24 ± 2.73	n = 16 (I+C = 8M/26F) 55.4 ± 8 27.97 ± 3.81	1000g Magnesium stearate/1500 mg capsule	*Lactobacillus acidophilus*, *L. bulgaricus*, *L. bifidum and L. casei*	2 × 1500 mg/d	6 weeks	↓MDA (§)	4.24 ± 0.44 μmol/L (I, 6w) vs 5.09 ± 0.53 μmol/L (I, B) (§)	(128)
								↓IL-6 (§)	3.83 ± 0.35 pg/ml (I, 6w) vs 4.51 ± 0.45 pg/ml (I, B) (§)	
								↑hs-CRP (§)	4333.81 ± 1256.6 ng/ml (I, 6w) vs 3174.87 ± 701.77 ng/ml (I, B) (§)	
Probiotic (Multi sp.)	R, DB, PC, CT (Iran)	n = 27 (Sex NS) 52.59 ± 7.14 30.17 ± 4.23	n = 27 (Sex NS) 50.51 ± 9.82 31.61 ± 6.36	100 mg fructo-oligosaccharide with lactose/capsule	Freeze-dried *Lactobacillus acidophilus* (2 × 10^9 CFU), L. casei (7 × 10^9 CFU), *L. rhamnosus* (1.5 × 10^9 CFU), *L. bulgaricus* (2 × 10^8 CFU), *Bifidobacterium breve* (2 × 10^{10} CFU), *B. longum* (7 × 10^9 CFU), *Streptococcus thermophilus* (1.5 × 10^{9}CFU), and 100 mg fructo-oligosaccharide with lactose/capsule	1 capsule/d	8 weeks	↓hs-CRP	−777.57 ± 441.7 ng/ml vs +878.72 ± 586.44 ng/ml	(124)
								↑TAC (§)	+379.97 ± 41.8 mmol/L vs. +84.94 ± 24.32 mmol/L (§)	
								↑GSH	+240.63 ± 101.29 μmol/l vs −33.46 ± 69.54 μmol/l	
Probiotic (Multi sp.)	R, DB, PG, PC (Brazil)	n = 22 (14M/8F) 50.95 ± 7.20 27.94 ± 4.15	n = 23 (12M/11F) 51.83 ± 6.64 27.49 ± 3.97	Conventional fermented goat milk with Streptococcus thermophilus TA-40	Probiotic fermented goat milk with *Lactobacillus acidophilus* La-5 (1.62-77.2 × 10^6 CFU/g) and *Bifidobacterium animalis* subsp. *lactis* BB-12 (1.56–44.5 × 10^7 CFU/g)	120 g/d	6 weeks	↑TAS (§)	+0.01 mM (−0.07 to +0.08) (§)	(146)
								↓F2-iso (§)	−2.59 pg/ml (−21.41 to 16.23) (§)	
Probiotic (Multi sp.)	DB, PC, PG, RCT (Ukraine)	n = 26 (NR) 55.73 ± 8.76 35.63 ± 7.76	n = 28 (NR) 56.29 ± 11.14 35.66 ± 5.35	Organoleptically similar formulation as intervention	Multiprobiotic symbiter forte omega combination of *Lactobacillus* (1.0 × 10^9 CFU/g), *Bifidobacterium* (1.0 × 10^9 CFU/g), *Lactococcus* (1.0 × 10^8 CFU/g), *Propionibacterium* (1.0 × 10^8 CFU/g), Acetobacter (1.0 × ^{10} CFU/g), 2.0% bentonite, 3.0% wheat germ oil feed, 2.5% flax seed oil and, 2.5% wheat germ with 0.5–5% omega-3	10 × 1 g/d	8 weeks	↓TNF-α	−5.81 ± 9.13 pg/ml vs +0.38 ± 9.05 pg/ml	(138)
								↓IL-1β	−4.1 ± 8.36 pg/ml	
								↓IL-1β (§)	−4.1 ± 8.36 pg/ml vs −1.67 ± 7.20 pg/ml (§)	
								↓IL-6	−6.77 ± 9.62 pg/ml	
								↓IL-6 (§)	−6.77 ± 9.62 pg/ml vs −4.70 ± 11.38 pg/ml (§)	

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TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
								↓ IL-8	−8.99 ± 21.11 pg/ml	
								↓ IL-8 (§)	−8.99 ± 21.11 pg/ml vs −1.83 ± 33.78 pg/ml (§)	
								↓ IFN-λ (§)	−3.11 ± 39.94 pg/ml vs +7.79 ± 40.63 pg/ml (§)	
Probiotic (Multi sp.)	DB, PC, PG, RCT (Ukraine)	Patients with T2D and NAFLD $n = 24$ (NR) 57.38 ± 9.92 32.55 ± 3.62	Patients with T2D and NAFLD $n = 26$ (NR) 53.23 ± 10.09 33.19 ± 4.93	Organoleptically similar formulation as intervention	Symbiter forte (combination of 250 mg smectite gel and *Bifidobacterium* (1×10^{10} CFU/g), *Lactobacillus* + *Lactococcus* (6×10^{10} CFU/g), acetobacter (1×10^6 CFU/g) and SCFAs producing *Propionibacterium* (3×10^{10} CFU/g) genera)	10 × 1 g/d	8 weeks	↓ IL-1β	−6.74 ± 15.59 pg/ml or −6.00 ± 33.0 %	(139)
								↓TNF-α	−6.75 ± 7.73 pg/ml or −12.17 ± 14.4 %	
Probiotic (Multi sp.)	SC, DB, PC, PG, RCT (Ukraine)	$n = 22$ (NR) 57.18 ± 2.06 35.65 ± 1.57	$n = 31$ (NR) 52.23 ± 1.74 34.70 ± 1.29	Organoleptically similar formulation as intervention	Multiprobiotic "Symbiter" combination of *Lactobacillus* + *Lactococcus* (6×10^{10} CFU/g), *Bifidobacterium* (1.0×10^{10} CFU/g), *Propionibacterium* (3×10^{10} CFU/g), *Acetobacter* (1.0×10^6 CFU/g)	10 × 1 g/d	8 weeks	↓TNF-α	−7.95 ± 1.27 pg/ml vs +1.03 ± 2.07 pg/ml	(136)
								↓IL-1β	−5.44 ± 1.51 pg/ml vs +0.45 ± 1.97 pg/ml	
								↓IL-6	−3.45 ± 1.48 pg/ml	
								↓IL-6 (§)	−3.45 ± 1.48 pg/ml vs −1.89 ± 1.28 pg/ml (§)	
								↓ IL-8	−3.80 ± 1.05 pg/ml	
								↓ IL-8 (§)	−3.80 ± 1.05 pg/ml vs −3.85 ± 1.66 pg/ml (§)	
								↓ IFN-λ (§)	−13.80 ± 7.04 pg/ml vs +6.16 ± 8.88 pg/ml (§)	
Probiotic (Multi sp.)	PC, DB, RCT (Ukraine)	$n = 27$ (Sex NR) 56.93 ± 9.88 32.28 ± 6.08	$n = 28$ (Sex NR) 53.82 ± 9.58 31.99 ± 6.02	Organoleptically similar formulation as intervention	Symbiter forte (combination of 250 mg smectite gel and *Bifidobacterium* (1×10^9 CFU/g), *Lactobacillus* (1×10^9 CFU/g), *Lactococcus* (1×10^8 CFU/g), *Acetobacter* (1×10^5 CFU/g) and *Propionibacterium* (1×10^8 CFU/g) genera	10 × 1 g/d	8 weeks	↓ IL-1β (§)	−4.91 ± 8.23 pg/ml vs −0.41 ± 9.61 pg/ml (§)	(137)
								↓ IL-1β	−4.91 ± 8.23 pg/ml	
								↓TNF-α	−5.02 ± 9.33 pg/ml vs +0.69 ± 10.01 pg/ml	
								↓IL-6	−4.11 ± 7.15 pg/ml vs −0.70 ± 5.80 pg/ml	
								↓ IL-8 (§)	−5.6 ± 13.92 pg/ml vs −2.16 ± 11.49 pg/ml (§)	
								↓ IL-8	−5.6 ± 13.92 pg/ml	

(Continued)

TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose x frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Probiotic (Multi sp.)	DB, R, PG, PC (Malaysia)	n = 68 (34M/34F) 54.2 ± 8.3 29.3 ± 5.3 n = 53 (PP analysis)	n = 68 (31M/37F) 52.9 ± 9.2 29.2 ± 5.6 n = 47 (PP analysis)	Organoleptically similar sachets without probiotic	Sachets containing viable microbial cell preparation of *Lactobacillus acidophilus*, *L. casei*, *L. lactis*, *Bifdobacterium bifdum*, *B. longum* and *B. infantis* (0.5×10^{10} CFU, each) in 250 mL water	2 sachets/d	12 weeks	↑hs-CRP (§)	+0.23 ± 2.7 mg/L vs −0.36 ± 3.0 mg/L (§)	(130)
Probiotic (Multi sp.)	R, DB, PC (Iran)	T2D and CHD patients* n = 27 (10M/17F)) 62.4 ± 13.1 29.9 ± 5.0	T2D and CHD patients* n = 27 (11M/16F) 64.8 ± 8.3 31.4 ± 5.8	"Barij Essence"	LactoCare containing *Lactobacillus acidophilus*, *L. reuteri*, *Limosilactobacillus fermentum* and *Bifidobacterium bifidum* (2×10^9 CFU/g each) and 200 μg/d selenium yeast	1/d	3 months	↑NO	+7.86 μmol/L	(125)
								↑GSH	+154.16 μmol/L	
								↑TAC	+119.30 mmol/L	
								↓hs-CRP	−1043.28 ng/mL	
								↑MDA (§)	+0.10 μmol/L (§)	
Probiotic (Multi sp.)	R, DB, PC (Iran)	Patients with T2D and CHD* n = 30 (Sex NS) 61.8 ± 9.8 29.3 ± 4.1	Patients with T2D and CHD* n = 27 (Sex NS) 60.7 ± 9.4 30.3 ± 5.2	NS	Supplements containing *Bifdobacterium bifdum* (2×10^9 CFU/d), *L. casei* (2×10^9 CFU/d), *Lactobacillus acidophilus* (2×10^9 CFU/d)	1/d	3 months	↑NO	+4.28 μmol/L	(126)
								↑GSH	+45.15 μmol/L	
								↑TAC	+108.44 mmol/L	
								↓hs-CRP	−0.88 mg/L	
								↓MDA (§)	−0.23 μmol/L (§)	
Probiotic (Multi sp.)	R, DB, PC (Iran)	Patients with T2D and CHD* n = 30 (14M/16F) 67.3 ± 11.0 28.2 ± 4.9	Patients with T2D and CHD* n = 30 (16M/14F) 71.5 ± 10.9 29.0 ± 6.2	NS	50,000 IU vitamin D3 every 2 weeks and *Lactobacillus acidophilus*, *L. reuteri*, *Limosilactobacillus fermentum* and *Bifidobacterium bifidum* (each 2×10^9 CFU/g)	1/d	12 weeks	↑NO	+1.7 ± 4.0 μmol/L vs −1.4 ± 6.7 μmol/L	(127)
								↑GSH (§)	+18.0 ± 112.7 μmol/L vs −12.2 ± 122.5 μmol/L (§)	
								↑TAC	+12.6 ± 41.6 mmol/L vs −116.9 ± 324.2 mmol/L	
								↓hs-CRP	−950.0 ± 1811.2 ng/mL vs +260.5 ± 2298.2 ng/mL	
								↓MDA	−0.1 ± 0.3 μmol/L vs +0.1 ± 0.7 μmol/L	

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TABLE 2 | (Continued)

Type of nutraceutical	Study design, country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total period of intervention/study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Probiotic (Sp. NS)	PG, R, CT (Iran)	(C1) C. ficifolia group $n = 20$ (12M/8F) 51.8 ± 2.24 28.95 ± 3.34 (C2) Dietary advice group $n = 20$ (9M/11F) 46.95 ± 9.34 29.75 ± 4.66	Probiotic yogurt group $n = 20$ (3M/17F) 54.1 ± 9.54 28.77 ± 4.59	(1) C. ficifolia (2) Dietary Advice	Probiotic (species NS) yogurt	(C1) 100 × 1 g/d (C2) NS (I) 150 × 1 g/d	8 weeks	↓hs-CRP	1.13 ± 0.29 mg/L (I, 2w) vs 1.29 ± 0.27 mg/L (I, B); significant vs dietary advice group, markers NS	(131)
					Probiotic (species NS) yogurt and *C. ficifolia*	150 × 1 g/d probiotic yogurt, 100 × 1 g/d *C. ficifolia*	8 weeks	↓hs-CRP	1.13 ± 0.34 mg/L (I, 2w) vs 1.69 ± 0.25 mg/L (I, B); significant vs dietary advice group, markers NS	

**All participants are T2D-diagnosed patients, unless otherwise stated; ↓ indicates a decrease in value; ↑ indicates an increase in value; · = No change in value; ↑(x)= indicates prevention of increase in value compared to control; Φ= Order of markers compared = those of Intervention (I) group first, Control (C) or baseline (B) second; Text color represents comparison body as follows, Blue = Comparison of effective change due to intervention by adjusted Mean Difference (MD) of changes in markers observed between I&C groups at end of the study from baseline; Green = Comparison of changes in I vs. C groups at the end of study from respective baselines; Red = Comparison of change (or difference) in markers at the end of study from baseline in I group; § = Non-significant Result; T2D = Type-2 Diabetes; NS = Not Specified; NR = Not Reported; Sp. = Species; SB = Single-Blinded; DB = Double-Blinded; R = Randomized; RCT = Randomized Controlled Trial; CC = Crossover Controlled; PC = Placebo-Controlled; PG = Parallel Group; CT = Clinical Trial;; OL = Open Label; MDA = Malondialdehyde; IL-6 = Interleukin 6; TNF-α = Tumor necrosis factor alpha; CRP = C-reactive protein; SCFA = Short-Chain Fatty Acid; NAFLD = Non-Alcoholic Fatty Liver Disease; GSH: Glutathione; GSSG = oxidized glutathione; Hs-CRP: High sensitivity C-Reactive Protein; CAT = Catalase; TAC = Total Antioxidant Capacity; TAS = Total Antioxidant Status; LPS = lipopolysaccharide; LBP = lipopolysaccharide binding protein; IL-1ra = IL-1 receptor antagonist; SOD = Superoxide Dismutase; GPX = Glutathione Peroxidase; GR = glutathione reductase; HP = High Performance;; F2-iso = F2-isoprostane; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; NGT = normal glucose tolerance; IGT = impaired glucose tolerance; CHD = Coronary Heart Disease.*

Effect on Interleukin-6

Probiotic supplementation has been investigated among diabetics in a total of nine studies, including both single and multi-species probiotics; results were found to be inconclusive with reporting of both positive and negative, significant, and insignificant findings, across multiple studies (**Table 2**).

Across three single-species probiotic administering studies, organisms of the (previously) *Lactobacillus* genus have been associated with slight, statistically non-significant increases in IL-6. These range from average changes of + 0.1 and + 0.4 pg/mL levels following 1 and 4 months of intervention, respectively, to as high as + 0.95 and + 1.55 pg/mL following 6 months of supplementation and 3 months of follow-up (132, 134, 135).

On the other, hand, the six studies investigating the change in IL-6 following multi-species probiotics have all reported mean reductions in average IL-6 levels from baseline. Three of these presented significant changes from baseline; Sabico et al. (123) reported a difference of –3.9 pg/mL (-76.5%), while Kobyliak et al. (136, 138) reported changes of 3.45 ± 1.48 and –6.77 ± 9.62 pg/mL across two studies. Another study by Kobyliak et al. (137) revealed statistically significant changes of –4.11 ± 7.15 pg/mL from baseline compared to control. Other studies have also reported decreasing averages in IL-6 levels post supplementation, however, these changes were not found to be statistically significant (128, 129).

Effect on IFN-γ

Two studies by Kobliyak et al. (136, 137) have investigated and reported the effect of multi-species probiotics on IFN-γ; no studies have investigated the effects of single species probiotic supplementation on this pro-inflammatory marker (**Table 2**). These two "Symbiter Forte" regimens were associated with net decreases in IFN- γ, although statistically non-significant.

Effect on IL-8

Three multispecies probiotic administering probiotic combinations have been associated with significant efficacies following an 8-week intervention (**Table 2**). Kobliyak et al. (136–138) have reported reductions in IL-8 levels ranging from –3.80 ± 1.05 to –8.99 ± 21.11 pg/mL, although it must be noted that all three interventions were not statistically significant from the effect seen in control.

Effect on Interleukin 17

Only Hsieh et al. (134) have reported on the association of IL-17 levels with probiotic administration (**Table 2**). However, the authors report statistically insignificant effects; changes of + 0.66 ± 3.24 and + 0.47 ± 2.91 ng/mL following live and heat-killed probiotic use.

Effect on Endotoxin

Two studies by Sabico et al. (123, 140) have investigated the effects of multi-species probiotics on blood endotoxin levels in diabetic patients (**Table 2**). While one study reported that following probiotic supplementation, endotoxin levels significantly dropped by 3.2 IU/mL (-69.6%) from baseline, another reported a significant change of –2.40 IU/mL (-52.2%) compared to the change seen in control.

Effect on Lipopolysaccharide-Binding Protein

The one study reporting on the effect of probiotic on LBP concluded that there was no change in the mean levels of marker following a 16-week intervention with *Lacticaseibacillus casei* (135).

Anti-inflammatory Markers in Type 2 Diabetes

Effect on Interleukin-10

A study by Hsieh et al. (134) investigated the effect of a 6-month single species probiotic course on diabetic patients; although there was a generally positive trend in the change post-intervention compared to baseline and control (+ 1.48 ± 3.09 and + 2.05 ± 3.25 ng/mL in the live and heat-killed bacteria groups, respectively), these results were not statistically significant.

Effect on IL-1RA

Andreasen et al. (132) have reported an insignificant increase of + 2 pg/mL in IL-1RA from baseline following a 4-week regimen of single-species probiotic; however, this change was much more pronounced in control.

Markers of Oxidative Stress in Type 2 Diabetes

Effect on Malondialdehyde

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. MDA has been extensively studied as a biomarker of inflammation in the context of metabolic disease. A total of seven studies investigated this inflammatory marker as a subject of change following supplementation with either single (2) or multispecies (5) probiotics (**Table 2**).

Miraghajani et al. (141) reported a beneficial 0.07 μmol/L change in mean MDA levels among a cohort of T2D patients with nephropathy after an 8-week course of single-species probiotics that was significantly different than the change observed among controls receiving conventional soy milk. On the other hand, another group reported an non-significant increase of 0.06 μmol/L following a course of another single-species probiotic in bread (142).

Among five multi-species probiotics investigated for their effects on MDA levels among diabetics, two have reported significant reductions in MDA levels, while three have presented statistically insignificant results. Ejtahed et al. (143) have reported a significant decrease of 0.26 μmol/L in the mean level of MDA from baseline following a 6-week course, while a more recent study by Raygan et al. (127) have presented a significant change of –0.1 ± 0.3 μmol/L from baseline compared to control following a twice-longer course. Mazloom et al. (128) and Raygan et al. (126) both reported net reductions in mean level of MDA markers; however, these were not found to be statistically significant.

Effect on Superoxide Dismutase

SOD levels post probiotic administration has been a subject of investigation under four studies included in this review (**Table 2**). All studies have reported positive trends, some

have been reported as significant versus control. Mirmiranpour et al. (144) have reported slightly higher SOD levels post-intervention than control after the authors report that no significant change existed between the groups at baseline. Hariri et al. (145) have also presented the significant effects of another single-species probiotic on diabetic nephropathy patients following an 8-week course. In another study, the same authors reported changes of + 0.93 ± 0.95 and + 0.04 ± 1.31 U/mL after administration of live and heat-killed bacterium, however, these were not found to be statistically significant (134). Lastly, Ejtahed et al. (143) reported a significant increase of 137.87 U/g Hb in erythrocyte SOD from baseline following a 6-week course of a multi-species probiotic yogurt.

Effect on Catalase

A few studies have reported on the effect of nutraceuticals on CAT levels (**Table 2**). While one study reported a significant result, an overall conclusion cannot be made in this regard as two others have found no statistically significant changes nor similar trends. Mirmiranpour et al. (144) have reported that after starting from similar baseline values, the group receiving 3 months of a single-species probiotic capsule (2.44 ± 0.50 U/mL) had markedly increased CAT levels compared to control (1.95 ± 0.34 U/mL), a difference in mean of 0.49 U/mL that was statistically significant. While a slight positive change from baseline was also found by Bahmani et al. (142) this was not significantly different from the change observed in control; on the other hand, Ejtahed et al. (143) found an overall insignificant decrease in mean CAT.

Effect on GSH Peroxidase and GSH Reductase

Multiple studies have investigated the effect of probiotics on GPX activity levels post-intervention, however, the results have been conflicting (**Table 2**). Ejtahed et al. (143) reported that a 6-week regimen of multi-species probiotic was associated with a significant increase in mean GPX activity to hemoglobin ratio of 0.78 U/g Hb compared to both baseline and control. Similar significant increases (of + 0.43 U/g Hb in GPX and + 0.38 U/g Hb in GR) have been reported by Miraghajani et al. (141) following an 8-week administration of a single-species probiotic. More recently, Mirmiranpour et al. (144) have reported that after starting from similar levels at baseline, the difference between an intervention group receiving single-species probiotic and control was + 7.26 U/mL in GPX activity. Interestingly, another group of researchers have shown fractional and statistically insignificant changes in GPX activity after single-species probiotic activity; however, these changes were smaller in magnitude than that seen in control (134).

Effect on GSH and GSSG

The effect of probiotics on reduced (GSH) and oxidized (GSSG) glutathione levels have been studied across multiple studies with interstudy consenting trends (**Table 2**). All studies report a positive change in mean plasma GSH following intervention periods, although not all have been found to be statistically significant. Miraghajani et al. (141) reported a statistically significant increase from baseline in GSH (732.96 ± 61.95 vs. 600.66 ± 69.61 μmol/L) and a similar decrease in GSSG (19.00 ± 0.70 vs. 30.37 ± 0.20 μmol/L) following an 8-week supplementation period with a single-species probiotic soymilk; these changes were also statistically significant compared to changes observed in a control group. Although Bahmani et al. (142) also investigated another single-species probiotic in this regard, the results were not statistically significant given a larger increase in the control group.

Among multispecies probiotics investigation, Asemi et al. (124) reported a significant increase of + 240.63 ± 101.29 μmol/L from baseline compared to control the earliest. Raygan et al. (125–127) through a series of three, 3-months studies investigating different multi-species probiotics, a change of + 18.0 ± 112.7 μmol/L from baseline in one of the studies was reported, and baseline-adjusted intergroup mean difference of changes of + 45.15 and + 154.16 μmol/L was reported in the two other studies; all three reports were statistically significant.

Effect on Total Antioxidant Capacity and Total Antioxidant Status

The association of probiotic supplementation on the levels of TAC has been extensively studied (**Table 2**). While many of these reports a significant increase in mean levels of TAC, few others have presented a mean positive change that are not statistically significant.

Miraghajani et al. (141) and Bahmani et al. (142) have reported respective changes of + 39 and + 78.6 mmol/L in mean TAC levels among diabetics following an 8-week supplementation with different single-species probiotics; however, these were not statistically significant. Among the other studies reporting on the effect of multispecies probiotics, only Asemi et al. (124) failed to show a statistically significant change despite a large mean increase of + 379.97 ± 41.8 mmol/L from baseline levels. On the other hand, Raygan et al. (127) have shown that a mean increase of just 12.6 ± 41.6 mmol/L was significant due to the control group having a large decrease of –116.9 ± 324.2 mmol/L. However, through a series of two other multispecies probiotic-investigating studies among diabetics with congestive heart disease, Raygan et al. (125, 126) have reported larger significant baseline-adjusted intergroup mean differences of + 108.44 and + 119.30 mmol/L. Ejtahed et al. (143) and Tonucci et al. (146) have also presented significant decreases in TAS levels compared to baseline and control group, despite a less pronounced change.

Effect on Nitric Oxide

Few studies have investigated changes in NO levels as a measure of oxidative stress following probiotic use (**Table 2**). Bahmani et al. (142) reported a statistically non-significant rise (+ 18.5 ± 36.2 μmol/L) in mean NO levels following 8 weeks of single-species probiotic bread supplementation. On the other hand, Raygan et al. (125–127) successfully reported, through three different studies each using multispecies probiotics, net increases in mean NO levels in the order of + 7.86 μmol/L and + 4.28 μmol/L (both as intragroup baseline adjusted intergroup mean difference) and + 1.7 ± 4.0 μmol/L (change from baseline).

Effect on Oxidized Low-Density Lipoprotein, 8-Hydroxy-2'-Deoxyguanosine and F_2-Isoprostanes

OxLDL, 8-OhdG and F_2-IsoP remain some of the lesser used measures of oxidative stress utilized to investigate oxidative stress (**Table 2**). Mirmiranpour et al. (144) recently reported that compared to a control group (17.07 ± 1.01 mU/L) that did not have significantly different mean OxLDL levels at baseline, an intervention of single-species probiotics was associated with a lower mean OxLDL (16.85 ± 1.53 mU/L) at 3 months; however, this was not statistically significant. Similarly, Tonucci et al. (146) reported a decrease in F_2-IsoP levels following a course of multispecies probiotics. Hariri et al. (145) described a significant intergroup mean decrease in 8-OhdG, however specific marker levels were not reported.

Pro-inflammatory Markers in Type 2 Diabetes

Effect on C-Reactive Protein and High-Sensitivity C-Reactive Protein

We found six studies investigating the effects of prebiotics on hs-CRP, with most studies reporting significant ($p \leq 0.05$) decreases in this marker of inflammation following administration of inulin, resistant dextrin, or resistant starch in T2D patients (**Table 3**). Dehghan et al. have reported that high performance (HP) inulin supplementation in T2D patients resulted in a significant baseline adjusted mean hs-CRP difference of −3.8 ng/mL compared to a control group consuming maltodextrin, while another recent study has confirmed this significant effect of HP inulin using a cohort of diabetic and overweight patients consuming either HP inulin or HP inulin plus butyrate (147, 148). Moreover, a recent study by Farhangi et al. (149) reported that resistant dextrin supplementation was responsible for a significant baseline-adjusted mean difference of −8.02 ng/mL (-54.00%) compared to a similar control. Resistant dextrin has also been showed to be associated with a significant reduction of −2.40 ng/mL in hs-CRP from baseline in one study by Aliasgharzadeh et al. (47) Administration of oligofructose-enriched inulin and a formula containing 60% resistant starch type 2 were also shown to result in promising baseline-adjusted mean differences of −3.9 ng/mL (-31.70%) and −4.6 ng/mL (-11.9%) compared to their control(s), respectively, although these reductions were not found to be statistically significant (150, 151).

Effect on Tumor Necrosis Factor-α

Five out of six studies investigating the effect of prebiotic supplementation on TNF-α have reported promising results, while another has reported a non-significant ($p > 0.05$) decrease in the levels of this inflammatory marker (**Table 3**) (38). Aliasgharzadeh et al. (47) have reported a significant baseline-adjusted mean difference of 5.40 pg/mL (-18.8%) between the prebiotic group receiving HP inulin and control. Two other studies have reported similar baseline-adjusted differences of 3.0 pg/mL (-19.80%) and 2.9 pg/mL between changes of prebiotic and control groups following supplementation with oligofructose-enriched and HP-inulin, respectively (147, 150). Similar results have been reported from the use of resistant starch by Gargari et al. while significant reductions of 25 and 9% in baseline have been reported following administration of inulin with starch or butyrate, respectively, by another group (148, 151).

Effect on Interleukin-6

Prebiotic supplementation on the levels of IL-6 has been investigated in four studies, two of which have reported significant decrease in levels compared to control after adjusting for baseline values, while two other studies investigating resistant starch and galacto-oligosaccharides have found statistically insignificant results (**Table 3**) (38, 151). Aliasgharzadeh et al. have reported an adjusted change of −1.45 pg/mL (-28.4%) in IL-6 compared to control following an 8-week course of resistant dextrin, while Dehghan et al. have reported a −1.3 pg/mL (-8.15%) in baseline-adjusted changes in IL-6 vs. control following an 8-week course of oligofructose-enriched Inulin (47, 150).

Effect on Interleukin-12

Two studies have reported the effect of prebiotics on the pro-inflammatory cytokine IL-12 with promising success (**Table 3**). Dehghan et al. reported that a 10 g/d regimen with prebiotics was associated with a significant reduction of 2.49 ± 1.60 pg/mL in IL-12 levels compared to the change of + 1.23 ± 0.60 pg/mL in those receiving placebo (152). Farhangi et al. also reported a significant change of −2.8 pg/mL from baseline in another group of diabetics receiving a different prebiotic; however, comparison with control after adjusting for baseline values did not yield significant results (153).

Effect on IFN-γ

Unlike the inconclusive results of the multispecies probiotic studies discussed before, of three studies investigating the effect of prebiotics on IFN-γ, two reported favorable results that were statistically significant. Only an early study by Dehghan et al. reported statistically insignificant mean difference of −0.3 pg/mL (16.50%) between intergroup effects (150). Later, Dehghan et al. and Farhangi et al. reported a change of −0.28 ± 0.06 pg/mL from baseline and a baseline-adjusted mean difference of −0.6 pg/mL between intervention and control, respectively (152, 153). The latter group also noted a significant reduction in the IFN- γ/IL-10 ratio of −0.01 (153).

Effect on Endotoxin or Lipopolysaccharide

A total of five studies have investigated the effects of prebiotics on blood endotoxin levels in diabetic patients, a cross-study trends point to prebiotics having a significant negative effect overall (**Table 3**). Aliasgharzadeh et al. have reported a significant baseline-adjusted mean difference of −6.2 units/mL following intervention with resistant dextrin (-17.8%) (47). Similar significant effects of resistant dextrin administration have been published by two other studies: −6.5 EU/mL (-23.40%), (149) −6.1 EU/mL (153). HP and oligofructose-enriched inulin were also shown to also be significantly negatively associated LPS levels: −4.2 and −6.0 EU/mL (-21.95%), respectively, (147, 150).

Effect on Lipopolysaccharide-Binding Protein

Gonai et al. are the only group that have reported the results of prebiotics on LBP; no mean statistically significant change was observed following a 4-week intervention with galacto-oligosaccharides (**Table 3**) (38).

TABLE 3 | Studies investigating the effect of prebiotics on markers of inflammation and oxidative stress in T2D.

Type of nutraceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total Period of intervention/ study	Effect on markers	Mean change in Φ	References
		Control/Placebo	Intervention							
Prebiotic	TB, PC, RCT Iran	*n* = 25 (25F) 49.6 ± 8.4 30.8 ± 5.2	*n* = 30 (30F) 49.2 ± 9.6 31.8 ± 4.5	Maltodextrin	Resistant dextrin	5 x 2 g/d	8 weeks	↓IL-6	−1.45 pg/ml or −28.4%	(47)
								↓TNF-α	−5.40 pg/ml or −18.8%	
								↓MDA	−1.21 nmol/ml or −25.6%	
								↓Endotoxin	−6.2 units/ml or −17.8%	
								↓hs-CRP (§)	−2.7 mg/L or 35.1 % (§)	
								↓hs-CRP	−2.40 mg/L	
Prebiotic	R, TB, PC (Iran)	*n* = 25 (25F) 48.40 ± 9.70 29.90 ± 4.10	*n* = 27 (27F) 48.45 ± 8.40 31.90 ± 4.00	Maltodextrin	Oligofructose-enriched inulin	5 x 2 g/d	8 weeks	↑TAC	+0.2 mmol/L or +20.0%	(155)
								↑CAT (§)	69.5 ± 20.2 U/mg Hb (I, 8w) vs 57.2 ± 16.0 U/mg Hb ↑(I, B)	
								↑GPX (§)	34.4 ± 5.4 U/mg Hb (I, 8w) vs 33.7 ± 5.1 U/mg Hb (I, B) (§)	
								↑SOD (§)	1684.7 ± 254.2 U/mg Hb (I, 8w) vs 1633.9 ± 237.3 U/mg Hb (I, B)	
								↓MDA	−1.7 nmol/mL or −39.7%	
Prebiotic	TB, RCT (Iran)	*n* = 25 (25F) 48.7 ± 9.7 29.9 ± 4.2	*n* = 27 (27F) 48.4 ± 8.4 31.9 ± 4.5	Maltodextrin	Oligofructose-enriched Inulin	5 × 2 g/d	8 weeks	↓hs-CRP (§)	−3.9 mg/L or −31.70% (§)	(147)
								↓TNF-α	−3.0 pg/ml or −19.80%	
								↓IL-6	−1.3 pg/ml or −8.15%	
								↓LPS	−6.0 EU/mL or −21.95%	
								↓IFN-λ (§)	−0.3 pg/ml or −16.50% (§)	
								↑IL-10 (§)	+0.4 pg/ml or +11.50% (§)	
Prebiotic	R, DB, PC, CT (Iran)	T2D and overweight patients* *n* = 15 (5M/10F) 51.73 ± 8.44 30.86 ± 5.41	T2D and overweight patients* Inulin group *n* = 15 (8M/7F) 51.47 ± 6.46 30.37 ± 2.82	Starch powder and starch capsules	HP inulin, starch capsules as placebo	(C) 6 x100 mg/d starch capsules, 5 x 2 g/d starch powder (I) 2 × 5 g/d HP inulin, 6 × 100 mg starch capsules/d	45 days	↓hs-CRP	3.80 ± 1.38 mg/L (I, 45d) vs 5.45 ± 2.28 mg/L (I, B) [−25.63%]; significant MD vs control, markers NS	(149)
								↓MDA	6.13 ± 1.93 nmol/mL (I, 45d) vs 6.40 ± 2.09 nmol/mL (I, B) [−3.39%]; significant MD vs control, markers NS	
								↓TNF-α mRNA	Ratio: 0.75 ± 0.18-fold vs baseline; also significant vs control, markers NS	

(Continued)

TABLE 3 | (Continued)

Type of nutraceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose x frequency	Total Period of intervention/ study	Effect on markers	Mean change in Φ	References
		Control/Placebo	Intervention							
			T2D and overweight patients*; Butyrate + Inulin group $n = 14$ (4M/10F) 47.14 ± 7.99 30.31 ± 4.25	Starch powder and starch capsules	NaBut, HP Inulin	(C) 6 x100 mg/d starch capsules, 5 x 2 g/d starch powder (I) 2 × 5 g/d HP inulin, 6 × 100 mg NaBut capsules	45 days	↓hs-CRP	2.44 ± 1.01 mg/L (I, 45d) vs 3.89 ± 1.14 (I, B) [−34.25%]; significant MD vs control, markers NS	
								↓MDA	5.51 ± 2.17 nmol/mL (I, 45d) vs 6.68 ± 2.27 nmol/mL (I, B) [−12.31%]; significant MD vs control, markers NS	
								↓TNF-α mRNA	Ratio: 0.91 ± 0.32 fold vs baseline; also significant vs control, markers NS	
Prebiotic	R, DB, C, CT (Iran)	Control Group $n = 27$ (12M/15F) 58.2 ± 11.8 BMI NR	Prebiotic Group $n = 28$ (14M/16F) 58.8 ± 12.8 BMI NR	Capsule containing 0.5 g of rice flour powder	Capsule containing 0.5 g of powdered cinnamon	1 capsule/d	3 months	↑CAT	2.44 ± 0.50 U/ml (I, 3m) vs 1.95 ± 0.34 U/ml (C, 3m)	(144)
								·GPX (§)	84.61 ± 13.43 U/mL (I, 3m) vs 84.89 ± 6.52 U/mL (C, 3m) (§)	
								↑SOD (§)	4.16 ± 0.60 U/mL (I, 3m) vs 3.99 ± 0.27 U/mL (C, 3m) (§)	
								↓OxLDL (§)	16.32 ± 1.21 mU/L (I, 3m) vs 17.07 ± 1.01 mU/L (C, 3m) (§)	
Prebiotic	TB, RCT (Iran)	$n = 32$ (32F) 49.6 ± 8.4 30.8 ± 5.2	$n = 28$ (28F) 49.5 ± 8.0 31.5 ± 4.5	Maltodextrin	Hi-Maize 260 (60% resistant starch type 2)	5 x 2 g/d	8 weeks	↓hs-CRP (§)	−4.6 mg/L or −11.9% (§)	(152)
								↓IL-6 (§)	−1.4 pg/ml or −14.2% (§)	
								↓TNF-α	−3.4 pg/ml or −18.9%	
Prebiotic	DB PC (Iran)	T2D and overweight patients* $n = 22$ (22F) 48.61 ± 9.16 29.98 ± 4.01	T2D and overweight patients* $n = 27$ (27F) 48.07 ± 8.70 31.43 ± 3.50	Maltodextrin	Oligofructose-enriched chicory inulin	5 x 2 g/d	2 months	↑IL-4	7.41 ± 1.38 pg/ml vs −2.96 ± 0.88 pg/ml	(153)
								↓IL-12	−2.49 ± 1.60 pg/ml vs +1.23 ± 0.60 pg/ml	
								↓IFN-λ	−0.28 ± 0.06 pg/ml vs +0.058 ± 0.03 pg/ml	

(Continued)

TABLE 3 | (Continued)

Type of nutraceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/Placebo and intervention dose × frequency	Total Period of intervention/ study	Effect on markers	Mean change in Φ	References
		Control/Placebo	Intervention							
Prebiotic	R, PC, CT (Iran)	*n* = 25 (25F) 48.7 ± 9.7 29.9 ± 4.2	*n* = 24 (24F) 47.8 ± 10.1 31.6 ± 4.1	Maltodextrin	HP inulin	5 x 2 g/d	8 weeks	↓hs-CRP	−3.8 mg/L	(148)
								↓TNF-α	−2.9 pg/ml	
								↑IL-10	+1.9 pg/ml	
								↓LPS	−4.2 EU/mL	
Prebiotic	TB, PC, RCT (Iran)	*n* = 25 (25F) 49.6 ± 8.4 30.8 ± 5.2	*n* = 30 (30F) 49.2 ± 9.6 31.8 ± 4.5	Maltodextrin	Resistant Dextrin	5 x 2 g/d	8 weeks	↓IL-12	−2.8 pg/ml	(**?**)
								↓IL-12 (§)	−0.7 pg/ml (§)	
								↑IL-4 (§)	+4.3 pg/ml	
								↓IL-4 (§)	−1.0 pg/ml (§)	
								↑IL-10	+2.6 pg/ml	
								↓IFN-λ	−0.6 pg/ml	
								↓IFN-λ/IL-10 ratio	−0.01	
								↓LPS	−6.1 EU/mL	
Prebiotic	R, DB, PC (S. Korea)	IFG, IGT and T2D patients* *n* = 25 (10M/15F) 56.0 ± 1.28 24.6 ± 0.50	IFG, IGT and T2D patients* *n* = 22 (4M/18F) 54.4 ± 1.31 23.8 ± 0.63	Powdered rice flour	Jerusalem artichoke (containing inulin and fructooligosaccharides) and fermented soybean powder mixture 1:1	40 g/d	12 weeks	↓MDA (§)	−0.41 ± 0.43 nmol/L vs −0.58 ± 0.35 nmol/L (§)	(156)
Prebiotic	R, DB, PC (Japan)	*n* = 25 (17M/8F) 54 ± 12 27.2 ± 4.6	*n* = 27 (21M/6F) 55 ± 11 27.9 ± 3.6	Maltodextrin syrup	Galacto-oligosaccharide syrup	10 g/d	4 weeks	ŭ IL-6 (§)	2.3 ± 4.3 pg/ml (I, 4w) vs 2.3 ± 4.8 pg/ml (I, B) (§)	(38)
								↓ IL-10 (§)	3.3 ± 7.7 pg/ml (I, 4w) vs 3.3 ± 7.7 pg/ml (I, B) (§)	
								↓ TNF-α (§)	2.2 ± 1.4 pg/ml (I, 4w) vs 2.5 ± 2.3 pg/ml (I, B) (§)	
								↓ LBP (§)	12.6 ± 2.2 μg/mL (I, 4w) vs 12.8 ± 1.8 μg/mL (I, B) (§)	
Prebiotic	R, PC, CT (Iran)	*n* = 33 (33F) 48.6 ± 7.9 32.0 ± 3.9	*n* = 32 (32F) 49.5 ± 8.0 31.5 ± 4.5	Maltodextrin	Resistant dextrin supplement (NUTRIOSE06)	5 x 2 g/d	8 weeks	↑TAC	+0.33 mmol/L or +36.25%	(150)
								↓hs-CRP	−8.02 mg/L or −54.00%	
								↓LPS	−6.5 EU/mL or −23.40%	
								↑SOD	+56.3 U/mg Hb	
								↑SOD (§)	+67.5 U/mg Hb (§)	
								↑GPX	+3.80 U/g Hb	
								↑GPX (§)	+0.85 U/g Hb (§)	
								↓CAT (§)	−5.3 U/g Hb	
								↓CAT (§)	−10.7 U/g Hb (§)	
								↓MDA	−1.21 nmol/mL or −25.58%	

**All participants are T2D-diagnosed patients, unless otherwise stated; ↓ indicates a decrease in value; ↑ indicates an increase in value; · = No change in value; Φ= Order of markers compared = those of Intervention (I) group first, Control (C) or baseline (B) second; Text color represents comparison body as follows, Blue = Comparison of effective change due to intervention by adjusted Mean Difference (MD) of changes in markers observed between I&C groups at end of the study from baseline; Green = Comparison of changes in I vs. C groups at the end of study from respective baselines; Red = Comparison of change (or difference) in markers at the end of study from baseline in I group; § = Non-significant Result; T2D = Type-2 Diabetes; NS = Not Specified; NR = Not Reported; SB = Single-Blinded; DB = Double-Blinded; TB = Triple-Blinded; R = Randomized; RCT = Randomized Controlled Trial; CC = Crossover Controlled; PC = Placebo-Controlled; PG = Parallel Group; CT = Clinical Trial;; OL = Open Label; MDA = Malondialdehyde; IL-6 = Interleukin 6; TNF-α = Tumor necrosis factor alpha; CRP = C-reactive protein; SCFA = Short-Chain Fatty Acid; NAFLD = Non-Alcoholic Fatty Liver Disease; GSH: Glutathione; GSSG = oxidized glutathione; Hs-CRP: High sensitivity C-Reactive Protein; CAT = Catalase; TAC = Total Antioxidant Capacity; TAS = Total Antioxidant Status; LPS = lipopolysaccharide; LBP = lipopolysaccharide binding protein; IL-1ra = IL-1 receptor antagonist; SOD = Superoxide Dismutase; GPX = Glutathione Peroxidase; GR = glutathione reductase; HP = High Performance; F2-iso = F2-isoprostane; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; NGT = normal glucose tolerance.*

Adaptive Immunity Markers in Type 2 Diabetes

Effect on IL-4

More research is required in context to the effect of pre- and synbiotics, on IL-4 post-intervention (**Table 3**). Dehghan et al. have reported that HP inulin significantly increased IL-4 levels by + 7.41 ± 1.38 pg/mL compared to control in a cohort of diabetic and overweight patients (152). However, more recent study by Farhangi et al. produced inconclusive results as the change due to intervention was positive compared to baseline, but negative compared to the baseline-adjusted change in control; both of these were statistically insignificant (153).

Anti-inflammatory Markers in Type 2 Diabetes

Effect on IL-10

Four studies have reported the effects of prebiotic supplementation in diabetic patients, with the majority following a trend of positive change compared to baseline and/or control (**Table 3**). Dehghan et al. reported a significant baseline-adjusted mean difference of + 1.9 pg/mL between changes in IL-10 post 8 weeks of intervention with HP inulin compared to control (147). Another study has also reported a similar significant mean difference of + 2.6 pg/mL using resistant dextrin (153). In addition, a study by Dehghan et al. also reported a positive change in IL-10 levels, however, this was not found to be statistically significant, similar to the results of Gonai et al. (38, 150).

Markers of Oxidative Stress in Type 2 Diabetes

Effect on Malondialdehyde

MDA levels have also been widely probed following prebiotic use; four studies have investigated and reported significant baseline-adjusted intergroup mean difference between intragroup changes, while another has reported changes from baseline (**Table 3**). Aliasgharzadeh et al. have reported net effects of intervention of –1.21 nmol/mL (-25.6%) and –1.7 nmol/mL (-39.7%) using two different prebiotics (47, 154). Farhangi et al. have recently confirmed the effects of resistant dextrin in another study, reporting an intervention effect of –1.21 nmol/mL (-25.58%) (149). Finally, Roshanravan et al. have interestingly also reported the effect of HP inulin with and without butyrate; both effects were significant with mean changes of 1.17 and 0.27 nmol/mL from baseline levels, respectively, after just 45 days (148). Finally, Ahn et al. reported a slight decrease in MDA levels from baseline using Jerusalem artichoke and soyabean mixture, but this was not found to be significant (155).

Effect on Superoxide Dismutase

Like probiotics, all three studies considered exploring the effect of prebiotics on SOD levels have reported positive changes (**Table 3**). Farhangi et al. have recently reported a significant increase of + 56.3 U/mg Hb from baseline in SOD levels after an 8-week course with resistant dextrin (149). Aliasgharzadeh et al. and Mirmiranpour et al. have also investigated the effects of other prebiotics on SOD levels; however, these were statistically insignificant (144, 154).

Effect on Catalase

Three trials administering prebiotics have reported on their effects on CAT levels, with little evidence to support any one hypothesis (**Table 3**). Mirmiranpour et al. have reported that a 3 months course of prebiotics was significantly associated with increased CAT activity levels (+ 2.44 ± 0.50 U/mL) compared to the control group (1.95 ± 0.34 U/mL) when baseline levels were not significantly different (144). Furthermore, a similar increase in mean CAT/Hb ratio (+ 12.3 U/g Hb) in the intervention group was reported by another group, this, however, was not statistically significant (154). Only Farhangi et al. reported a non-significant decrease in mean CAT following prebiotic use (149).

Effect on GPX

Three distinct clinical studies have reported the use of prebiotics to investigate changes in GPX levels among diabetic patients (**Table 3**). Of these, only Farhangi et al. have presented results that were significant; following an 8-week course of prebiotics, a difference of + 3.80 U/g Hb was observed from baseline in the intervention group (149). Aliasgharzadeh et al. and Mirmiranpour et al. have also reported on GPX levels post-prebiotic administration; however, almost no change was observed in both cases following supplementation using their respective prebiotics (144, 154).

Effect on Total Antioxidant Capacity

Two studies have investigated the effect of prebiotics on TAC levels, with each reporting a significant increase post-supplementation (**Table 3**). Aliasgharzadeh et al. reported a mean difference of + 0.2 mmol/L (+ 20.0%) associated with the prebiotic intervention (154), while Farhangi et al. more recently have reported a larger change of + 0.33 mmol/L (36.25%) (149).

Effect on OxLDL

Mirmiranpour et al. reported that compared to a control group without significantly different baseline OxLDL levels, an intervention of prebiotic was associated with a lower mean OxLDL (16.32 ± 1.21 vs. 17.07 ± 1.01 mU/L) at 3 months; however, this was not statistically significant (**Table 3**) (144).

Effect of Synbiotics on Inflammatory and Oxidative Stress Markers in Type 2 Diabetes

Pro-inflammatory Markers in Type 2 Diabetes

Effect on CRP and hs-CRP

A total of eight studies reported the association between synbiotic consumption in diabetics and either CRP or hs-CRP, with many studies reporting significant desired results (**Table 4**). Moreover, like the trend observed in probiotics, multispecies synbiotic supplementation outperform single-species probiotics in their ability to result in desired changes in CRP and hs-CRP levels.

Asemi et al. reported that a single species synbiotic mixture of *Bacillus coagulans* (previously *Lactobacillus sporogenes*) and inulin significantly correlated to hs-CRP level changes from baseline of –1.057 ± 0.283 mg/L compared to a slight mean increase of 0.0054 ± 0.385 mg/L in control, whereas another intervention group from a study using *B. coagulans* and inulin

TABLE 4 | Studies investigating the effects of synbiotics on markers of inflammation and oxidative stress.

Type of nutra-ceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/placebo and intervention dose x frequency	Total period of intervention/ study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
Synbiotic (Single sp.)	R, DB, CC, CT (Iran)	*n* = 62 (19M/43F) 53.1 ± 8.7 29.90 ± 5.18	*n* = 62 (19M/43F) 53.1 ± 8.7 29.60 ± 4.53	0.38 g isomalt, 0.36 g sorbitol and 0.05 g stevia per 1g	Heat-resistant Bacillus coagulans (1 × 10^7 CFU), 0.04 g inulin (HPX), 0.38 g isomalt, 0.36 g sorbitol and 0.05 g stevia per 1g	9 × 3 g/d	6 × 2 weeks	↓hs-CRP	−1.057.86 ± 283.74 ng/ml vs +95.40 ± 385.38 ng/ml	(157)
								↑GSH	+319.98 μmol/L vs. +19.73 μmol/L	
								↑TAC (§)	+69.48 ± 38.13 mmol/l vs +60.06 ± 40.76 mmol/l (§)	
Synbiotic (Single sp.)	DB, R, CC, CT (Iran)	*n* = 51 (16M/35F) 52.9 ± 8.1 30.15 ± 5.07	*n* = 51 (16M/35F) 52.9 ± 8.1 29.88 ± 4.77	0.38 g isomalt, 0.36 g sorbitol and 0.05 g stevia per 1 g	*Bacillus coagulans* (1 × 10^7 CFU), 0.1 g inulin HPX), 0.05 g beta-carotene with 0.38 g isomalt, 0.36 g sorbitol and 0.05 g stevia per 1 g	9 x 3 g/d	6 x 2 weeks	↑NO	+6.83 ± 16.14 μmol/L vs. −3.76 ± 16.47 μmol/L	(159)
								↑GSH	+36.58 ± 296.71 μmol/L vs. −92.04 ± 243.05 μmol/L	
								↓TAC (§)	−6.97 ± 203.51 mmol/L vs. −10.03 ± 170.15 mmol/L	
								↓hs-CRP (§)	−274.70 ± 3560.67 ng/mL vs −212.02 ± 2943.09 ng/mL (§)	
								↓MDA	−1.28 ± 1.33 μmol/l	
								↓MDA (§)	−1.28 ± 1.33 μmol/l vs −0.95 ± 0.88 μmol/l (§)	
Synbiotic (Single sp.)	R, DB, C, CT (Iran)	Control Bread (CB) *n* = 25 (Sex NS) 54.60 ± 0.83 27.04 ± 0.50 Lactic Acid Bread (LAB) *n* = 25 (Sex NS) 55.00 ± 0.97 26.33 ± 0.46	Synbiotic group *n* = 25 (Sex NS) 54.92 ± 1.02 26.39 ± 0.51	Bread containing beta- glucan (3g) ± lactic acid (4 g)/ 40g package	Bread containing beta-glucan (3 g), *Bacillus coagulans* (1 × 10^8 CFU), and inulin (10 g) /40g package	40g x 3 packages/d	8 weeks	↓TAC (§)	−0.007 ± 0.01 mmol/L vs – 0.01 ± 0.01 mmol/L (CB) & +0.02 ± 0.02 mmol/L (LAB) (§)	(158)
								↑SOD	+0.40 ± 0.13 mmol/L vs +0.18 ± 0.17 mmol/L (CB) & −0.54 ± 0.40 mmol/L (LAB)	
								↑GPX (§)	+0.85 μmol/L vs +0.47 U/mL (CB) & +1.23 U/mL (LAB) (§)	
								↓hs-CRP	−689.76 ± 368.98 ng/mL vs +33.80 ± 237.60 ng/mL (LAB)	
			Synbiotic + Lactic Acid group *n* = 25 (Sex NS) 53.88 ± 1.09 26.83 ± 0.42	Bread containing beta-glucan (3g) ± lactic acid (4 g)/ 40g package	Bread containing beta-glucan (3 g), *Bacillus coagulans* (1 × 10^8 CFU), inulin (10 g) and lactic acid (4 g) /40g package	40g x 3 packages/d	8 weeks	↑TAC (§)	+0.03 ± 0.01 mmol/L vs – 0.01 ± 0.01 mmol/L (CB) & +0.02 ± 0.02 mmol/L (LAB) (§)	
								↑SOD	+0.87 ± 0.22 mmol/L vs +0.18 ± 0.17 mmol/L (CB) & −0.54 ± 0.40 mmol/L (LAB)	
								↑GPX	+19.02 ± 17.10 U/mL vs. −24.05 ± 12.17 U/mL (LAB)	

(Continued)

TABLE 4 | (Continued)

Type of nutra-ceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/placebo and intervention dose x frequency	Total period of intervention/ study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
								↓hs-CRP (§)	−575.96 ± 268.60 ng/mL vs +519.35 ± 304.35 ng/mL (CB) & +33.80 ± 237.60 ng/mL (LAB) (§)	
Synbiotic (Single sp.)	R, DB, C, CT (Iran)	Control Bread (CB) *n* = 27 (5M/22F) 53.4 ± 7.5 30.5 ± 4.1	Synbiotic Bread *n* = 27 (5M/22F) 51.3 ± 10.4 30.8 ± 5.9	Control bread	Bread containing viable and heat-resistant *Bacillus coagulans* (1 × 10^8 CFU) and 0.07g inulin / 1g	40 x 3 g/d	8 weeks	↓hs-CRP (§)	−983.5 ± 2,066.1 ng/ml vs −586.9 ± 2,009.2 ng/ml (§)	(160)
Synbiotic (Single sp.)	R, DB, C, CT (Iran)	Control Group *n* = 27 (12M/15F) 58.2 ± 11.8 BMI NR	Synbiotic Group *n* = 30 (Sex NS) 58.4 ± 11.4 30.8 ± 5.9 BMI NR	Capsule containing 0.5 g of rice flour powder	Capsule containing *Lactobacillus acidophilus* (10^8 CFU) and 0.5 g of powdered cinnamon	1 capsule/d	3 months	↑CAT (§)	2.20 ± 0.31 U/ml (I, 3m) vs 1.95 ± 0.34 U/ml (C, 3m) (§)	(144)
								↑GPX (§)	89.71 ± 9.04 U/mL (I, 3m) vs 84.89 ± 6.52 U/mL (C, 3m) (§)	
								↑SOD (§)	4.13 ± 0.64 U/mL (I, 3m) vs 3.99 ± 0.27 U/mL (C, 3m) (§)	
								↓OxLDL	15.88 ± 1.98 mU/L (I, 3m) vs 17.07 ± 1.01 mU/L (C, 3m)	
Synbiotic (Single Sp.)	R, DB, C, CT (Iran)	Control Bread (CB) *n* = 27 (5M/22F) 53.4 ± 7.5 30.5 ± 4.1	Synbiotic Bread *n* = 27 (5M/22F) 51.3 ± 10.4 30.8 ± 5.9	Control bread	Bread containing viable and heat-resistant *Bacillus coagulans* (1 × 10^8 CFU) and 0.07g inulin / 1g	40 x 3 g/d	8 weeks	↑NO	+40.6 ± 34.4 μmol/L vs −0.8 ± 24.5 μmol/L	(142)
								↑TAC (§)	+3.6 ± 247.2 mmol/L vs. -45.7 ± 240.3 mmol/L (§)	
								↑GSH (§)	+25.0 ± 528.2 μmol/L vs +18.8 ± 417.8 μmol/L	
								↑CAT (§)	+2.2 ± 25.7 U/mL vs +2.7 ± 14.9 U/mL	
Synbiotic (Multi Sp.)	R, DB, PC, CT (Iran)	Overweight and obese patients with T2D and CHD* *n* = 30 (11M/19F) 64.0 ± 11.7 29.6 ± 4.6	Overweight and obese patients with T2D and CHD* *n* = 30 (11M/19F) 64.2 ± 12.0 32.3 ± 6.0	Capsules containing placebo	Capsules containing *Lactobacillus acidophilus* T16, L. casei T2 and Bifidobacterium bifidum strain T1 (2 × 10^9 CFU/g each) and 800 mg inulin	1 capsule/d	12 weeks	↓hs-CRP	−2632.3 ± 743.2 ng/ml vs – 433.3 ± 743.2 ng/ml	(162)
								↑NO	+7.6 ± 1.7 μmol/L vs – 3.4 ± 1.7 μmol/L	

(Continued)

TABLE 4 | (Continued)

Type of nutra-ceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/placebo and intervention dose x frequency	Total period of intervention/ study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
								↑TAC (§)	+49.8 ± 33.6 mmol/L vs. +30.0 ± 33.6 mmol/L (§)	
								↑GSH (§)	+23.6 ± 17.1 μmmol/l vs +12.2 ± 17.1 μmmol/l (§)	
								↓MDA	−0.6 ± 0.1 μmmol/l vs – 0.1 ± 0.1 μmmol/l	
Synbiotic (Multi Sp.)	R, DB, PC (Iran)	T2D and non-obese patients* n = 23 (14M/9F) 60.39 ± 6.74↑↑ 28.27 ± 2.54	T2D and non-obese patients* n = 20 (12M/8F) ↑59.10 ± ↑9.71 27.32 ± 4.34	Sachet containing 2g starch and 0.7% Natural Orange flavor	2g sachet containing 10^{11} spores of *Bacillus coagulans* Ganeden BC30, 10^{10} CFU *Lacticaseibacillus rhamnosus* GG,↑10^9 CFU *Lactobacillus acidophilus*, 500↑mg fructooligosaccharides and 0.7% Natural orange flavor	1 x 2 g/d	12 weeks	↓hs-CRP	−2.41 ± 2.48 mg/L↑vs +0.89 ± 3.21 mg/L	(163)
Synbiotic (Multi Sp.)	RCT, OL (Japan)	T2D and obese patients* n = 42 (34M/8F) 55.9 ± 10.7 29.1 ± 3.	T2D and obese patients* n = 44 (31M/13F) 61.1 ± 11.0 29.5 ± 4.4	NS, no pre- pro- or synbiotics	Dry powder (dp) containing *Lacticaseibacillus paracasei* YIT 9029 (3 × 10^8 CFU), *Bifidobacterium breve* YIT 12272 (3 × 10^8 CFU), and 7.5g galacto-oligosaccharides (GOS)	(2gdp, 5gGOS) + (1gdp, 2.5gGOS) /d	24 weeks	↓IL-6 (§)	−0.2 ± 1.8 pg/ml vs +0.4 ± 2.0 pg/ml	(161)
								↑CRP (§)	+40.0 mg/dL vs -3.5 mg/dL (§)	
								↑LBP (§)	+2.0 ± 4.2 μg/mL vs 3.4 ± 4.2 μg/mL (§)	
Synbiotic (Multi Sp.)	R, PG, DB, PC (Iran)	Patients with T2D and chronic periodontitis* n = 24 (8M/16F) 50.1 ± 3.6 25.5 ± 2.7	Patients with T2D and chronic periodontitis* n = 23 (6M/17F) 48.6 ± 5.8 24 ± 3.6	Same substance as intervention without bacteria and fructo-oligosaccharide	500 mg capsule containing 7 viable and freeze-dried strains: *Lactobacillus acidophilus* UBLA-34 (2 × 10^9 CFU), L. casei (7 × 10^9 CFU), *L. rhamnosus* (1.5 × 10^9 CFU), *L. bulgaricus* (2 × 10^9 CFU), *Bifidobacterium breve* (2 × 10^9 CFU), *B. longum* (7 × 10^9 CFU), *Streptococcus thermophilus* (1.5 × 10^9 CFU) and 100 mg fructo-oligosaccharide	1 capsule/d	8 weeks	↓IL-1β	−0.45 ± 0.42 pg/ml vs −0.13 ± 0.47 pg/ml	(165)
								↓MDA	−1.02 ± 0.95 μM vs −0.13 ± 0.39 μM	
								↑TAC	+0.04 ± 0.06 mM	

(Continued)

TABLE 4 | (Continued)

Type of nutra-ceutical	Study Design, Country	Participant* demographics size/sex (n, F/M) age (Mean ± SD; yrs.) BMI (Mean ± SD; kg/m^2)		Control/Placebo substance administered	Interventional nutraceutical administered	Control/placebo and intervention dose x frequency	Total period of intervention/ study	Effect on markers	Mean change in markers Φ	References
		Control/Placebo	Intervention							
								↑TAC (§)	+0.04 ± 0.06 mM vs +0.01 ± 0.12 mM (§)	
								↑SOD	+1.75 ± 2.49 U/mL vs +0.16 ± 0.48 U/mL	
								↑CAT (§)	+0.44 ± 5.33 U/mL vs +0.44 ± 5.33 U/mL	
								↑GPX	+14.72 ± 24.9 U/mL vs −3.27 ± 22.86 U/mL	
Synbiotic (Multi sp.)	R, DB, PC, Pilot (Austria)	Diabesity patients* $n = 14$ (8M/6F) 59 34	Diabesity patients* $n = 12$ (11M/1F) 61 33	Probiotic matrix containing maize starch, maltodex-trins, vegetable protein, potassium chloride, magnesium sulphate, amylases and manganese sulphate and prebiotic matrix containing maltodextrin, natural elderflower flavoring and Gum Arabic	Probiotic Ecologic Barrier containing g B. bifidum W23, *Bifidobacterium animalis* subsp. lactisW51, *Bifidobacterium animalis* subsp. lactisW52, *Lactobacillus acidophilus* W37, *L. casei* W56, *L. brevis* W63, *L. salivarius* W24, *L. lactis* W58 and *L. lactis* W19 (1.5×10^{10} CFU total) and 6 g matrix and 10 g, and Prebiotic 'Omnilogic Plus' containing 8 g active Galacto-oligosaccharides and Fructo-oligosaccharides, konjac glucomannan, calcium carbonate, zinc citrate 3-hydrate, vitamin D3 (cholecalciferol) and vitamin B2 (riboflavin) and 2g matrix	1 each/d	6 months	↑LPS (§)	0.69 EU/mL (I, 6m) vs 0.64 EU/mL (mg/dL) (§)	(166)
								↑LBP (§)	20.5 ng/mL (I, 6m) vs. 19 ng/mL (I, B) (§)	
Synbiotic (Sp. NS)	R, DB, PC (Iran)	$n = 22$ (8M/14F) 54.5 ± 11.10 22.47 ± 2.38	$n = 22$ (8M/14F) 53.45 ± 10.8 22.79 ± 2.7	Placebo tablet	Synbiotic tablet	1 tablet/d	8 weeks	↓hs-CRP	4.15 ± 1.96 mg/L (I, 8w) vs 4.94 ± 2.36 mg/L (I, B); significant MD vs control, markers NS	(164)
								↓IL-6	8.12 ± 5.02 ng/L (I, 8w) vs 9.19 ± 5.97 ng/L (I, B); significant MD vs control, markers NS	
								↓TNF-α	7.36 ± 2.61 ng/L (I, 8w) vs 8.03 ± 2.73 ng/L (I, B); significant MD vs control, markers NS	

**All participants are T2D-diagnosed patients, unless otherwise stated; ↓ indicates a decrease in value; ↑ indicates an increase in value; • = No change in value; Φ = Order of markers compared = those of Intervention (I) group first, Control (C) or baseline (B) second; Text color represents comparison body as follows, Blue = Comparison of effective change due to intervention by adjusted Mean Difference (MD) of changes in markers observed between I&C groups at end of the study from baseline; Green = Comparison of changes in I vs. C groups at the end of study from respective baselines; Red = Comparison of change (or difference) in markers at the end of study from baseline in I group; § = Non-significant Result; T2D = Type-2 Diabetes; NS = Not Specified; NR = Not Reported; Sp. = Species; SB = Single-Blinded; DB = Double-Blinded; TB = Triple-Blinded; R = Randomized; RCT = Randomized Controlled Trial; CC = Crossover Controlled; PC = Placebo-Controlled; PG = Parallel Group; CT = Clinical Trial; OL = Open Label; MDA = Malondialdehyde; IL-6 = Interleukin 6; TNF-α = Tumor necrosis factor alpha; CRP = C-reactive protein; SCFA = Short-Chain Fatty Acid; NAFLD = Non-Alcoholic Fatty Liver Disease; GSH: Glutathione; GSSG = oxidized glutathione; Hs-CRP: High sensitivity C-Reactive Protein; CAT = Catalase; TAC = Total Antioxidant Capacity; TAS = Total Antioxidant Status; LPS = lipopolysaccharide; LBP = lipopolysaccharide binding protein; IL-1ra = IL-1 receptor antagonist SOD = Superoxide Dismutase; GPX = Glutathione Peroxidase; GR = glutathione reductase; HP = High Performance; F2-iso = F2-isoprostane; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; NGT = normal glucose tolerance.*

rich bread presented a significant change of –689.76 ± 368.98 μg/L compared to an increase of 33.80 ± 237.60 μg/L in a control group consuming lactic acid-enriched bread (156, 157). Similar studies investigating the effects of single species synbiotics using other formulations of *B. coagulans* and inulin have all showed a trend of decrease in the levels of either CRP or hs-CRP, although these results were not significant (157–159).

Among multi-species synbiotic nutraceuticals, only Kanazawa et al. recently reported a non-significant increase of 40.0 mg/dL in CRP from baseline following supplementation with *Lacticaseibacillus paracasei, Bifidobacterium breve* and galacto-oligosaccharides (GOS) in a cohort of diabetic and obese patients in Japan (160). However, two other studies based in Iran investigating cohorts with similar comorbidities reported significant inter-group reductions in the levels of hs-CRP compared to controls following supplementation consisting of *Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum* and inulin, and *B. Coagulans, Lacticaseibacillus rhamnosus, Lactobacillus acidophilus* and fructooligosaccharides, respectively, (161, 162). A study by Kooshki et al. (163) reported significant decrease in hs-CRP from both baseline vs. control, although the composition of the tablet was not specified.

Effect on TNF-α

It is interesting to note that the effect of synbiotics on TNF-α is minimally reported (**Table 4**). Kooshki et al. (163) have found that multi-species synbiotic supplementation led to a mean decrease of ∼0.67 pg/mL following administration of a synbiotic tablet; however, the composition of this supplement was not reported.

Effect on IL-1β

Like TNF-α, the data on synbiotic supplementation on IL-1β is currently limited and requires further research (**Table 4**). Nonetheless, Bazyar et al. (164) have reported significant changes of –0.45 ± 0.42 pg/mL compared to the effect of control following an 8-week supplementation with a 7-species plus fructo-oligosaccharides synbiotic in a cohort of diabetics with chronic periodontitis from Iran.

Effect on IL-6

There is a limitation in the literature exploring the effects of synbiotic supplementation on IL-6, with only two studies reporting an overall average decrease in IL-6. However, only one of those reports was found to be statistically significant (**Table 4**). Kooshki et al. have reported a significant ∼1 ng/L change from baseline in IL-6 compared to control following an 8-week supplementation of synbiotic (composition unreported) (163). A further statistically insignificant change of –0.2 ± 1.8 pg/mL in IL-6 was reported by another group following a 24-week regimen of galacto-oligosaccharide and multispecies synbiotic (160).

Effect on Endotoxin (LPS)

Endotoxin (LPS) levels have not been widely investigated in the context of synbiotic administration (**Table 4**). Nevertheless, it is interesting to note that Horvath et al. have recently described a mean increase of 0.05 EU/mL from a baseline of 0.64 EU/mL in LPS levels following a 6-month intervention with a multi-species probiotic; however, this was not found to be significant with respect to intragroup baseline or the intergroup (control), perhaps owing to the small sample size in the pilot study (165).

Effect on Lipopolysaccharide-Binding Protein

Multispecies synbiotic intervention was associated with a larger change in mean LBP levels than either pro- or synbiotics, although both studies investigating their association failed to show statistically significant results (**Table 4**). Kanazawa et al. and Horvath et al. reported an increases in average marker levels from baseline (+ 2.0 ± 4.2 μg/mL and + 1.5 ng/mL, respectively) following a 6-month intervention (160, 165).

Markers of Oxidative Stress in Type 2 Diabetes

Effect on Malondialdehyde

MDA levels have been investigated as a marker of oxidative stress following synbiotic use in three studies; all three have reported some form of significant decrease in mean levels of MDA following supplementation (**Table 4**). Asemi et al. reported that a single-species probiotic was associated with a significant decrease of –1.28 ± 1.33 μmol/L from baseline; however, this was not significant compared to the change seen in control (158). However, two recent studies by Farrokhian et al. and Bazyar et al. have yielded significant changes of –0.6 ± 0.1 and –1.02 ± 0.95 μmmol/L following multi-species probiotic use (161, 164).

Effect on Superoxide Dismutase

The effect of synbiotics on SOD levels in diabetics were investigated by three studies, all of whom have reported an increasing effect following intervention (**Table 4**). Ghafouri et al. reported significant rise in SOD levels of + 0.40 ± 0.13 and + 0.87 ± 0.22 mmol/L following an 8-week supplementation with bread containing single-species probiotics without and with lactic acid, respectively (157). Mirmiranpour et al. presented a statistically insignificant increase of 0.14 U/mL in mean SOD levels between the two groups (144). While Bazyar et al. reported a large increase of + 1.75 ± 2.49 U/mL following a similar time frame of intervention using a multi-species probiotic in diabetic patients with chronic periodontitis (164).

Effect on Catalase

While three trials have reported the effect of various synbiotics on CAT activities, these were not found to be statistically significant, despite all showing upward slopes following intervention (**Table 4**). Mirmiranpour et al. reported that the mean CAT activities between the group receiving single-species synbiotic and the control differed by 0.25 U/mL at the end of trial, while another study reported a change of + 2.2 ± 25.7 U/mL from baseline in the intervention group (142, 144). Finally, although Bazyar et al. reported a slight increase in CAT activity from baseline, the change was found not to differ from control (164).

Effect on GPX

The results of synbiotic use on diabetics with respect to GPX has been investigated in three studies showing promising results (**Table 4**). Bazyar et al. has recently presented a significant promising change of + 14.72 ± 24.9 U/mL from baseline compared to control (164). On the other hand, Ghafouri et al. has reported another significant change of + 19.02 ± 17.10 U/mL

in GPX following administration of a bread containing another synbiotic combination (157). The results of Mirmiranpour et al. show promise in their large mean increase from baseline compared to control, however, it was not statistically significant (144).

Effect on GSH

The effect of synbiotics on mean reduced glutathione (GSH) levels have also been investigated across literature; while all of five such studies report an increase in the mean GSH, only two have shown to be statistically significant (**Table 4**). Through two similar single-species synbiotics, Asemi et al. have shown increases of + 319.98 (vs. + 19.73 μmol/L in control) and + 36.58 ± 296.71 (vs. −92.04 ± 243.05 μmol/L in control) (156, 158). A more recent study by Raygan et al. demonstrated an increase in mean GSH (+ 18.0 ± 112.7 vs. −12.2 ± 122.5 μmol/L in control) following another single-species synbiotic supplement, these effects, however, were not statistically significant (127). In another study investigating multi-species synbiotic use among diabetics failed to show significant results given a similar rise in mean GSH in control (142). In addition, a recent study among severely comorbid diabetics had also failed to reach statistical significance in the findings (161).

Effect on Total Antioxidant Capacity

While multiple studies have investigated the effect of synbiotic use on TAC, the overall trends are not conclusive, with only a single study reporting a statistically significant association (**Table 4**). Bazyar et al. have reported a significant increase in TAC of + 0.04 ± 0.06 mM from baseline following a multispecies probiotic supplementation; however, this was not significant when compared to the change in control (164). On the other hand, Farrokhian et al. reported a larger absolute increase in mean TAC level (+ 49.8 ± 33.6 mmol/L), however, it was also not significant compared to a the change of + 30.0 ± 33.6 mmol/L in control (161).

Among single-species probiotics, Asemi et al. reported, across two studies, mean changes of + 69.48 ± 38.13 and −6.97 ± 203.51 mmol/L following different formulations; however, in both instances, the average change was positive when compared to control, although statistically insignificant (156, 158). A similar (yet statistically insignificant) trend is seen in the case of Bahmani et al. wherein a change of + 3.6 ± 247.2 mmol/L was found among the intervention group compared to −45.7 ± 240.3 mmol/L among the controls (142). Finally, Ghafouri et al. compared the effects of synbiotic bread with or without a metabolite, however, the effect of both on TAC levels were found to be both statistically and numerically insignificant (157).

Effect on Nitric Oxide

Very few studies have reported on the effect of NO following synbiotic supplementation among diabetics; however, of the available studies, all have shown significant associations following synbiotic supplementation (**Table 4**). Bahmani et al. reported an increase of + 40.6 ± 34.4 μmol/L in NO levels following an 8-week course of single-species synbiotic bread (142), while Asemi et al. reported an increase of + 6.83 ± 16.14 μmol/L following a 6 × 2 weeks crossover trial of another singles-species synbiotic (158). On the other hand, Farrokhian et al. showed a significant increase of + 7.6 ± 1.7 μmol/L after a 12 week intervention with a multi-species synbiotic (161).

Effect on Oxidized Low-Density Lipoprotein

Mirmiranpour et al. reported that compared to a baseline matched control group, an intervention of single-species synbiotic was significantly associated with a lower mean OxLDL (15.88 ± 1.98 vs. 17.07 ± 1.01 mU/L) at 3 months (**Table 4**) (144).

DISCUSSION

This systematic review collectively pooled data from forty-seven randomized controlled trial (RCT) studies to investigate the effect of probiotic, prebiotic and synbiotic supplementation on various markers of inflammation and oxidative stress among patients with T2D, with or without other comorbidities. Our results point toward the successful capacity of gut-microbiome promoting therapeutics to have beneficial effects on multimodal inflammation and oxidative stress inducing factors in the pathogenesis of T2D. Here we discuss intervention-specific trends in our findings that may add to growing evidence of currently researched questions and/or incentivize novel discoveries.

The Promise of Probiotics

Described as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance," (166) probiotics have been widely studied across literature given their capacity to have clinical therapeutic potential by antagonizing pathogenic or "harmful" bacteria and/or reversing pathogenic dysbiosis of the microbiome (166–170). Despite their role in metabolic diseases only being described from 2007 after a description of their potential role in obesogenesis in mice (171), a large array of mechanisms listing their potential against T2D and clinical trials testing their efficacies have surfaced.

A recent systematic review of association of different bacterial gut microbiome species in the T2D pathophysiology has reported that most species of *Bifidobacterium* are associated with a protective function in T2D, with lower levels of this probiotic compared to healthy controls being reported in T2D patients (172). Strain and species-specific associations of (previously) *Lactobacillus* have also been reported, although with more complexity in interpretation, given apparent opposite effects of different strains. Among studies investigating the effect of probiotics on inflammatory and oxidative stress biomarkers, multi-species probiotic supplementation has shown to be largely consistently more effective compared to single-species or monostrain probiotic administration. This can be observed in pro-inflammatory markers like hs-CRP (and CRP), TNF-α, IL-β, IL-6, IL-8, and LPS (endotoxin) across multiple trials (123, 136–140). Among these, (previously) *Lactobacillus* and *Bifidobacterium* strains such as *L. casei, L. rhamnosus, L. gasseri, L. plantarum, B. infantis, B. longum,* and *B. breve*, often in synergism, and to a lesser extent, *Lactococcus, Propionibacterium,*

and *Acetobacter* strains, have been reported to be consistently promising. *Bifidobacterium* have been shown across multiple animal studies to have promising probiotic effects in multiple metabolic dysfunctions through a variety of mechanisms; these include restoring the lymphocyte-macrophage balance and gut microbiota structure, reducing B-cell infiltration and increasing Treg activity (173–175), and modulating gut microbiome resulting in higher acetate SCFA levels (176). Animal models have also cemented that *Bifidobacterium* and (previously) *Lactobacillus* strains including *B. adolescentis* N3, *B. adolescentis* 7-2, *B. bifidum* M2, *L. rhamnosus* 7-1, and *L. rhamnosus* YC, were independently correlated with reduced levels of inflammatory biomarkers such as TNFA, IL1B, and IL-6 (177). However, given that (previously) *Lactobacillus* single-species administration has been associated with insignificant differences across multiple clinical trials reported in this review (124, 129, 143, 178), we may conclude that their probiotic effects are best attained in humans when used in combination with other strains and species of the same or different genus. This superiority of multistrain and multispecies probiotic is not new and has been described across other diseases and trials of the gut, such as pouchitis and ulcerative colitis (179). This is likely due to the enhanced probability of at least one of the many strains/species administered to survive, adapt, and produce anti-pathogenic and dysbiosis-attenuating response upon administration and survival in diseased microbiome consisting of harmful bacteria, whereas a single species is more vulnerable to endogenous microflora. In addition, single-species probiotics are limited in their therapeutic potential given that they are limited to the species-specific ability of the probiotic to render beneficial changes, whereas a collection of multispecies bacteria may not only have multiple mechanisms of completing similar beneficial pathways but may also use different pathways to achieve similar end effects.

Additionally, probiotics like *Lactobacillus acidophilus*, *L. reuteri, L. fermentum, L. bulgaricus, Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *Lactis*, and *Streptococcus thermophilus* have shown significant promise with respect to markers such as MDA (127), SOD, (143), GPX (144), GSH (124), TAC, and NO (125). Possible mechanisms reported include their effects on ascorbate autoxidation, metal-ions chelation, antioxidant enzymes system, and various antioxidant metabolites such as GSH (180), butyrate, and folate (181), and activity reduction and excretion of free radicals such as superoxide anion and hydrogen peroxide (182, 183). Their mode of delivery includes fermented milk, yogurt, bread, or simply as supplemental capsules. Hence a combination with sources that are shown to have probiotic-independent antioxidant abilities such as that of casein-derived peptides should be considered to maximize effectiveness (184). Using animal models, Hsieh et al. have shown that multi-strain probiotic composed of *Lactobaccilus salivarius* subsp. *salicinius* AP-32, *L. johnsonii* MH-68, *L. reuteri* GL-104, and *Bifidobacterium animalis* subsp. *lactis* CP-9 improved not only inflammatory markers, but also reduced MDA and increased SOD levels (185), potentially having β-cell protective function which would otherwise be disrupted due to oxidative stress (186).

The Promise of Prebiotics

Prebiotics refer to non-digestible and fermentable food ingredients serving as substrates that are selectively utilized by host gut-microbiota to provide health benefits through encouraging the growth of beneficial bacteria (187, 188). They are abundantly found in multiple fruits, vegetables, and cereals, including bananas, beans, garlic, onions, peas, and artichoke in the form of polysaccharides such as inulin, oligosaccharides, including both galactooligosaccharides and fructooligosaccharides, resistant starch, and even cinnamon (144, 167). By traveling undigested through the upper GI system, they are available for fermentation by the beneficial bacteria in the colon (189), leading to the production of beneficial metabolites such as SCFAs (acetate, butyrate, propionate) and lactic acid, which has significant effects on inflammation and intestinal membrane integrity (26), along with other mechanisms.

In our review, although we have highlighted the promise offered by a variety of prebiotic supplementations, resistant dextrin has been shown to be the most widely effective in both categories of markers (147, 149, 152, 153). Resistant dextrins are non-sweet short-chain glucose polymers with high resistance to digestive enzymes of the human gut, up to 75% of which is available for fermentation (172, 190). Ślizewska et al. have shown that resistant dextrin supplementation among mice was associated with lower levels of *Clostridium* strain (191), which is found in elevated levels among diabetics compared to normal gut (192). Resistant dextrin also led to higher levels of both the beneficial *Bifidobacterium* and prior *Lactobacillus* strains in the faces and cecum of rats without changing the overall bacterial count significantly (191). Similar results have been reported from clinical trials involving dextrin supplementation in humans (193). Valcheva et al. reported that IL-10 deficient mice fed with fiber dextrin diets over the course of 12 weeks secreted 47–88% less colonic IL-1β, TNF-α, IL-23, IL-12p70, IL-6, and CXCL1, with lower enterocyte injury scores and an increase in butyrate SCFA production (194). It has been priorly described (150) that through production of NF-κβ, butyrate modulates inflammation, controls macrophage and neutrophil activators and chemoattractant (195), and increases the expression of cytokine signaling 3 suppressor (196), all of which serve to promote anti-inflammatory Th2-lymphocyte differentiation rather than into Th1, ultimately increasing IL-10 levels among other mechanisms (197).

The prebiotic is also associated with beneficial changes in serum insulin, lipid, and gut microbial composition, with the promotion of the insulin signaling and the fatty acid β oxidation pathways in high-fat-high-fructose diet-fed rats and the enhancement of *Parasutterella* and *Parabacteroid* relative abundances and prevention of further harmful gut dysbiosis (198). Total SCFA concentrations and those of acetate, butyrate, and propionate individually were found to be dose-dependently higher among a group of rats fed increasing amounts of resistant dextrin compared to control (199). Finally, among two placebo-controlled RCTs investigating NUTRIOSE, a commercial resistant dextrin formula, increased levels of *Bacteroides* and SCFAs were observed (199). Resistant dextrin has

also shown to significantly increase GSH/GSSG ratio (by 33%) in rat models (200). By promoting growth of acid-resistant bacteria (such as *Roseburia*) that produce butyrate, resistant dextrin has shown to reduce ROS levels and increase plasma antioxidant enzymes through an LPS-mediated process that includes multiple factors such as NF-κβ (201–203). In addition, inhibiting the growth of *Clostridium perfringens* has been shown to reduce ROS through PKC, MEK/ERK, and NFκB pathways (149). Other mechanisms through which prebiotics including resistant dextrin but also oligo-fructose-enriched inulin, may modulate oxidative stress markers include reducing advanced glycation end -products and by serving as scavengers of ROS (150). Our findings are consistent with prior reviews (188) in this field.

The Promise of Synbiotics

Synbiotics are combinations of probiotic and prebiotic that are administered together. The rationale behind acceptance of their co-administration has been multifaceted: it has been shown to improve probiotic survival through provision of metabolic substrates that facilitate gastrointestinal tract transition, increase viability (204) and possible synergistic effects that may be independent of but parallel to the effects of the probiotic itself (205). Such combinations have shown to have beneficial effects on insulin resistance and glucose metabolism (25). Moreover, adding a prebiotic such as inulin to probiotic milk, yogurt, ice cream, and cheese formulations was found to increase survival in storage, increase apparent viscosity (206). Green et al. have also argued that the rationale of specific matching of prebiotic and probiotic can also include the possibility that certain prebiotics promote the growth of some bacteria more than others, if at all, based on the findings of Scott et al. who report that chain length of fructans (in prebiotic) is an important factor determining fermentation-specificity between species (207). Perhaps the most significant effect of a synbiotic is conferred from the study by Nazzaro et al. where the authors show that *Lactobacillus acidophilus* growth using inulin was associated with 14.5 times more butyrate production than in the presence of pectin, both of which were still significantly higher than administration of the probiotic with just glucose, with undetectable levels of butyrate (208). In addition to the priorly discussed roles of SCFAs such as butyrate, it has also been shown that butyrate inhibits IFN-γ production and has an active role in regulating peroxisome proliferator activated receptor-g (209). Clinical trials have reported more effective beneficial effect of synbiotic administration than in the case of probiotics alone in the case of prediabetic individuals with focus on prediabetes (210).

In our study, we have shown that across multiple studies investigating probiotics, prebiotics and synbiotics, the latter group of supplements have shown to be more effective compared to the former two primarily when comparing the change in oxidative stress biomarkers and that of antioxidant enzymes, namely MDA (164), plasma (164) and erythrocyte SOD (143), GPX (157), GSH (156), NO (142), and OxLDL (144). A combination of multispecies probiotic of *Lactobacillus acidophilus, L. casei, L. rhamnosus, L. bulgaricus, Bifidobacterium breve, B. longum, Streptococcus thermophilus,* and the prebiotic fructo-oligosaccharide has shown exemplary promise among the greatest number of markers. *Bacillus coagulans* with inulin was the most effective single species synbiotic. Given the previously described rising importance to "mix-and-match" prebiotic with a probiotic that can derive maximal benefit from it, the work of Fuhren et al. with respect to synbiotic matchmaking using inulin, fructooligosaccharides and multiple strains of *Lactiplantibacillus plantarum* show an effective screening model that may enable researchers and industrial manufacturers to create the ideal synbiotic (211). One such study by Nagpal and Kaur revealed that *L. casei,* one of the seven probiotic species of the most promising synbiotic identified in this review, had higher viability in inulin compared to oligosaccharide media (212); it would be interesting to compare the other strains and species with various prebiotics, especially resistant dextrin, which was identified as the most promising in this review.

Intestinal Dysbiosis and Inflammation

Apart from the direct impact of poor nutrition on the immune response, changes in the intestinal microbiota due to obesogenic environmental factors can also stimulate inflammation (213). The altered composition of the gut microbiota due to the consumption of a high-fat, low-fiber diet has been directly correlated with a pronounced low-grade inflammatory state linked to T2D (214). This is because consumed fibers are broken down and fermented by the gut microbiota in the large intestine into short chain fatty acids (SCFAs), including butyrate, propionate, and acetate (215). These metabolites are recognized by GPR41 and GPR43, both of which are G protein-coupled receptor. SCFAs are considered a main source of nutrients and energy for colonocytes and microbes, are involved in the regulation of energy and pH levels, and stimulate the release of glucagon-like peptide-1 (GLP-1) (216). Lower fiber intake results in a decrease in metabolites such as SCFAs and eventually lead to cases of intestinal dysbiosis. Microbial metabolites also have significant implications on inflammatory responses. Butyrate controls the maturation of dendritic cells (DC) as well as preventing associations between adipocytes and macrophages (217). Propionate, on the other hand, decreases synthesis of adipokine in adipocytes (218). Acetate also participates in the maintenance of balance in the gut by suppressing the expression of inflammatory cytokines while upregulating the synthesis of anti-inflammatory cytokines (26). Thus, a decrease in these metabolites due to reduced fiber intake can stimulate pro-inflammatory responses in patients with T2D. Additionally, dysbiosis allows potentially pathogenic microbes to shift locations and replicate faster in the gut epithelium. Large numbers of translocated bacteria are recognized as invaders by the immune system (219), thereby evoking a chronic inflammatory response through the activation of toll-like receptors (TLRs) and upregulation of pro-inflammatory cytokines (220).

Since there is a proven alteration in the gut microbiota of T2D, researchers have investigated the merit of correcting this dysbiosis as a potential cure for T2D. It is unclear if measurable alteration in the gut microbiota directly correlates to or is needed for pro/prebiotics to exert their effect for treating T2D (165). The short study duration of some of the clinical studies can explain

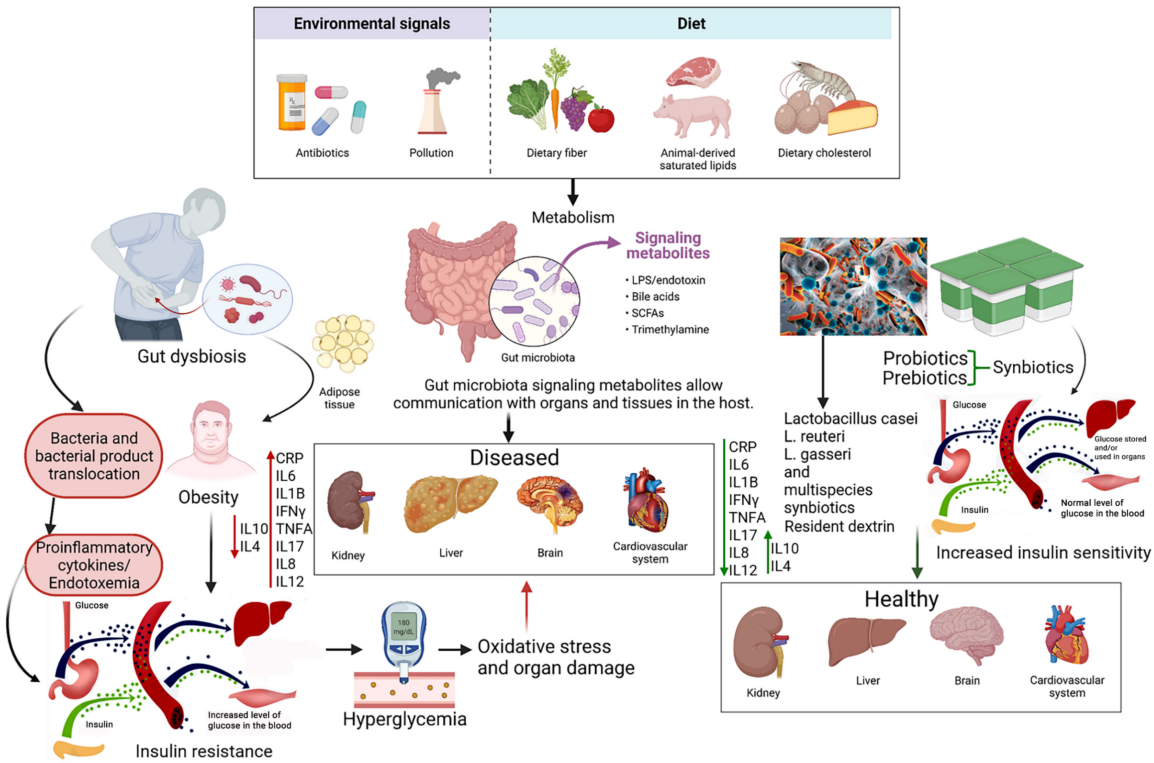

FIGURE 2 | Summary diagram illustrating the effect of diet, pre and probiotics as a potential safe therapy in treating T2D.

this; for example, Gonai et al. (38) have shown that while the prebiotic galacto-oligosaccharide (GOS) ameliorated the decrease of *Bifidobacteriaceae* in T2D, LPS- binding protein (LBP) and glucose tolerance did not improve during the trial period. LPB stimulates inflammatory cytokines through TLR4 and thus it provides a potential mechanism of how the gut microbiota dysbiosis causes T2D (38). However, in another small study examining the effect of GOS on gut permeability no significant improvement has been found (221). It is important to note that the first study had a bigger sample size and a larger administered dose of GOS than the second. Another prebiotic fiber that is extensively studied in the treatment of T2D is inulin-type fructans. Increased production of SCFAs improves T2D and a 50/50 mixture of inulin and oligofructose increases *bifidobacteria* and SCFAs in feces, while butyric acid and the microbial diversity are not affected (41).

Intestinal Permeability and Inflammation

The intestinal barrier is a semipermeable membrane that regulates the absorption of nutrients and electrolytes from the lumen into the blood stream, while preventing the entrance of infectious microorganisms as well as antigens, endotoxins, and proinflammatory substances (222). Intestinal permeability, therefore, is an intrinsic characteristic of the intestinal epithelium that allows for the exchange of luminal substances whilst maintaining an immunological barrier. However, intestinal hyperpermeability, commonly referred to as a "leaky gut," has been linked to several disorders, including gastrointestinal, such as celiac disease and colon carcinoma, in addition to other extra-intestinal diseases, including diabetes (223). Even though defective intestinal barriers could be a result of disease aggravation, clinical studies hypothesize that it could also be a causal factor in the progression of disease and the initiation of autoimmune destruction (224). Altered intestinal barrier function leads to an unrestricted influx of antigens or toxins into the gut, consequently instigating an inflammatory response in the lumen and other proximal organs (225). Increased intestinal permeability has, thus, become a new target for disease prevention and therapy of type 1 and 2 diabetes (226). Considering the close relationship between intestinal permeability and gut dysbiosis, we can conclude that meticulous dietetic and probiotic approaches to recover healthy microbiota have the potential to make a breakthrough in the management of these diseases in the near future.

An epidemiological study has shown that the increased levels of 16S ribosomal DNA from gut bacteria in the blood is a risk factor for diabetes (227). Thus, it is hypothesized that bacterial translocation could play a role in T2D pathophysiology. It has been found that the administration of the probiotic *Lacticaseibacillus casei* strain Shirota could reduce gut bacterial translocation and can alter the gut microbiota in patients with T2D (135). Additionally, *L. reuteri* and *L. gasseri* both were found in higher levels in the probiotic fed subjects' fecal samples. This may explain the decreased bacterial translocation as these bacteria improve the membrane integrity through

mucus production expression of tight junctions and reduction of apoptosis (135). Further, it was also shown by Horvath et al. (165) that multispecies synbiotic strengthens the gut barrier function and thus reduces levels of c-peptide, LPS and bacterial DNA in the participants' serum, indicating a decreased translocation of bacterial products to the blood.

Gut Microbiota, Nutraceuticals, COVID-19, and Diabetes

While age remains to be the most significant predictor of COVID-19 related morbidity and mortality, diabetes, along with other chronic conditions, was identified early as a significant comorbidity of the disease (228), with diabetes being present in over one-third of hospitalized individuals in one New York City cohort (11). Low-grade inflammation, characterized by chronically increased levels of inflammatory cytokines such as IL-6, TNF-α, and IL-1β, is present as a common feature of many metabolic diseases including T2D, as well as COVID-19 (229). Since diabetics with low-grade inflammation compared to healthy controls have shown notable reductions in serum inflammatory markers following insulin therapy (230) and COVID-19 associated mortality among diabetics was also significant reduced in a subgroup with controlled blood glucose (231), it may very well be the case that control of low-grade inflammation through modulation and correction of gut dysbiosis may serve as a significant therapeutic strategy against COVID-19 associated mortality and morbidity among those with pre-existing T2D. Perhaps unsurprisingly, COVID-19 hospitalized patients have been shown to have "significant alterations in fecal microbiomes, characterized by enrichment of opportunistic pathogens (such as *Coprobacillus, Clostridium ramosum*, and *Clostridium hathewayi*) and depletion of beneficial commensals (such as *Faecalibacterium prausnitzii*, an anti-inflammatory bacterium, and multiple *Bacteroides* species responsible for downregulation of ACE2 expression)" (232). It is then expected that probiotics may be able to assist host innate and adaptive immunity in COVID-19 struck patients as a form of adjuvant strategy (233). In addition to resolution of gut dysbiosis, probiotic have the potential to contribute to a healthy gut-lung axis by reducing translocation of pathogens through the intestinal mucosa, thereby reducing the potential of simultaneous infections, which may lead to poorer prognosis. The effects of bacteria from the previously *Lactobacillus* and *Bifidobacterium* genus have been elucidated in our systematic review; it is very encouraging to also note that these species have shown great promise in reducing the incidence of Ventilator associated pneumonia and upper-respiratory tract infections in venerable cohorts (234). In fact, probiotics have been shown to have a larger beneficial effect in inflammatory biomarker levels such as IL-6 and CRP as a measure of low-grade inflammation than even Angiotensin Receptors Blockers, omega-3, metformin, resveratrol, and vitamin D (235). These gut-microbiome modulating nutraceuticals have shown to serve also as immunomodulators leading to downregulation of the low-grade inflammation state (236, 237), leading to an overall reduction or attenuation of COVID-19 related symptoms such as "diarrhea, abdominal pain, vomiting, headache, cough, sore throat, fever, and viral infection complications such as acute respiratory distress syndrome (ARDS)" (238). By close monitoring of potential interactions with diabetes and antiviral drugs, especially conserving that many antibiotics given during COVID-19 infection may further lead to gut microbiome dysbiosis (233), probiotics, along with pre- and synbiotics, may serve as therapeutic agents with low adverse event incidence for treatment of diabetics with COVID-19 (239).

CONCLUSION

Systematic review of current literature showed that T2D patients have a different gut microbiome composition than healthy individuals which may result from dysbiosis due to the pathogenic state. On the other hand, it is also plausible that this altered gut microbiota has the potential to lead early onset and development of T2D. By altering the gut microbiota using pre-, pro-, and synbiotics, it is possible to modify factors causing inflammation and oxidative stress. In this review, we have reported on multiple promising and effective pre-, pro-, and synbiotics for their association to changes observed in markers of inflammation and oxidative stress. We have identified significant trends and observations between single and multistrain probiotics, identified the most promising rising synbiotic as resistant dextrin, and showed how synbiotics may be more effective compared to the other two types of nutraceuticals among oxidative stress markers. Furthermore, we have elucidated and reviewed the role of metabolites, signaling pathways, low-grade inflammation, gut permeability, and dysbiosis with respect their ultimate roles in the pathogenesis and possible therapy in T2D, as well as the potential of these nutraceuticals to attenuate COVID-19 infection related symptoms. **Figure 2** provides a generalized summary of the role of microbiome-targeted nutraceuticals in T2D pathogenesis and potential correction of dysbiosis.

FINAL REMARKS, PROSPECTS AND CLINICAL TRIALS

While human clinical trials in literature have elucidated the great potential and promise of using pro-, pre-, and synbiotics as therapeutic agents for the treatment of T2D, further large-scale and multicenter trials and investigations are required given their controversial results in the past. What also needs to be assessed and delivered is the type of biological agents that are most likely to be accepted widely, wildtype or recombinant, single or multiple strain/species. Multi-center and longer-term trials that are coordinated in their research methodology and analysis of data need to be undertaken to compare the effects of variable factors such as genetic susceptibility to the disease and genetic acceptance to the biotic therapy suggested here. In the interim, clinicians and researchers alike should follow ongoing clinical trials such as NCT05110703 that investigates the effect of a daily prebiotic fiber meal replacement shake on

the quality of life in T2D patients. In addition, trials such as NCT04089280 go a step beyond and aim to elucidate the effect of an 8-strain multispecies probiotic in their capacity to reduce metformin-induced GI adverse effects. Finally, NCT04769687 is perhaps the most promising trial that will attempt to investigate the effect of a twice-daily 8-strain multispecies, oligofructose and Raftiline HP synbiotic on a variety of parameters among T2D plus CKD patients, with the primary outcome of CRP levels, followed by inflammatory cytokines, circulating monocytes, microbial metabolome, membrane permeability, bacterial translation, quality of life and frailty.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AC designed the study, critically supervised the project, revised and reviewed the manuscript, and initially screened studies. RS performed the database search, references extraction, methodology section draft and flowchart, and reviewed and edited the manuscript. BA screened the study abstracts and full-texts. PP and RK further critically screened full-texts, extracted, and analyzed the data, updated the search, wrote majority of the manuscript, generated most of the tables, and further edited the manuscript. MA wrote parts of the manuscript and generated a table. MS and RU reviewed and edited the manuscript, and generated one figure. All authors have read and agreed to the published version of the manuscript.

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Fecal Microbiota Transplantation for Patients With Irritable Bowel Syndrome: A Meta-Analysis of Randomized Controlled Trials

Hui-jun Zhao [1,2†], Xiao-jing Zhang [1,3†], Na-na Zhang [1], Bin Yan [1], Ke-ke Xu [1,3], Li-hua Peng [1] and Fei Pan [1*]*

[1] Department of Gastroenterology and Hepatology, The First Medical Center, Chinese PLA General Hospital, Beijing, China, [2] Department of Gastroenterology, National Center for Children's Health, Beijing Children's Hospital, Capital Medical University, Beijing, China, [3] School of Medicine, Nankai University, Tianjin, China

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Tarek Mazzawi,
Al-Balqa Applied University, Jordan

***Correspondence:**
Fei Pan
panfei@plagh.org
Li-hua Peng
penglihua301@126.com

[†] These authors have contributed equally to this work and share first authorship

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Background: Gut microbiota has been identified as an imbalance in patients with irritable bowel syndrome (IBS). Fecal microbiota transplantation (FMT) is a novel method to restore microbiota and treat IBS patients.

Objective: To conduct a meta-analysis and estimate the efficacy and safety of FMT for the treatment of IBS patients with subgroup analyses to explore the most effective way of FMT for IBS.

Methods: All eligible studies were searched from PubMed, Embase, Web of Science, and the Cochrane Library through multiple search strategies. Data were extracted from studies comprising the following criteria: double-blind, randomized controlled trials (RCTs) that compared the efficacy of FMT with placebo for adult patients (≥18 years old) with IBS. A meta-analysis was performed to evaluate the summary relative risk (RR) and 95% confidence intervals (CIs).

Results: A total of seven RCTs comprising 489 subjects were eligible for this meta-analysis. Pooled data showed no significant improvement of global IBS symptoms in patients with FMT compared with placebo (RR = 1.34; 95% CI 0.75–2.41, $p = 0.32$). A significant heterogeneity was observed among the studies ($I^2 = 83\%$, $p < 0.00001$). There was no significant evidence of funnel plot asymmetry (Egger's test, $p = 0.719$; Begg's test, $p = 1.000$), indicating no existence of publication bias. Subgroup analyses revealed that FMT operated by invasive routes, including gastroscope, colonoscope, and nasojejunal tube, significantly improved global IBS symptoms (RR = 1.96; 95% CI 1.23–3.11, $p = 0.004$) with heterogeneity ($I^2 = 57\%$, $p = 0.06$) and an NNT of 3 (95% CI 2–14). However, FMT delivered *via* oral capsules showed a negative impact on patients with IBS (RR = 0.56; 95% CI 0.33–0.96, $p = 0.03$) with a low heterogeneity ($I^2 = 39\%$, $p = 0.2$) and an NNH of 3 (95% CI 2–37).

Conclusion: The current evidence from RCTs with all routes of FMT does not show significant global improvement in patients with IBS. However, FMT operated by invasive routes significantly improved global IBS symptoms.

Keywords: irritable bowel syndrome, fecal microbiota transplantation, meta-analysis, randomized controlled trials, global IBS symptoms, subgroup analysis

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most diagnosed GI conditions and a symptom-based functional bowel disorder, characterized by recurrent defecation-related abdominal pain, accompanied by altered bowel habits including stool form and frequency (1). The possible pathophysiological mechanisms include genetics, low-grade bowel inflammation, injured mucosal barrier, increased gut permeability, abnormal bile acids metabolism, aberrant serotonin metabolism, altered motility, visceral hypersensitivity, activated immune response, and central neurologic dysfunction (2, 3).

The current evidence suggests that the gut microbiota could be a significant factor in the pathogenesis of IBS patients who are always identified with dysbiosis. Various studies have proven the difference between the gut microbiota of IBS patients and that of healthy people (4, 5). Increased Firmicutes, decreased Bacteroidetes, and an increased Firmicutes/Bacteroidetes ratio were found in IBS patients (4). The gut microbiota is also related to the severity, symptoms, and subtypes of IBS. The severity of IBS was found negatively correlated with microbial richness, exhaled CH_4, presence of methanogens, and the prevalence of Prevotella enterotype (6). IBS-D and IBS-M patients had a higher prevalence of Bacteroides enterotype in comparison with the IBS-C patients (6). Infections with some bacterial pathogens like *Campylobacter jejuni*, *Escherichia coli*, and *Salmonella enterica serovar Typhimurium*, *Clostridioides difficile*, and *Vibrio cholerae* have been found associated with post-infection IBS (7). Exposure to antibiotics is also considered to increase the risk of developing IBS (8). Additionally, the dysfunction of the bidirectional communication between gut microbiota and the brain, known as the brain–gut axis, is widely regarded as a vital reason for the occurrence of IBS (9).

Due to the impact of gut microbiota on the occurrence and development of IBS, several interventions targeting gut microbiota are commonly used to treat IBS. These include dietary interventions, prebiotics, probiotics, synbiotics, and antibiotics. Except for the traditional interventions above, fecal microbiota transplantation (FMT) provides a creative way to restore the abnormal gut microbiome in patients with IBS directly. FMT has been widely accepted as an effective and safe treatment for recurrent and refractory *Clostridioides difficile* infection (10). However, studies on the role of FMT in IBS are limited and inconsistent. Some current clinical studies confirm the effectiveness of FMT in the treatment of IBS, but some come up with the opposite conclusion. The research on the changes in the gut microbiota of IBS patients after being treated by FMT are also far from consistent. Differences in the dosage, frequency, delivery, and preparation method of donor stool make huge heterogeneity in these studies. Hence, a meta-analysis and systematic review with subgroup analyses were conducted to estimate the efficacy and safety, as well as to explore the most effective way of FMT for the treatment of IBS.

METHODS

Search Strategy

We first conducted a systematic search of the PubMed, Embase, Web of Science, and the Cochrane Library from inception to January 2022. Then, we manually searched the clinicaltrials.gov and relevant gastrointestinal conferences, including Digestive Disease Week, United European Gastroenterology Week, and American College of Gastroenterology, for relevant trials up to January 2022.

The following medical subject headings (MeSH) or free-text terms were used: "Irritable Bowel Syndrome," "IBS," "Syndrome, Irritable Bowel," "Colon, Irritable," "Mucous Colitis" (free-text terms) were for IBS; "Fecal Microbiota Transplantation," "Microbiota Transplantation, Fecal," "Intestinal Microbiota Transfer," "Fecal Transplantation," "Transplantation, Fecal," "Fecal Transplant," "Donor Feces Infusion," "Infusion, Donor Feces" were for FMT. The search results of IBS and FMT were combined using the Boolean term "AND." HJZ and FP independently reviewed all titles and abstracts for eligibility based on predefined inclusion and exclusion criteria. The searching protocol was restricted to publications with human subjects, but no language limitations.

Study Selection

The articles included should satisfy the following criteria: double-blind, randomized controlled trials that compared the efficacy of FMT with placebo for adult patients ($\geq$18 years old) with IBS were eligible, including crossover RCTs reporting the data of the first treatment period; patients with IBS should meet the accepted symptom-based criteria (Manning, Kruis, Rome I, Rome II, Rome III, or Rome IV) or a physician's opinion. Studies had to report whether there was a global improvement of IBS symptoms after therapy. Minimum duration of 8-week follow-up was required. When studies did not offer the dichotomous data but were eligible for inclusion, we contacted the first authors or corresponding authors of these studies to obtain additional information.

Outcome Measures

The primary outcomes were the efficacy of FMT compared with placebo for response to therapy assessed by global improvement in IBS symptoms. Global improvement was defined as a self-report improvement of overall IBS symptoms or the reduction of IBS-related symptom questionnaires, including the IBS Severity Scoring System (IBS-SSS) or gastrointestinal symptom rating scale, IBS version (GSRS-IBS). Secondary outcomes were the change in IBS-specific quality of life (IBS-QOL), adverse events (AEs, total AEs, or individual AEs, including diarrhea, constipation, abdominal pain, bloating, and nausea), and microbiota alterations following FMT.

Data Extraction

Two authors (HJZ and XJZ) independently extracted all data into a Microsoft Excel spreadsheet. We collected the general information and outcomes from all eligible studies, including country of origin, the number of centers and population,

study design, IBS criteria and subtypes, preparation of fecal microbiota and placebo, FMT route and frequency, follow-up, primary outcomes, and AEs as dichotomous data, the change of IBS-SSS and IBS-QOL as continuous data. Dichotomous data were extracted by intention-to-treat analysis and dropouts were regarded as treatment failures. For continuous data, if the studies did not report the mean and standard deviations (SD), they were estimated based on the previous methods (11, 12).

Assessment of Risk of Bias

Two investigators (HJZ and XJZ) independently performed the quality assessment using the Cochrane Risk of Bias tool (11), and disagreements were discussed with the third investigator (FP). We documented the following information to evaluate the risk of bias of the included RCTs: the generation of randomization schedule, concealment of allocation, blinding of participants, personnel and outcome assessment, incomplete outcome data, selective reporting, and other bias. We further evaluated the quality of evidence using the GRADE (Grading of Recommendations Assessment, Development and Evaluation) method (13).

Data Analysis

Data analysis was performed using a random-effects model based on the DerSimonian and Laird (14) method. Summary relative risk (RR) and 95% confidence intervals (CIs) were calculated for

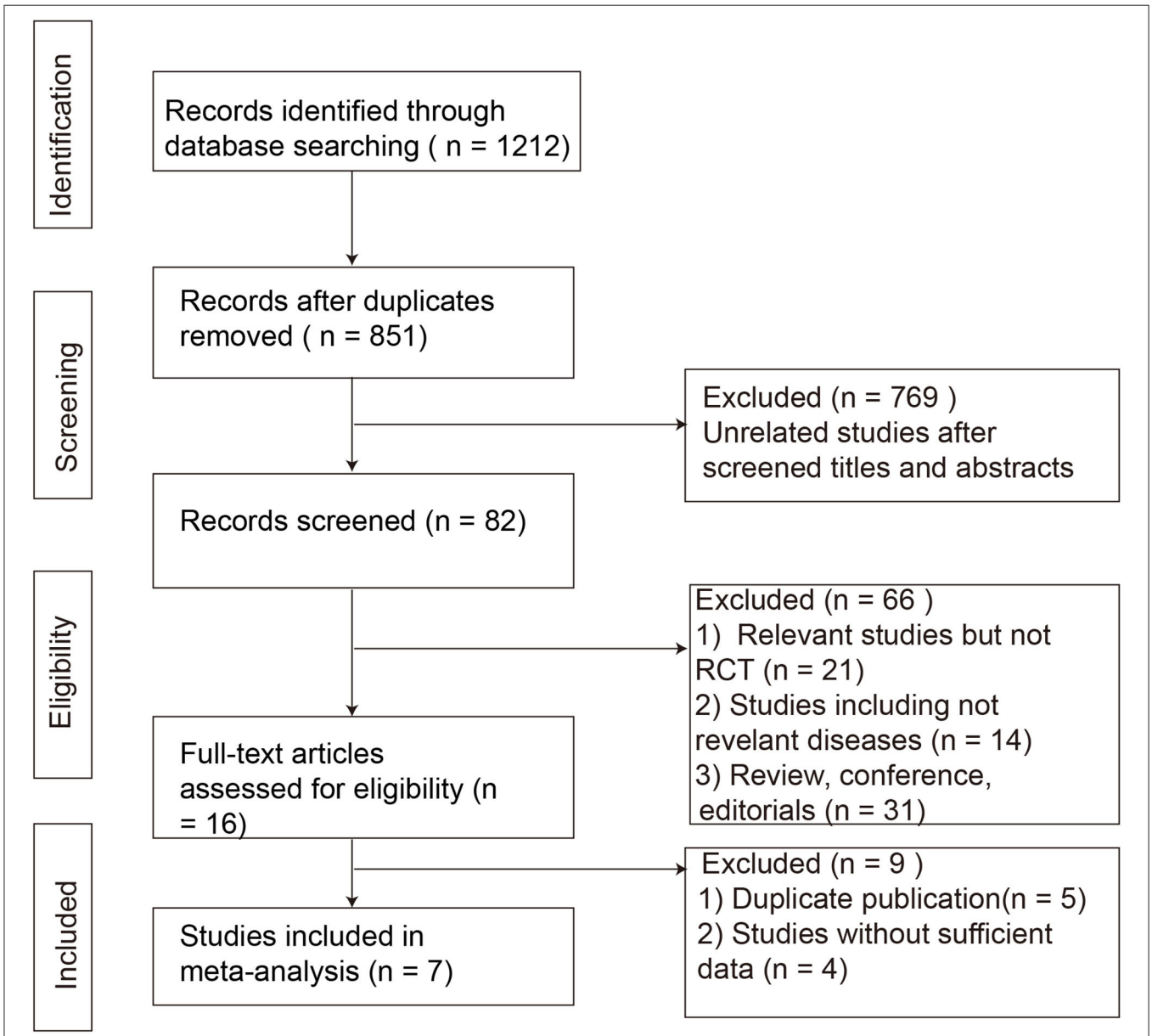

FIGURE 1 | Flowchart of study selection strategy in the systematic review and meta-analysis. RCTs, randomized controlled trials.

dichotomous outcomes, including the global improvement, and total and individual AEs. Pooled mean difference (MD) and 95% CIs were reported for continuous outcomes, including IBS-SSS and IBS-QOL. Significant heterogeneity was defined using the Cochrane Q statistic with a p-value < 0.10 and I^2 statistic with a cutoff of $\geq$50%. Subgroup analyses were performed to evaluate the effects of different factors on the efficacy of FMT in patients with IBS. Publication bias was assessed by funnel plot, Egger's and Begg's tests (15). The number needed to treat (NNT) or the number needed to harm (NNH) was calculated by the equation: NNT or NNH = 1/[control event rate × (1 – RR)]. RevMan 5.3 (Oxfordshire, United Kingdom) and Stata 14 (StataCorp, College Station, TX) were used for data analysis and to generate plots.

RESULTS

General Information, Assessment and Quality

A total of 1,212 citations were identified by the combination of key terms (**Figure 1**). A total of 361 articles were excluded as duplicates, and 769 were excluded as unrelated studies after cross-referencing the titles and abstracts; 66 references were further excluded for various reasons. Finally, 16 full manuscripts were reviewed and only seven RCTs containing 489 subjects were eligible according to the predetermined inclusion criteria (16–22). The general information of the included RCTs is represented in **Table 1**. A total of six studies (16–19, 21, 22) adopted Rome III as the diagnosis criteria except one study using Rome IV (20). One study included patients with IBS-D only (18), three studies included IBS-D/M (17, 21, 22), and three studies included IBS-D/M/C (16, 19, 20). Among them, two studies performed FMT administration through repeatedly frozen oral capsules with donor stool or placebo-mimics (16, 18), and other five studies through a single invasive method (gastroscopy, colonoscopy, or nasojejunal tube) with the suspension of the donor stool or autologous stool (17, 19–22).

The risk of bias was summarized using the Cochrane Collaboration tool (**Figure 2**). In addition, the overall quality of evidence using the GRADE method was "very low" for the inconsistency and imprecision of the primary outcome (**Supplementary Table 1**).

Primary Outcome: Global Improvement

Seven RCTs involving 420 subjects reported global improvement, which was defined as the reduction of IBS-SSS total score in five trials (16–18, 20, 21), the reduction of GSRS-IBS in one trial (19), or self-reported improvement of overall IBS symptoms and abdominal bloating (22). There were 136 (58.12%) of 234 patients receiving FMT who achieved clinical response, compared with 75 (40.32%) of 186 assigned to placebo. Pooled data showed no significant improvement of global IBS symptoms in patients with FMT compared with placebo [RR = 1.34; 95% CI 0.75–2.41, $p = 0.32$ from random effects] (**Figure 3A**). A significant heterogeneity was observed among studies ($I^2 = 83\%$, $p < 0.00001$). There was no significant evidence of funnel plot asymmetry (Egger's test, $p = 0.719$; Begg's test, $p = 1.000$), indicating no existence of publication bias (**Figures 3B,C**).

Given the high heterogeneity observed after pooling the results, we further conducted a subgroup analysis based on different designs of administration (**Figure 4**). FMT operated by invasive routes, including gastroscopy, colonoscopy and nasojejunal tube, significantly improved global IBS symptoms (RR = 1.96; 95% CI 1.23–3.11, $p = 0.004$) with heterogeneity ($I^2 = 57\%$, $p = 0.06$) and an NNT of 3 (95% CI 2–14). However, FMT delivered *via* oral capsules showed a negative impact on patients with IBS (RR = 0.56; 95% CI 0.33–0.96, $p = 0.03$) with a low heterogeneity ($I^2 = 39\%$, $p = 0.2$) and an NNH of 3 (95% CI 2–37). Studies under the invasive routes simultaneously adopted a single infusion of suspension from donor stool in the FMT group or from autologous stool in the placebo group, whereas studies *via* oral capsules adopted multiple doses of capsules from donor stool in the FMT group or from microbe-free mimics in the placebo group. Single FMT was found more beneficial compared to placebo (RR = 1.96; 95% CI 1.23–3.11, $p = 0.004$) with heterogeneity ($I^2 = 57\%$, $p = 0.06$) and an NNH of 3 (95% CI 2–14), whereas multiple FMT was less beneficial (RR = 0.56; 95% CI 0.33–0.96, $p = 0.03$) with a low heterogeneity ($I^2 = 39\%$, $p = 0.2$) and an NNH of 3 (95% CI 2–37). Donor stool was observed more effective than autologous stool (RR = 1.96; 95% CI 1.23–3.11, $p = 0.004$) with heterogeneity ($I^2 = 57\%$, $p = 0.06$) and an NNH of 3(95% CI 2–14), but less effective than microbe-free mimics (RR = 0.56; 95% CI 0.33–0.96, $p = 0.03$) with a low heterogeneity ($I^2 = 39\%$, $p = 0.2$) and an NNH of 3(95% CI 2–37). We further analyzed the influence of donor mixed or not, whether do a bowel cleansing before treatment and the first location of FMT, but no significant effect was observed on the global IBS improvement.

Secondary Outcome: IBS-SSS and IBS-QOL

The change of IBS-SSS was reported in six RCTs (16–21). A total of three trials, El-Salhy et al., Johnsen et al., and Lahtinen et al. (17, 20, 21), showed a significantly improved tendency of IBS-SSS total score in the FMT group compared to the placebo group, especially in El-Salhy et al.'s study ($p < 0.001$). A total of two studies (18, 19), Aroniadis et al. and Holster et al., found great improvement in IBS-SSS within groups after treatment, but no difference between groups. Only Halkjær et al. (16) observed a significant relief in the placebo group at 3 months, not in the FMT group (-125.71 ± 90.85 vs. -52.45 ± 97.72, $p = 0.012$). The raw data of IBS-SSS were not available in Johnsen et al. and Holster et al., so we extracted data from the other four RCTs containing 121 participants in the FMT group and 128 in the placebo (16, 18, 20, 21), however, no significant difference was observed between the FMT and placebo groups (mean difference = 15.58, 95% CI −66.74 to 97.91, $p = 0.71$ from random effects, $I^2 = 94\%$) (**Figure 5A**).

The change of IBS-QOL was compared in six RCTs (16, 18–22). Compared with placebo, one study, El-Salhy et al.'s (20), showed a significant increase in the IBS-QOL total score after FMT. A total of four other studies, Aroniadis et al., Holster

TABLE 1 | General information of included seven RCTs.

References	Country, Number of centers	Diagnostic criteria	IBS subtypes	Sample size	Allocation	Donors	Bowel cleansing	FMT route and location (upper/lower GI tract)	Dosage of FMT group
Aroniadis et al. (18)	USA, 3 centers	Rome III	100% IBS-D	48	1:1	Four donors, not mixed	No	Oral capsules, Upper	25 frozen capsules (0.38 g FMT) per day
El-Salhy et al. (20)	Norway,1 center	Rome IV	62 (37.8%) IBS-C; 63 (38.4%) IBS-D; 39 (23.8%) IBS-M	165	1:1:1	One donor, not mixed	No	Gastroscopy, Upper	Frozen 30 g FMT and 60 g FMT
Halkjær et al. (16)	Denmark, 2 centers	Rome III	17 (33.3%) IBS-C; 15 (29.4%) IBS-D; 19 (37.3%) IBS-M	52	1:1	Four donors, mixed	Yes	Oral capsules, Upper	25 frozen capsules (50 g FMT) per day
Holster et al. (19)	Sweden, 1 center	Rome III	4 (25%) IBS-C; 9 (56.3%) IBS-D; 3 (18.8%) IBS-M	17	1:1	Two donors, not mixed	Yes	Colonoscopy, Lower	Frozen 30 g FMT
Holvoet et al. (22)	Belgium, 1 center	Rome III	100% IBS-D or IBS-M	62	2:1	Two donors; not mixed	No	Nasojejunal tube, Upper	Donor fresh feces
Johnsen et al. (17)	Norway, 1 center	Rome III	44 (53%) IBS-D; 39 (47%) IBS-M	90	2:1	Two donors, mixed	Yes	Colonoscopy, Lower	Frozen or fresh 50–80 g FMT
Lahtinen et al. (21)	Finland, 4 centers	Rome III	25 (51%) IBS-D; 7 (14.3%) IBS-M; 14 (28.6%) IBS-unsubtyped; 3 (6.1%) other	55	1:1	One donor, not mixed	Yes	Colonoscopy, Lower	Frozen 30 g FMT

References	Dosage of control group	Frequency	Follow-up (months)	Primary outcome	Secondary outcome	Microbial analysis
Aroniadis et al. (18)	25 placebo capsules per day	Multiple: lasting 3 days	3	Difference in the IBS-SSS total score at 3 months	Reduction in the IBS-SSS total score of at least 50 points at 3 months; the assessment of differences in QOL, depression, anxiety, stool consistency and microbiome profiles at 3 months	16S rRNA
El-Salhy et al. (20)	Frozen 30 g autologous feces	Single	3	Reduction in the IBS-SSS total score of ≥50 points at 3 months	Reduction in the dysbiosis index (Di) and a change in the intestinal bacterial profiles at 1 month	16S rRNA
Halkjær et al. (16)	25 placebo capsules per day	Multiple: lasting 12 days	6	Reduction in the IBS-SSS total score of ≥50 points at 3 months	Change in IBS-QOL scores at 3 months and changes in microbiota diversity before and after FMT	16S rRNA
Holster et al. (19)	Frozen 30 g autologous feces	Single	6	Reduction in the GSRS-IBS total score of ≥ 30%	Change of the IBS-SSS, their general health and quality of life (36-item Short Form Survey (SF-36), IBS-QOL, anxiety and depression status	Human Intestinal Tract Chip (fecal and mucosa)
Holvoet et al. (22)	Autologous feces	Single	3	Self-reported improvement of overall IBS symptoms and abdominal bloating at 3 months	Changes in daily assessed IBS symptoms, IBS-QOL, change of IBS-related symptoms scores and fecal microbiota transplantation	16S rRNA
Johnsen et al. (17)	Frozen or fresh 50–80 g autologous feces	Single	12	Reduction in the IBS-SSS total score of ≥75 points at 3 months	Reduction in the IBS-SSS total score of ≥ 75 points at 12 months	NA
Lahtinen et al. (21)	Fresh 30 g autologous feces	Single	3	Reduction in the IBS-SSS total score of ≥50 points at 3 months	Changes in IBS-QOL, gut microbiota, fecal water content, intestinal microbiota composition, and stool dry weight.	16S rRNA

IBS, irritable bowel syndrome; IBS-C/D/M, IBS with predominant constipation, predominant diarrhea, predominant mixed diarrhea/constipation; GI tract, gastrointestinal tract; FMT, fecal microbiota transplantation; IBS-SSS, IBS Severity Scoring System; GSRS-IBS, gastrointestinal symptom rating scale, IBS version; IBS-QOL, IBS-specific quality of life; NA, not applicable.

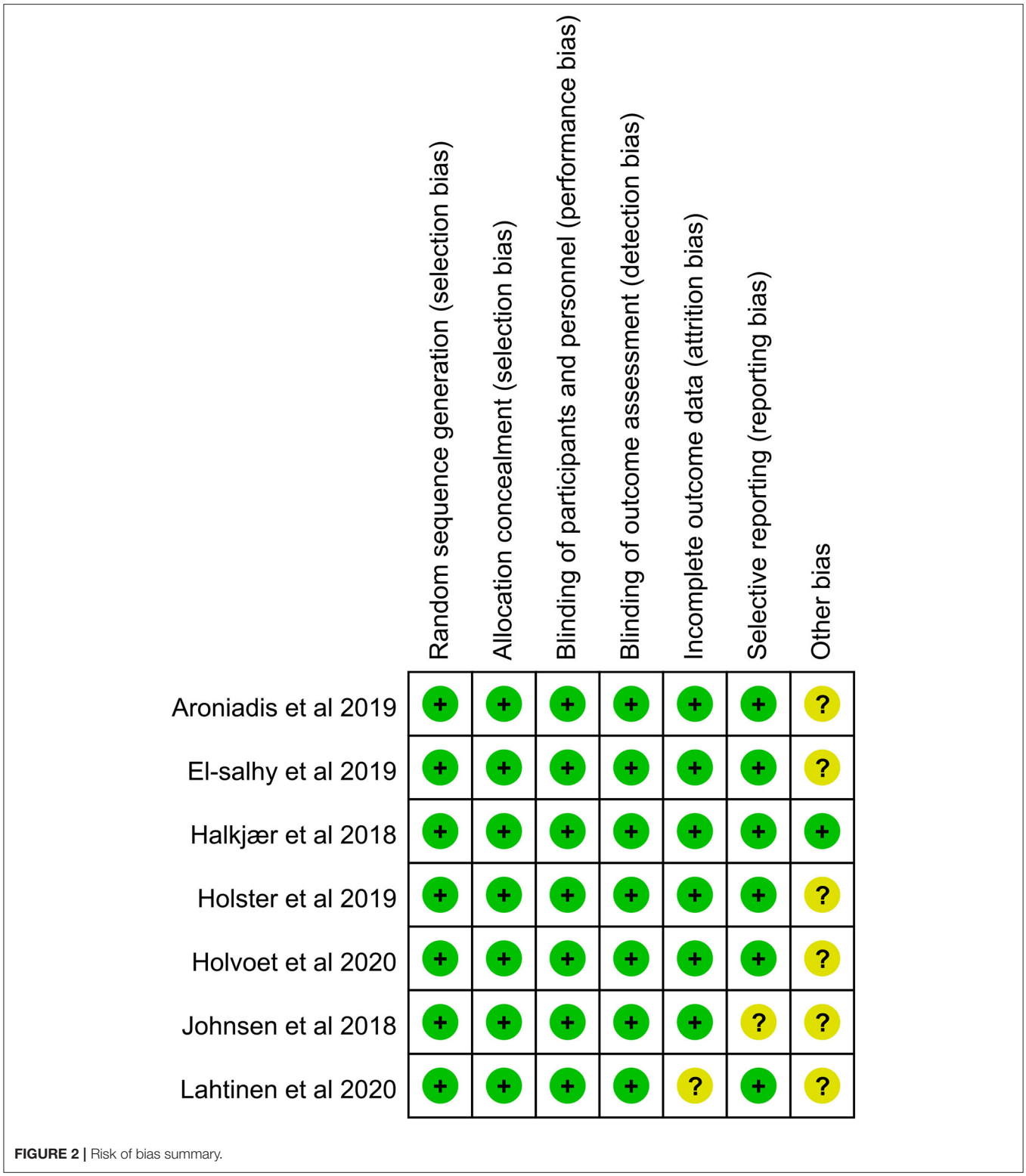

FIGURE 2 | Risk of bias summary.

et al., Lahtinen et al., and Holvoet et al. (18, 19, 21, 22), observed no dramatic difference between groups. One study, Halkjær et al.'s (16), showed a greater improvement in the placebo group than that in the FMT group. The raw data of four RCTs were available (16, 18, 20, 22) and the pooling analysis, including 143 subjects in the FMT group and 123 in the placebo, showed no significant difference between the FMT and placebo group (mean difference = 3.41, 95% CI −18.24 to 25.07, $p = 0.76$ from random effects, $I^2 = 95\%$; **Figure 5B**).

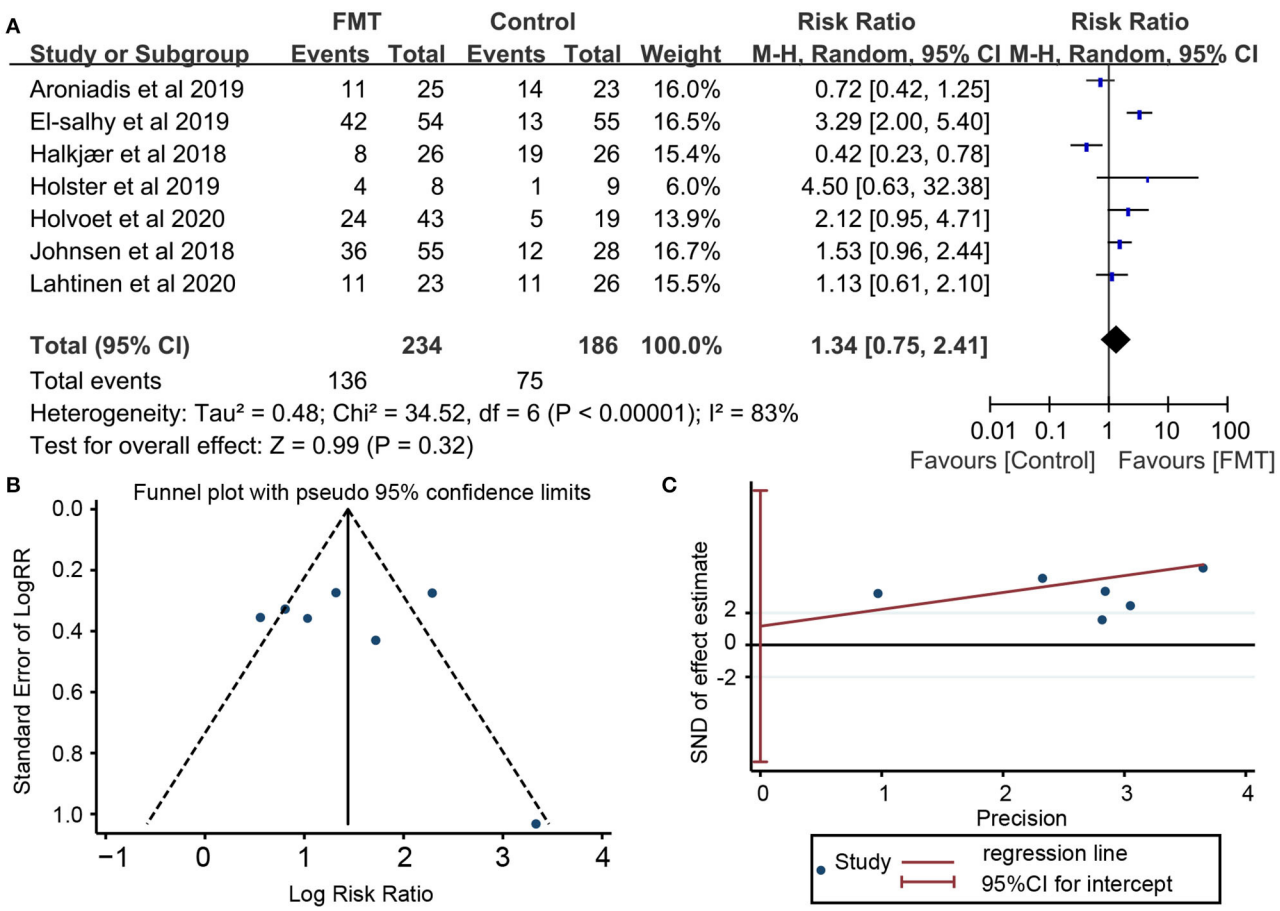

FIGURE 3 | Forest plot and publication bias for the effect of FMT on the primary outcome. **(A)** Forest plot: random-effects meta-analysis of the effect of FMT on the primary outcome of irritable bowel syndrome (IBS) compared with placebo; **(B)** Funnel plot of global improvement of IBS for publication bias; **(C)** Egger's test global improvement of IBS for publication bias.

Microbiota Analysis

A total of six of the seven RCTs reported the results of fecal microbiota analyses (16, 18–22). A total of two studies (16, 18) in which FMT was delivered by oral capsules, two studies by colonoscope (19, 21), one study by nasojejunal tube (22), found that the bacterial composition of FMT recipients shifted closer to that of the donors. The study by the nasojejunal tube showed a higher diversity of microbiota in the fecal samples from responders before FMT than that from non-responders (22). However, no significant difference in specific bacteria between responders and non-responders was observed. One study delivered by gastroscope showed that *Eubacterium biforme*, *Lactobacillus* spp., and *Alistipes* spp. were increased in responders following FMT, and *Bacteroides* spp. was decreased. *Lactobacillus* spp. was negatively correlated with the clinical outcome of IBS-SSS (20).

Adverse Events

A total of six of the seven RCTs provided the total or individual adverse events (AEs) data (16–21). The total AEs data from five RCTs, including 59 (35.8%) of 165 patients in the FMT group compared with 59 (42.8%) of 138 in the placebo group, were pooled (16–19, 21). No significant difference in the number of total AEs was found between the above two groups [RR = 0.97; 95% CI 0.68–1.39, $p = 0.89$ from random effects, $I^2 = 51\%$]. The most common individual AEs included diarrhea, constipation, nausea, abdominal pain, and bloating. Pooled data of AEs found a higher risk of constipation following FMT compared with placebo [RR = 4.66; 95% CI 1.05–20.74, $p = 0.04$ from random effects, $I^2 = 20\%$] (**Figure 6**). No significant differences were observed in other common individual AEs. Additionally, Only Johnsen et al. reported that one participant suffered transient vertigo and nausea following FMT, belonging to one serious AE, and needed to be hospitalized for several hours for observation (17).

DISCUSSION

This systematic review and meta-analysis evaluated the efficacy of FMT in the treatment of IBS and conducted subgroup analyses to explore the influencing factors for the effectiveness of FMT in IBS. According to our findings, the pooled data

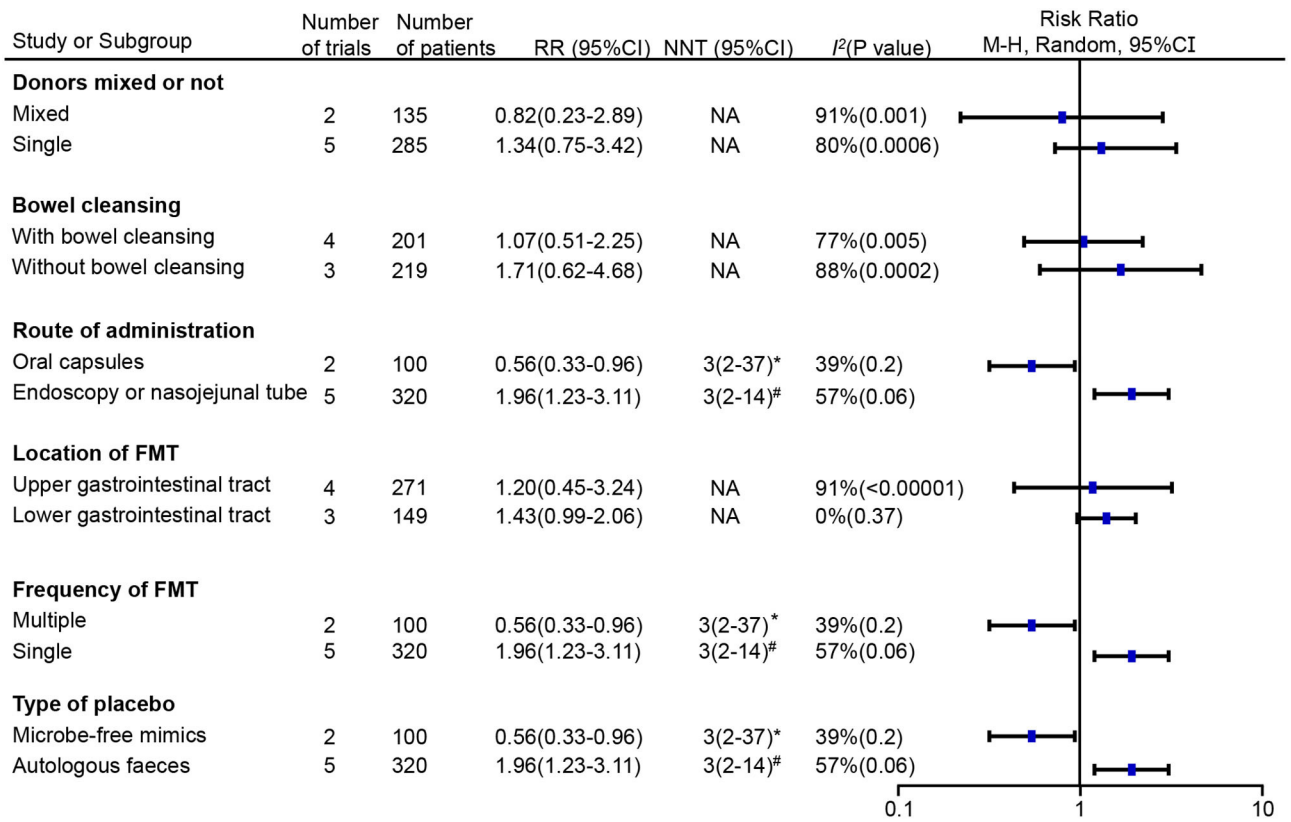

FIGURE 4 | Subgroup analyses of the effect of FMT on the primary outcome of irritable bowel syndrome (IBS) compared with placebo. RR, risk ratio; 95% CI, 95% confidence interval; NNT, number needed to treat; *number needed to harm (NNH); #number needed to treat (NNT); NA, not applicable.

showed no significant improvement in global symptoms in IBS patients treated with FMT compared with placebo, in line with the conclusions of the previous studies (23, 24). It was shown by pooled data that neither IBS-SSS nor IBS-QOL was reduced by FMT. However, due to the differences in the dosage, frequency, delivery, and preparation method of donor stool, as well as selected donors among the selected research, there has been huge heterogeneity in these studies on the efficacy of FMT in IBS. A total of five of the seven RCTs reported a trend for the bacterial composition of IBS patients to get close to the microbiota of the donor after FMT, including two studies in which FMT was operated *via* oral capsules (16, 18), one study *via* colonoscope (19), one study *via* gastroscope (20), and one study *via* nasojejunal tube (22), revealing the modification of gut microbiota after FMT. As for safety, the pooled data showed a higher risk of constipation after FMT compared with placebo, without showing significant differences in other common individual AEs like diarrhea, nausea, bloating, or abdominal pain. Only one serious AE was reported that one participant suffered transient vertigo and nausea after FMT and needed hospitalized observation (17).

When we further conducted subgroup analysis, a significant improvement of global IBS symptoms was observed in patients treated with FMT *via* invasive routes, including gastroscopy, colonoscopy, and nasojejunal tube, whereas a negative impact was found in IBS patients with FMT *via* oral capsules. In a study by Aroniadis et al., no significant symptom relief was found in the oral FMT group compared with the placebo group (18). Similarly, in the study by Halkjær et al., patients in the placebo group had more obvious symptom improvement than those treated with FMT *via* oral capsules, although increased microbial diversity was observed in the FMT group (16). They further analyzed the effect of oral FMT on abdominal pain, stool frequency, and stool form, but found no clinical beneficial effect (25). In their following study, they found long-term increased anaerobic bacteria in the FMT group, such as *Faecalibacterium*, *Prevotella*, and *Bacteroides* (26). It is presumed that the changes in the microbiota induced by oral FMT are not significant enough to improve the IBS symptoms. It is likely that invasive FMT can deliver a higher dose of donor stool to patients' bowels at a time than oral capsules, which is supposed to contribute to improving the abundance and diversity of microbiome reaching patients' bowels. A more expansive contact area is available through invasive ways, which may be helpful in the landing of donor stool and the rebuilding of gut microbiota. Considering the things mentioned above, it seems more advisable to choose invasive FMT routes in preference to oral ways.

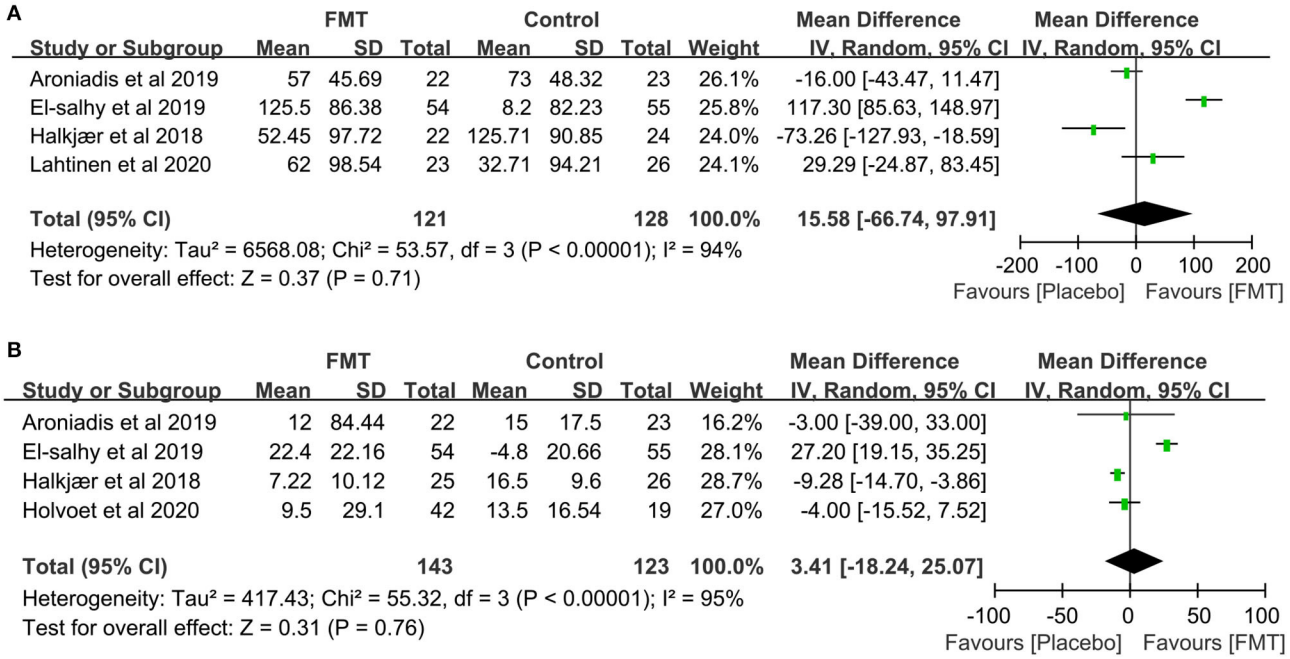

FIGURE 5 | Forest plot of the effect of FMT on IBS-SSS and IBS-QOL. **(A)** Forest plot of the effect of FMT on IBS-SSS compared with placebo; **(B)** Forest plot of the effect of FMT on IBS-QOL compared with placebo.

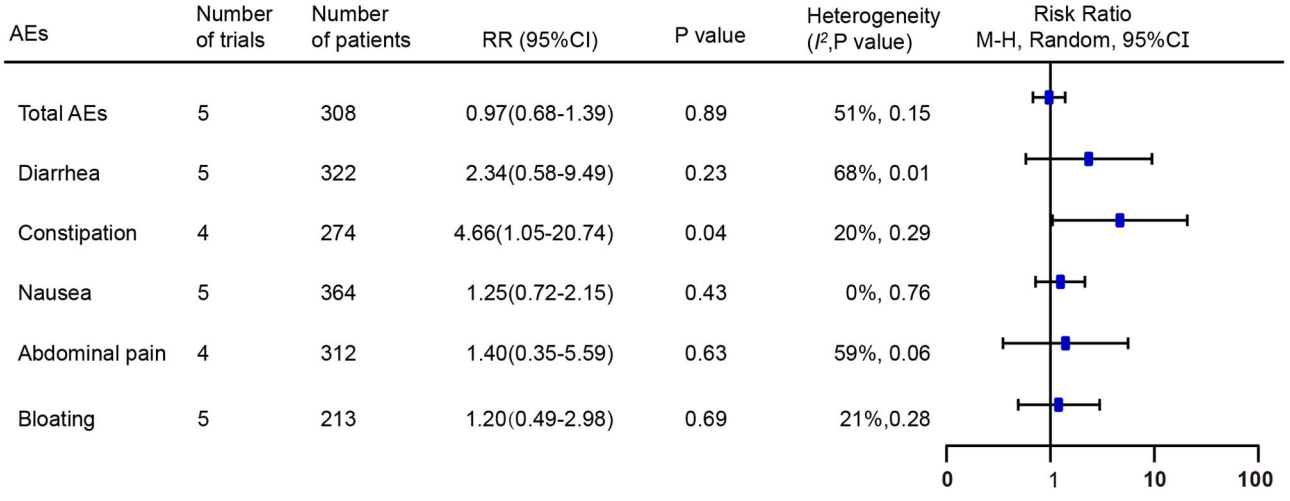

FIGURE 6 | Forest plot of the effect of FMT on total and individual adverse events compared with placebo. RR, risk ratio; 95% CI, 95% confidence interval.

As for invasive ways for FMT, only in one study by El-Salhy et al., FMT was operated by gastroscopy, using stool from a super donor (20). Definite improvements in abdominal pain, fatigue, and the quality of life were observed in a positive correlation with the dose. Changes in microbiota were observed in this study, like a higher abundance of *Eubacterium biforme*, *Lactobacillus* spp., and *Alistipes* spp., as well as a lower abundance of *Bacteroides* spp. Relationships between the symptoms and gut microbiota were also detected, such as an inverse correlation between the IBS-SSS score and the concentrations of *Lactobacillus* spp. and *Alistipes* spp., as well as a negative correlation between the Fatigue Assessment Scale (FAS) score and the concentration of *Alistipes* spp. (20). Guo et al.'s study, which was excluded from our meta-analysis for the lack of the standard of global improvement, also reported altered gut microbiota in IBS patients treated with oral FMT (27). Enriched α- and β- bacterial diversity, increased concentrations of the beneficial Bacteroidetes and Firmicutes, as well as decreased toxic releaser Enterobacteriaceae, *Bacteroides*,

and *Escherichia coli Shigella* were detected (27), which was partly similar with the result of El-Salhy et al.'s study (20). In El-Salhy et al.'s further study, they detected an increased fecal butyric acid level in the responders to FMT, which could be explained by the above changes in microbiota, indicating that changes in fecal short-chain fatty acids (SCFAs) may be a potential mechanism by which FMT could treat IBS (28).

FMT was conducted by colonoscopy in three studies from Johnsen, Holster, and Lahtinen respectively (17, 19, 21). All of them showed improvements in the IBS symptoms, despite different assessment methods. However, FMT only induced a transient relief for 3 months in accordance with Lahtinen's research, coinciding with the result of Johnsen's study (17, 21). Interestingly, relatively long-term conversions of the composition of gut microbiota were also detected in their studies (21, 29). It may attribute to the complex integrated effects of multiple factors. For example, the low FODMAP diet which IBS patients commonly take can inversely impact the maintaining of functions of planted microbiota, instead of the composition, due to the lack of fermentation substrate. Other factors like drugs and comorbidities may also impact the functions of gut microbiota. But it is a pity that the related information has not been recorded and thus cannot be analyzed.

According to the result of Holster's study, single FMT *via* colonoscopy after bowel cleansing could improve symptoms and quality of life in IBS patients without significant difference compared to autologous FMT (19). Perhaps it could be explained by that bowel cleansing before treatment could improve the restoration of microbiota and the improvement of IBS symptoms after FMT, no matter allogenic or not. There are increasing pieces of evidence for bowel cleansing altering gut microbiota (30, 31). In Freitags' study conducted in mice, pre-interventions with antibiotics before FMT were found useless for the overall plantation of donor microbiota, but helpful for the plantation of *Bifidobacterium*, which was commonly considered as a probiotic (32). However, the specific role of bowel cleansing is still not fully understood. In our subgroup analysis, we also observed that the efficacy of donor stool was superior to that of autologous stool but inferior to that of microbe-free mimics, which is as per that of another meta-analysis from Ianiro G (23). However, when we took all the selected RCTs into account, pooled data showed no significant effect of bowel cleansing before treatment. It was also found that bowel cleansing merely without FMT did not improve the overall restoration of microbiota (32), suggesting that FMT could help rebuild gut microbiota. In Holster's further study, increased expression of the immune-related gene was found induced by allogenic FMT, with a significant difference compared to autologous FMT (33). The utmost response was observed at the time of 2 weeks after FMT, which could partly account for the transient effect of FMT on IBS mentioned above.

Another study conducted by Holvoet et al. in which FMT was operated in the IBS patients with predominant abdominal bloating by nasojejunal tube, reported statistically significant reductions in discomfort, the number of stools, urgency, abdominal pain and flatulence as well as an improvement in quality of life after FMT (22). Additionally, they mentioned high diversity and overall bacterial composition at baseline as an important biomarker to predict successful FMT (22). The effectiveness of FMT was also found positively related to the stability of the microbial composition in the donors and the similarity of the microbiota composition between patients and donors. However, no significant difference in specific bacteria between responders and non-responders was discovered. Repetitive FMT using stool of another successful donor was found effective in a fair number of non-responders to single FMT, suggesting an advantage of multiple FMTs. However, some non-responders still failed to respond to repetitive FMT (22). When we took all the data from selected RCTs into consideration to analyze the influence of FMT frequency on the effectiveness in IBS patients, we even came up with a contrary conclusion to Holvoet's study. It suggests a potential resistance for some IBS patients to FMT. Moreover, it would be a different result after eliminating research in which FMT was conducted *via* oral ways.

We further analyzed the influence on the FMT effectiveness of donor stool mixed or not, but found nothing significant. As for the location of FMT, our subgroup analysis observed no significant effect on the global IBS improvement. However, the study from Ianiro demonstrated a latent benefit for lower-gastrointestinal-tract FMT compared to upper-gastrointestinal-tract FMT (23).

There are some limitations to our study. All the selected RCTs are small-sample studies, thus it is necessary to expand the sample size. Differences in FMT administration routes, doses, frequency, preparation of stools, patient inclusion criteria, and donor selection among RCTs resulted in huge heterogeneity in our study. Therefore, the establishment of a more standard FMT experimental process is still needed. Influencing factors like diet and drugs were not recorded or analyzed in these studies, which may affect the result to a certain extent. Due to the distinct inclusion criteria, it is hard to assess the influence of FMT on various IBS subtypes and symptoms. Thus, larger and more standard RCTs on FMT treating IBS are still required.

In conclusion, pooled data from current RCTs of FMT showed no significant relief for global IBS symptoms, but a lasting alteration of gut microbiota. However, invasive FMT significantly improved global symptoms in IBS patients compared with oral FMT.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FP designed research. N-nZ, BY, K-kX, L-hP, and X-jZ performed research. H-jZ and FP analyzed data. H-jZ and X-jZ wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.890357/full#supplementary-material

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Effects of Multispecies Synbiotic Supplementation on Anthropometric Measurements, Glucose and Lipid Parameters in Children With Exogenous Obesity: A Randomized, Double Blind, Placebo-Controlled Clinical Trial (Probesity-2 Trial)

Gonca Kilic Yildirim[1,2], *Meltem Dinleyici*[1,3], *Yvan Vandenplas*[4] *and Ener Cagri Dinleyici*[1*]

[1] *Department of Pediatrics, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey,* [2] *Pediatrics Nutrition and Metabolism Unit, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey,* [3] *Department of Social Pediatrics, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey,* [4] *UZ Brussel, KidZ Health Castle, Vrije Unversiteit Brussel, Brussels, Belgium*

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University of Foggia, Italy

***Correspondence:**
Ener Cagri Dinleyici
timboothtr@yahoo.com

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Studies on the effects of synbiotics on obesity in children are limited. The objective of this randomized double-blind placebo-controlled trial was to test the effects of a multispecies synbiotic during 12 weeks on anthropometric measurements, glucose metabolism and lipid parameters in 61 children with exogenous obesity. All children were treated with a standard diet and increased physical activity and received once daily a synbiotic supplement (probiotic mixture including *Lactobacillus acidophilus, Lacticaseibacillus rhamnosus, Bifidobacterium bifidum, Bifidobacterium longum, Enterococcus faecium* and fructo-oligosaccharides) or daily placebo for 12 weeks. At baseline, no statistically significant differences existed in anthropometric measurements, glucose and lipid parameters between both groups. We observed changes for anthropometric measures (% reduction comparing to baseline) in both synbiotic and placebo groups. After 12 weeks; changes (% reduction comparing to baseline) in weight ($p < 0.01$), BMI ($p < 0.05$), waist circumference ($p < 0.05$) and waist circumference to height ratio ($p < 0.05$) were significantly higher in the children receiving the synbiotic supplement. There is no difference in glucose metabolism, lipid parameters, presence of non-alcoholic fatty liver disease between both groups after 12 weeks. The daily intake of a multispecies synbiotic in addition to diet and increased physical activity did improve anthropometric measurements: body weight, BMI, waist circumference and waist/height ratio. The supplementation of this synbiotic is an efficient weight-loss strategy above diet and exercise in pediatric obesity (Trial identifier: NCT05162209).

Keywords: obesity, children, adolescent, probiotic, synbiotic

INTRODUCTION

Obesity is a critical public health concern that affects ∼20% of the world's population and is linked to a number of serious comorbidities including metabolic, cardiovascular, respiratory, and cancer illnesses in both developed and developing countries (1, 2). An estimated 38 million children under the age of five years and over 340 million children and adolescents aged 5–19 years were overweight or obese. The prevalence of overweight and obesity among children was 18% in 2016 (1). Childhood obesity is classified as exogenous or endogenous, depending on the etiology. Exogenous obesity is produced by a long-term imbalance in energy intake and expenditure, whereas endogenous obesity is caused by a variety of genetic, syndromic, and endocrine factors (3). The majority of obese children and adolescents grow up to be obese adults. Obesity in childhood not only leads to long-term health issues that manifest in adulthood, but it also leads to secondary complications such as dyslipidemia, insulin resistance, and non-alcoholic fatty liver disease (1, 2, 4, 5). Obesity in children is typically treated by reducing energy intake through food regulation and increasing energy expenditure through increased activity (2).

Many disorders (obesity, metabolic syndrome, diabetes, asthma, and atherosclerosis) have been demonstrated to have altered microbiota compositions (dysbiosis), but research is currently ongoing to identify whether these changes are cause or effect. As a result, it's suggested that manipulating the gut microbiota could be a therapeutic target for reducing host energy storage (4). There have been studies on the use of probiotics and prebiotics as a support for obesity treatment, however the majority of these studies involved adults (6, 7). The synbiotic is a mixture, comprising live microorganisms and substrate(s) selectively utilized by host microorganisms, that confers a health benefit on the host, according to the International Scientific Association of Probiotics and Prebiotics. Complementary and synergistic synbiotics are the two types of synbiotics (8). Probiotics and synbiotics, specifically certain strains of *Lactobacillus gasseri, L. rhamnosus, and L. plantarum,* associated with other *Lactobacillus species* and/or species from the Bifidobacterium genus, have the potential to aid in weight and fat mass loss in overweight and obese populations, according to the systematic review (5). *L. acidophilus* in combination with *L. casei and Bifidobacterium*, or *L. acidophilus* in combination with *Bifidobacterium infantis*, had positive benefits on body weight loss in participants who maintained their usual lifestyle (9, 10). It has also been reported that daily ingestion of diet-enriched prebiotics, such as fructooligosaccharide, enhances satiety. However, while experimental studies indicate the positive effect of prebiotics in obesity, clinical trial outcomes are mixed (11). The standard treatment of obesity in children is based on a reduction of the energy intake by regulating the diet and increasing the energy expenditure by increasing the activity. There are studies on the use of probiotics and prebiotics as a support for treatment in obesity, but most of these studies were conducted in adult age groups. Studies on the effects of synbiotics on obesity in children are limited (12, 13). The goal of this randomized, double-blind, placebo-controlled trial was to see how this particular multi-strain synbiotic affected anthropometric measurements, glucose metabolism, and lipid markers in children with exogenous obesity.

PATIENTS AND METHODS

Study Design

This is a single-center, prospective, randomized, double-blind, placebo-controlled clinical study in children aged between 8 and 17 years with exogenous obesity who admitted for the first time to Eskisehir Osmangazi University Faculty of Medicine, Department of Pediatrics, Nutrition and Metabolism Department between January 2019-June 2021. This clinical study was planned and performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, patient rights regulation and ethical committees. Permission for the study was obtained from the Clinical Research Ethics Committee of Eskisehir Osmangazi University Faculty of Medicine with the Decision Number 54 on September 27, 2018. This study is registered in ClinicaTrials.gov under the Identifier number NCT05162209. The study protocol was explained to all participants and their families, and a written informed consent was obtained from all parents and children prior to the inclusion.

Study Population, Inclusion and Exclusion Criteria

Children and adolescents, aged 8–17 years, with a body mass index (BMI) equal to or higher than the age- and sex-specific 95th revised percentiles of the Centers for Disease Control and Prevention (CDC) were evaluated according to the study criteria (14). Patients who had no pathological findings other than obesity in their physical examination were considered as "exogenous obese" and included in the study (3). Patients with secondary obesity or endogenous obesity, history of any chronic diseases and/or chronic medication use and/or monogenic syndromes and other genetic syndromes, or those under special diets were excluded from the study. Patients who used probiotics/synbiotics/fibers or antibiotics in the 8 weeks before possible inclusion, were also excluded. The flow chart of the study shown in **Figure 1**.

Physical Examination, Anthropometric Measurements and Calculation

The participants' age and gender were recorded. A detailed nutritional history of the patients was collected. A full physical examination (including blood pressure measurement, presence of striae and acanthosis nigricans) have been performed. All anthropometric measurements (body weight, height, waist and hip circumference) were made by a trained nurse using standard protocols and calibrated instruments. Weight and height of participants were measured with light clothes and without shoes. Body weights were measured with a classical scale and height was measured in centimeters, standing upright, using a wall mounted stadiometer. CDC data were used to evaluate body weight and height measurements (14). BMI was calculated as the ratio of body weight (kilograms) to height squared (meter square). The

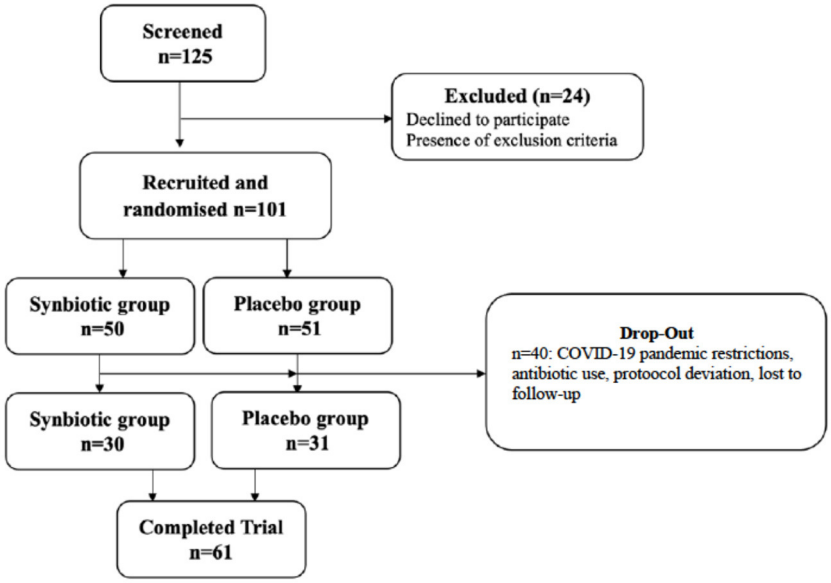

FIGURE 1 | Flow chart of the study.

circumference of the middle arm was measured at the left arm at the middle of the distance between shoulder and elbow; skinfold thickness was measured at the midpoint of the left shoulder and elbow over the triceps using a skinfold caliper. Waist and hip circumferences were measured with a non-elastic tape. Waist circumference was measured while the child was standing, with the abdomen relaxed, arms at the sides, and feet together, by wrapping a non-elastic tape measure around the thinnest part of the body, at a point midway between the lower border of the rib cage and the iliac at the end of expiration. The hip measurement was taken by holding it parallel to the ground, at the maximum girth of the buttocks. Waist-to-height and waist-to-hip ratio was calculated.

Laboratory Tests

Laboratory tests including serum glucose, insulin, serum liver markers (aspartate aminotransferase-AST; alanine aminotransferase-ALT, serum lipid parameters including triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) after following 12 h of fasting from all patients have been noted at baseline and 12 weeks later. The diagnosis of dyslipidemia was made according to the criteria $\geq$95th percentile of each serum TG, TC, LDL-C level or below 5th percentile of HDL-C level by comparing with reference values according to age and gender (15). The degree of insulin resistance was estimated with the homoeostatic model assessment for insulin resistance (HOMA-IR) score was calculated with the formula (glucose) x (insulin)/405. The diagnosis of non-alcoholic fatty liver disease (NAFLD) was based on ultrasonographic findings and sex-specific ALT reference ranges (normal ALT <26 U/L in males and <22 U/L in females) (16).

Diet and Increasing the Physical Activity

The definition of obesity, its effects on the body, complications and the course of the intervention were explained in detail to the patients and their families during about 30 min. A dietary intervention and increased physical activity were recommended in all cases. All obese children recorded their daily food consumption during last three days. The diets of the patients were reduced with 10% from their habitual caloric intake. No foods were banned, but cutting back on high-energy foods and drinks were recommended. The total energy content was composed so that 55% came from complex carbohydrates, 30% from fats and 15% from proteins. Daily cholesterol intake was regulated not to exceed 300 mg. The intake of saturated fats was planned to be <10% of the total energy intake and the intake of trans fats was planned to be <1% of the total energy intake. The intake of total polyunsaturated fatty acids was limited to 15%. Free sugar intake was reduced below 10% of total energy intake. All enrolled children monitored *via* a phone call once per 15 days and the patients were called to the outpatient clinic controls once a month. The diet was checked according to the verbal statements of the individual about the diet menus prepared for the individual. In addition, the declared dietary content was calculated by the same dietitian at each visit. In addition to their normal activities, the patients were advised to exercise moderate and heavy for at least 30 min daily. At each visit, they were questioned about their compliance with the exercise.

Randomization, Intervention and Masking

The patients were divided in two groups by a computer-generated randomization sequence which assigned participants in a 1:1 allocation ratio to treatment with synbiotic or placebo with blocks of 8, blinding the study team, patients and their relatives. Interventional products were numbered, and all investigators

and patients were blinded for all the duration of the study. Treatment duration was 12 weeks. In the first group, 1 sachet each day for 12 weeks (*Lactobacillus (L.) acidophilus* ($4.3x10^8$ CFU/sachet), *Lacticaseibacillus (L.) rhamnosus* (4.3×10^8 CFU/sachet), *Bifidobacterium (B.) bifidum* ($4.3x10^8$ CFU/sachet), *B. longum* ($4.3x10^8$ CFU/ sachet), *Enterococcus faecium* (8.2×10^8 CFU/sachet), total 2.5×10^9 CFU per sachet, fructooligosaccharide (FOS) 625 mg, lactulose 400 mg, Vitamin A (6 mg), Vitamin B1 (1.8 mg), Vitamin B2 (1.6 mg), Vitamin B6 (2.4 mg), Vitamin E (30 mg), Vitamin C (75 mg) were given. The second study group was given a placebo consisting of a similar sachet with shape, taste, and smell identical to the synbiotic sachet for 12 weeks.

Follow-Up

Anthropometric measurements and biochemical indices were evaluated in all participants at baseline and after 12 weeks of intervention. Study compliance was monitored *via* a phone call once per 15 days and the patients were called to the outpatient clinic controls once a month. The patients were asked to record and contact the study team in case of an undesirable effect associated with the use of interventional products. Patients were asked to report if antibiotic therapy was started during the study; these children were also excluded from the study.

Outcomes

The primary outcome of the study was to evaluate the effects of synbiotics on the anthropometric measurements after 12 weeks. Secondary end points were the effects of synbiotics on lipid parameters, presence of hyperlipidemia, glucose metabolism, and NAFLD.

Statistical Analysis

Statistical Package for Social Sciences (SPSS) version 28.0 for Windows (SPSS - Chicago, IL, United States) was used for statistical analysis. Statistical analyses were performed according to per protocol. Continuous variables were expressed as mean, given as standard deviation. After assessment of the normal distribution by the Kolmogorov-Smirnov test, anthropometric measurements, glucose and lipid parameters were compared among the groups by using the independent sample *t*– test for continuous data and a chi-square test for categorical data. Paired Student's *t*-tests were used for comparing baseline and 12 weeks of intervention in synbiotic and placebo group. *p*-Values lower than 0.05 were considered as statistically significant.

RESULTS

Data from 61 children; 33 girls (18 synbiotic/15 placebo) and 28 boys (12 synbiotics; 16 placebo), aged between 8 and 17 years with exogenous obesity, who completed the 12 study-weeks (30 in the placebo group and 31 in the placebo group) were available. There was no statistically significant difference in gender ($p > 0.05$). The mean age of the patients in the synbiotic group was 11.8 ± 3.1 years, and 12.4 ± 2.7 years in the placebo group ($p > 0.05$). The anthropometric parameters of the synbiotic and placebo group at baseline and at the end of the 12th weeks of intervention are provided in **Table 1**.

Comparison of Anthropometric Measurements

At baseline, there was no statistically significant difference between the two groups for anthropometric measurements and calculations ($p > 0.05$).

In the synbiotic group, at the end of the 12th week, all the following parameters were lower compared to baseline: body weight ($p < 0.001$), body weight Z-score ($p < 0.001$), BMI ($p < 0.001$), BMI Z-score ($p < 0.001$), triceps skinfold ($p < 0.001$) thickness ($p < 0.001$), upper arm circumference ($p < 0.001$), waist circumference ($p < 0.001$), hip circumference ($p < 0.001$), and waist-to-height ratio ($p < 0.001$) were lower.

In the placebo group, compared to baseline, at the end of the 12th week, there was no statistically significant decrease in body weight ($p > 0.05$), while body weight Z-score ($p < 0.05$), BMI ($p < 0.05$), BMI Z-score ($p < 0.01$), triceps skinfold thickness ($p < 0.05$), upper arm circumference ($p < 0.05$), waist circumference ($p < 0.01$), hip circumference ($p < 0.01$), and waist-to-height ratio ($p < 0.01$) decreased. There was no statistical difference in the waist/hip ratio ($p > 0.05$).

At the end of the 12th week, the following parameters were significantly lower in the synbiotic group compared to the placebo group: body weight ($p < 0.01$), body weight Z-score ($p < 0.05$), BMI ($p < 0.05$), BMI Z-score ($p < 0.01$), waist circumference ($p < 0.05$), waist circumference-height ratio ($p < 0.05$). There was no statistical difference between the other anthropometric parameters after 12 weeks.

Comparison of Laboratory Parameters

At baseline, there was no statistically significant difference between the two groups for glucose and lipid parameters ($p > 0.05$). There was no statistical difference in serum glucose, insulin, HOMA-IR, AST, ALT, total cholesterol, triglyceride, HDL and LDL values in the synbiotic and placebo groups at baseline and after 12 weeks of intervention ($p > 0.05$). Similarly, the percentage of patients with dyslipidemia, mean systolic and diastolic blood pressure values, and the presence of NAFLD were found to be similar between the groups at the beginning of the study and at the end of the 12th week ($p > 0.05$) (**Table 2**). The percentage of patients with NAFLD were found to be similar between the synbiotic and placebo group at the beginning of the study (14/30, 46.6% vs. 14/31, 45.1%; $p > 0.05$) and also at the end of the 12th week (12/30; 40% vs. 14/31; 45.1%; $p > 0.05$). No adverse events have been reported during the study period related with synbiotic or placebo group.

DISCUSSION

In this study, daily multispecies synbiotic intake, along with diet and exercise, had a more substantial favorable effect on anthropometric parameters (body weight, BMI, waist circumference, and waist/height ratio) in children and adolescents with exogenous obesity than placebo. A significant reduction in (i) body weight of 4%, (ii) BMI of 5.1 percent, (iii)

TABLE 1 | Evaluation of the change in anthropometric measurements of the synbiotic and placebo groups at the beginning of the study and at the end of the 12th week.

Parameters	Synbiotic group $n = 30$				Placebo group $n = 31$				p3
	Baseline	12^{th} Weeks	% Reduction	p1	Baseline	12^{th} weeks	% Reduction	p2	
Weight (kg)	67.6 ± 18.6	64.4 ± 18.3	4.0 ± 3.1	***p < 0.001***	75.4 ± 23.1	74.2 ± 21.9	1.2 ± 4.19	ns	***p < 0.01***
Weight z-score	2.12 ± 0.37	1.97. ± 0.36	8.7 ± 7.3	***p < 0.001***	2.22 ± 0.75	2.08 ± 0.79	5.1 ± 6.5	***p < 0.01***	***p < 0.05***
Height (cm)	152.3 ± 14.1	153.1 ± 14.0	-	**ns**	157.2 ± 11.8	158.3 ± 11.5	-	ns	ns
Weight-for-Height (%)	151.2 ± 16.1	146.4 ± 14.7	3.0 ± 3.9	***p < 0.01***	149.1 ± 18.9	148.9 ± 20.3	0.3 ± 0.7	ns	ns
BMI (kg/m^2)	28.2 ± 3.7	26.7 ± 3.7	5.1 ± 3.1	***p < 0.001***	29.8 ± 6.0	29.0 ± 5.8	1.1 ± 3.4	***p < 0.05***	***p < 0.01***
BMI Z-score	2.06 ± 0.26	1.88 ± 0.28	8.78 ± 7.31	***p < 0.001***	2.08 ± 0.39	2.04 ± 0.43	5.1 ± 6.56	***p < 0.05***	***p < 0.001***
TSFT (mm)	28.2 ± 5.3	24.5 ± 6.3	13.6 ± 13.9	***p < 0.01***	28.6 ± 7.4	26.5 ± 6.8	6.1 ± 16.7	***p < 0.05***	ns
UAL (cm)	30.4 ± 3.4	29.0 ± 3.3	4.4 ± 4.3	***p < 0.001***	32.7 ± 5.7	31.5 ± 5.68	3.4 ± 6.0	***p < 0.01***	ns
Waist circumference (cm)	92.5 ± 8.5	86.8 ± 8.7	6.0 ± 4.8	***p < 0.001***	96.7 ± 15.0	93.1 ± 14.7	3.7 ± 3.4	***p < 0.001***	***p < 0.05***
Hip circumference (cm)	107.0 ± 14.4	104.0 ± 13.8	2.7 ± 3.5	***p < 0.001***	102.6 ± 12.7	98.8 ± 12.1	3.6 ± 3.7	***p < 0.001***	ns
Waist/height ratio	0.60 ± 0.03	0.56 ± 0.04	4.43 ± 3.25	***p < 0.001***	0.61 ± 0.03	0.56 ± 0.03	6.58 ± 4.77	***p < 0.001***	***p < 0.05***
Waist/hip ratio	0.90 ± 0.02	0.88 ± 0.06	2.44 ± 4.90	ns	0.90 ± 0.06	0.89 ± 0.07	0.89 ± 5.11	ns	ns

The data were expressed as mean ± standart deviation. p1: Baseline vs. 12^{th} week in synbiotic group, p2, Baseline vs. 12^{th} week in placebo group; p3: Synbiotic group vs. Placebo group at 12 weeks. BMI, body mass index; TSFT, triceps skinfold thickness; UAL, upper arm length.
Bold values are statistically significant.

TABLE 2 | Evaluation of biochemistry and lipid parameters of the synbiotic and placebo groups at the beginning of the study and at the end of the 12th week.

Parameters	Synbiotic group $n = 30$		Placebo group $n = 31$		p
	Baseline	12^{th} Weeks	Baseline	12^{th} Weeks	ns
Glucose (mg/dl)	83.4 ± 6.12	86.0 ± 6.68	81.6 ± 5.24	83.9 ± 7.48	ns
Insulin	20.2 ± 10.6	17.5 ± 8.12	19.9 ± 11.8	17.1 ± 7.64	ns
HOMA-IR	4.23 ± 2.29	3.69 ± 1.73	4.32 ± 2.93	3.60 ± 1.72	ns
AST (IU/L)	23.6 ± 11.1	21.3 ± 7.0	21.0 ± 5.0	20.0 ± 4.1	ns
ALT (IU/L)	26.6 ± 18.5	26.1 ± 7.7	23.9 ± 13.2	20.5 ± 8.6	ns
Total cholesterol (mg/dl)	161.6 ± 28.9	163.1 ± 30.0	158.1 ± 37.8	157.3 ± 35.4	ns
HDL-C (mg/dl)	43.7 ± 7.1	43.4 ± 6.1	44.5 ± 11.9	44.8 ± 10.4	ns
LDL-C(mg/dl)	108.3 ± 24.7	108.8 ± 28.5	106.8 ± 35.0	101.0 ± 35.4	ns
Triglyceride (mg/dl)	128.1 ± 49.0	121.1 ± 62.0	142.5 ± 91.6	104.7 ± 95.2	ns
Presence of NAFLD (%, *n*)	46.% (14/30)	40% (12/30)	45.1% (14/31)	45.1% (14/31)	ns

The data all data were expressed as mean ± standart deviation, except the presence of NAFLD has been shown as percentage. There is no statistical significance between baseline and 12^{th} weeks results and also between synbiotic and placebo group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HOMA-IR, homoeostatic model assessment for insulin resistance; NAFLD, non-alcholic fatty liver disease.

waist circumference of 6%, and (iv) hip circumference of 2.4 percent was achieved after 12 weeks of synbiotic use.

In our clinic between 2011 and 2012, the efficacy of the identical synbiotic mixture was tested in children with exogenous obesity during a one-month intervention (not placebo controlled, single blind research) (13). After therapy, 71.4 percent of the adolescents in the synbiotics group lost weight, and body weight, body mass index, and triceps skinfold thickness values all decreased statistically significantly (13). In this new study, when compared to the trial baseline, the synbiotic group had a significant drop in body weight (all patients), body weight Z-score, BMI, BMI Z-score, triceps skinfold thickness, upper arm circumference, waist and hip circumference, and waist-to-height ratio at the conclusion of the 12^{th} week. The fact that synbiotic therapy was provided for 3 months in the new trial explains the better results when compared to our prior study using the same preparations. When the synbiotic group and the placebo group were compared at the end of the 12^{th} week, it was discovered that the synbiotic group had a greater percent decrease in body weight, percent decrease in BMI value, decrease in BMI Z-score, decrease in waist circumference, and decrease in waist circumference-height ratio. The considerable reduction in waist circumference, which is directly linked to cardiovascular risk, is quite noteworthy (17).

Perna et al. (7) included 20 randomized controlled trials in their study of the effectiveness of probiotics for the management of body weight and anthropometric parameters in adults (n = 1,411) with overweight and obesity (7). Despite no substantial reduction in body weight, probiotic administration was found to have a favorable effect on BMI (6). Another comprehensive analysis of the benefits of probiotics and synbiotics on weight loss in overweight and obese people found that 23 of 27 trials showed positive results in terms of weight loss and other anthropometric measurements (6). The administration of biotics was commonly combined with energy restriction and increased physical activity (11 studies), like in our investigation. In these studies that reveal favorable effects of pro- and synbiotics on anthropometric parameters (6, 12). Twenty-four of the 27 investigations were undertaken in adult populations, while three were conducted in children (6). Two lactobacilli (*L. acidophilus and L. rhamnosus*) and two bifidobacteria strains were included in the synbiotic formulation evaluated in our investigation (*B. bifidum and B. longum*). The combination of Lactobacillus and Bifidobacterium had a favorable effect on body weight loss in subjects who also maintained their usual lifestyle (9). There are few studies on the effects of probiotics and synbiotics in the treatment of childhood obesity (8). In a meta-analysis research involving nine randomized trials from Iran, Italy, Turkey, Denmark, Spain, and the United States, Mohammadia et al. evaluated the effects of probiotic and synbiotic use on anthropometric and metabolic markers in overweight and obese children and adolescents (8). They examined data from 410 kids in the study (215 probiotic/synbiotic, 195 controls) (six probiotic and three synbiotic studies). The usage of probiotics for 4–16 weeks had no statistically significant impact on body weight, BMI Z-score, hip circumference, blood sugar, or lipid markers in overweight and obese children, according to this meta-analysis (8). Subgroup analyses, on the other hand, revealed that synbiotics had an influence on the BMI Z-score (8). When compared to a placebo group, children receiving synbiotics had a lower BMI Z-score and higher levels of cytokines and adiponectin, and synbiotic supplementation was expected to have a favorable effect on inflammation (18).

One of the most frequent liver illnesses in children is non-alcoholic fatty liver disease (16). Recent research suggests that the interaction between the liver and the gut, known as the "gut-liver axis," may play a key role in the phenotypic flip from NAFLD to a more aggressive liver disease such as non-alcoholic steatohepatitis (NASH) and NASH-related fibrosis. Dysbiosis has been linked to the development of NAFLD in children in recent research (19, 20). As a result, numerous writers have proposed modulating gut microbiota with pre-/pro-/synbiotics as a potential treatment for obesity-related NAFLD (21–24). At the start of the study and at the end of the 12th week, the percentage of patients with NAFLD was found to be similar in the synbiotic and placebo groups. We also found no effect of probiotics and synbiotics on glucose and lipid metabolism. This could be linked to the intervention's short duration. Probiotics can affect the lipid profile and insulin sensitivity, two processes that can improve body weight, BMI, waist, and hip circumference (6–9). Probiotics have been proven to lower total cholesterol, triglycerides, and LDL-C levels while increasing HDL-C levels (7, 25).

The gut microbiota may influence whole-body metabolism through influencing energy balance, glucose metabolism, and low-grade inflammation linked to obesity and metabolic diseases. The effect mechanisms of pre/pro/synbiotics on preventing weight gain or loss in obesity have been the subject of numerous hypotheses. Reduction of inflammation, strengthening of the intestinal epithelial barrier, prevention of bacterial translocation, modulation of intestinal enzyme activity, effects on neuroendocrine and immunological functions, inhibition of energy storage and food intake, reduction of dietary cholesterol absorption, prevention of bile acid reabsorption in the small intestines, and reduction of intestinal inflammation are some of the benefits. The association between the microbiome and obesity is complicated, and many factors remain unknown (4, 6). The effects of probiotics and synbiotics on the composition of the gut microbiota are strain-specific.

A limitation of our study is that the outcome is based on the patients' reporting on dietary compliance and exercise recommendations. Despite our patients' great compliance, the majority of those who were enrolled were unable to visit our clinic due to mitigation strategies (stay-at-home orders or hospital reorganization) implemented during the first year of the COVID-19 epidemic. According to International Scientific Association of Probiotics and Prebiotics (ISAPP), studies on a "synergistic synbiotic" that compare the synbiotic to the control can provide supportive evidence but do not constitute a direct evidence that confirms a synergistic effect. Instead, a study including the combination, the substrate alone, the live microorganisms alone and a control should be conducted (8).

CONCLUSION

This randomized placebo controlled study found that taking a specific synbiotic for 12 weeks in addition to dietary and physical activity recommendations had a positive effect on anthropometric measurements, resulting in a 4 percent reduction in body weight, 5.1 percent reduction in BMI, 6 percent reduction in waist circumference, and 2.4 percent reduction in hip circumference. Twelveweeks use of synbiotics have some beneficial effects of anthropometric measurements, and these effects might be explained potential effects of synbiotics on microbiota composition. In childhood obesity, the administration of this specific multistrain synbiotics is an effective weight-loss method in addition to diet and exercise.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Eskisehir Osmangazi University Faculty of Medicine Local Ethical Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GK, MD, YV, and ED conceptualized and designed the study. GK recruited participants and collected samples. ED interpreted the analyses. All authors contributed to drafting and critical review of the manuscript.

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Gabriel Vinderola,
Universidad Nacional del Litoral (FIQ-UNL), Argentina
Junling Shi,
Northwestern Polytechnical University, China

*CORRESPONDENCE
Hung-Chih Lin
d0373@mail.cmuh.org.tw
Yao-Tsung Yeh
glycosamine@yahoo.com.tw

†These authors have contributed equally to this work

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A multi-strain probiotic blend reshaped obesity-related gut dysbiosis and improved lipid metabolism in obese children

An-Chyi Chen[1,2†], Tzu-Jung Fang[3,4†], Hsieh-Hsun Ho[5], Jui-Fen Chen[5], Yi-Wei Kuo[5], Yen-Yu Huang[5], Shin-Yu Tsai[5], Shu-Fen Wu[1,2], Hung-Chih Lin[6,7,8*] and Yao-Tsung Yeh[9,10*]

[1]Division of Pediatric Gastroenterology, China Medical University Children's Hospital, Taichung City, Taiwan, [2]School of Medicine, China Medical University, Taichung City, Taiwan, [3]College of Medicine, Graduate Institute of Clinical Medicine, Kaohsiung Medical University, Kaohsiung City, Taiwan, [4]Division of Geriatrics and Gerontology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung City, Taiwan, [5]Department of Research and Design, Glac Biotech Co., Ltd., Tainan City, Taiwan, [6]Division of Neonatology, China Medical University Children's Hospital, Taichung City, Taiwan, [7]School of Chinese Medicine, China Medical University, Taichung City, Taiwan, [8]Asia University Hospital, Asia University, Taichung City, Taiwan, [9]Aging and Disease Prevention Research Center, Fooyin University, Kaohsiung City, Taiwan, [10]Department of Medical Laboratory Science and Biotechnology, Fooyin University, Kaohsiung City, Taiwan

Background and aims: Obese children are more prone to becoming obese adults, and excess adiposity consequently increases the risk of many complications, such as metabolic syndromes, non-alcoholic fatty liver disease, cardiovascular disease, etc. This study aimed to evaluate the effects of multi-strain probiotics on the gut microbiota and weight control in obese children.

Methods: A double-blind, randomized, placebo-controlled trial was carried out on overweight and obese children. Subjects received 12 weeks of treatment with supplementary probiotics that contained three strains: *Lactobacillus salivarius* AP-32, *L. rhamnosus* bv-77, and *Bifidobacterium animalis* CP-9, plus diet and exercise guidance. A total of 82 children were enrolled, and 53 children completed the study.

Results: The supplementation of multi-strain probiotics resulted in a significant effect demonstrating high-density lipoprotein (HDL) and adiponectin elevation. At the same time, body mass index (BMI) and serum total cholesterol, low-density lipoprotein (LDL), leptin, and tumor necrosis factor-alpha (TNF-α) levels were reduced. *Lactobacillus* spp. and *B. animalis* were particularly increased in subjects who received probiotic supplements. The abundance of *Lactobacillus* spp. was inversely correlated with the ether lipid metabolism pathway, while that of *B. animalis* was positively correlated with serum adiponectin levels.

Conclusion: Our results show that obesity-related gut dysbiosis can be reshaped by the supplementation of a multi-strain probiotic to improve lipid metabolism. The regular administration of a multi-strain probiotic supplement may be helpful for weight control and health management in overweight and obese children.

KEYWORDS

childhood obesity, probiotics, gut microbiota, high-density lipoprotein (HDL), adiponectin

Introduction

According to the estimations of the World Health Organization (WHO), the prevalence of overweight or obese children and adolescents aged 5–19 years increased by more than four-fold, from 4 to 18%, globally from 1975 to 2016 (1). Compared to people with normal weight, obese people have a risk of diabetes, metabolic syndrome, and dyslipidemia that is 3 times higher compared to their normal-weight counterparts and a risk of hypertension, cardiovascular disease, knee arthritis, and gout that is 2 times higher (2). Childhood obesity has a number of negative health effects, such as early onset diabetes, high blood pressure, hyperlipidemia, and cardiovascular disease. Moreover, studies have found that obese children have a higher blood vessel stiffness, faster heartbeat, and poorer vascular endothelial function than normal-weight children (3). Long-term follow-up studies have revealed that the long-term effects of childhood obesity may cause abnormal heart structures and may result in a higher risk for cardiovascular disease in adults (4).

Although the main cause of obesity is excessive calorie intake and insufficient physical activity, recent studies have indicated that lack of sleep, poor eating habits, and the dysbiosis of intestinal bacteria can be attributed to obesity (5). Studies have further demonstrated that the composition of the intestinal flora in obese and normal-weight individuals is distinctly different. For instance, *Firmicutes* and *Staphylococcus aureus* were increased while *Bacteroidetes* and *Bifidobacterium* were decreased in obese children compared to lean children (6, 7). At present, it is not yet clear how the intestinal flora modulates obesity. Hypothetical mechanisms include changing the absorption, storage, and utilization of nutrients, and the regulation of energy metabolism and body inflammation (8).

According to the FAO/WHO, probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (9, 10). The most commonly used probiotic genera are *Bifidobacterium* and *Lactobacillus*. Numerous studies have revealed that obese adults who use probiotic-related products such as prebiotics, dairy products containing probiotics, and synbiotics or who take probiotic bacteria directly can effectively control their body weight (11). Nevertheless, it is critically noted that the effect of probiotics in obese children may not be the same as those observed in obese adults. Clinical investigations in this field remain very limited. In 2013, Safavi's group reported the positive effect of a synbiotic product containing probiotics, which reduced the body weight, body mass index (BMI), and serum lipid levels in obese children (12). Contrastively, Jones' group reported the null effect of a probiotic supplement (VSL#3®) intervention in obese Hispanic adolescents in 2018 (13). Therefore, there is a keen need to clarify specific probiotic strains or products that can provide positive effects on weight control in obese children. In our previous study, the weight-reducing effect of a multi-strain probiotic supplement was validated in high-fat diet (HFD)-induced obese rats (14). The multi-strain probiotic supplement contained three strains: *L. rhamnosus* bv-77, *B. lactis* CP-9, and *L. salivarius* AP-32. These three probiotic strains were able to reduce the ketone body level and alleviate body fat formation in rats when administered separately or together.

In this study, a clinical trial in overweight and obese children was carried out to explore whether an oral supplement of the three aforementioned multi-strain probiotics can help obese or overweight children to lose weight and improve metabolic disorders. In addition to weight control, the effect of this probiotic mixture was evaluated according to physiological observations of the subject, such as height, waist circumference, hip circumference, body fat, and blood pressure. Blood chemistry, including blood sugar, blood fat, liver function, and kidney functions were investigated. Serum cytokine and adipokine levels, such as those of TNF-α, leptin, and adiponectin were also analyzed. Eventually, next generation sequencing (NGS) was performed to reveal the influence of probiotic supplements on the gut microbiota.

Materials and methods

Ethics, informed consent, and permissions

This randomized, double-blind, placebo-controlled clinical study was carried out in the China Medical University Children's

Hospital. According to documentation from the Ministry of Health and Welfare, children with a BMI equal to or higher than the age- and sex-specific 85th percentile were overweight, and those with a BMI that was equal to or higher than the 95th percentile were obese. Overweight/obese children aged 6–18 years old were recruited through meetings with their parents. Children were excluded if they (1) had underlying conditions and other severe chronic diseases, (2) were on anti-obesity medication, (3) had already been taking probiotic products for a long time, and (4) were taking antibiotics. All of the qualified children whose parents had given informed consent were placed into randomized groups for the trial (n = 82). The study protocol (IRB No. CMUH105-REC2-096) was approved by the ethics committee of China Medical University Hospital. The trial is registered with the trial registry under code NCT03883191.

Study design and subjects

The intervention lasted 3 months. Every day, the subjects in the study group took three packages of the supplement, which contained functional ingredients (i.e., white kidney bean extract: 300 mg, Psyllium husk: 100 mg, and Garcinia cambogia extract: 100 mg) and probiotics. The subjects were advised to take one package 30 min prior to each meal and to take three packages in total per day. Every package contained a total number of 10^{10} colony-forming units (CFU) including *Lactobacillus salivarius* AP-32 (10^9 CFU), *Lactobacillus rhamnosus* bv-77 (10^9 CFU), and *Bifidobacterium animalis* CP-9 (8×10^9 CFU). *L. salivarius* AP-32 was isolated from a healthy human gut and deposited as BCRC910437 and CCTCC M 2011127. *L. rhamnosus* bv-77 was isolated from human breast milk and deposited as BCRC 910533 and CCTCC M 2014589. *B. animalis* CP-9 was isolated from human breast milk and deposited as BCRC 910645 and CCTCC M 2014588. At the same time, the subjects in the placebo group took three packages of the supplement containing the same ingredients without any of the probiotics. The doctors, parents, children, and investigators were unaware of which set of packages contained the probiotic mixtures until the end of the intervention and after the analysis was performed. During the intervention, other products containing probiotics were forbidden.

A detailed participant flowchart showing the baseline visit to the end visit is shown in **Figure 1**. Subjects visited Dr. An-Chyi Chen at the special clinic for pediatric obesity at the China Medical University Children's Hospital. Information on the child's family, living environment, child's nutrition habits, exercise habits, and illnesses was collected. The child's body height, body weight, blood pressure, heart rate, waistline, and hip circumference were also recorded. Body fat was measured at four sites: biceps, triceps, lower scapula, and thighs using Baseline Skinfold Calipers (Fabrication Enterprises, Inc., Pakistan). Symptoms such as acanthosis nigricans, obesity lines, gynecomastia, and snoring were diagnosed by the doctor. Fecal samples were collected at the beginning and the end of the study.

Blood biochemical analysis

Blood collection and examination

Blood samples were collected from the subjects at the Medicine Laboratory Department of China Medical University Hospital. Serum indicators for thyroxine (TSH, T3, and T4), blood glucose (fasting blood glucose, HbA1c, C-peptide, and insulin), blood lipids [total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides], liver function (GOT and GPT), and renal function (uric acid) were analyzed. Values were compared, and change rates were investigated before and after the 3-month intervention.

Enzyme-linked immunosorbent assay

The blood serum was obtained by centrifugation and analyzed by commercial enzyme-linked immunosorbent assay (ELISA) kits. The ELISA kits for the human inflammatory cytokine TNF-α were purchased from BioLegend, Inc., United States, and those for human leptin and adiponectin were purchased from R&D Systems, Inc., United States and LifeSpan BioSciences Inc., United States, respectively. All samples are tested in at least three replicates.

Statistical analysis

Due to the potential dispersion of data, the value of the continuous variable is expressed as the mean ± standard error deviation (SEM). Wilcoxon signed-rank test and Mann–Whitney U test was used to compare the differences within or between groups; the variable is expressed as an N value or percentage was analyzed by Fisher's exact test. The percentage of the test item was presented as the standardized average percentage (Endpoint/Baseline). The heat map demonstrating the fecal microbial richness was produced by using Graphpad prism 8 (Graphpad Software, San Diego, CA, United States). SPSS 18 (IBM, United States) was used for statistical analysis, and $p < 0.05$ were considered to be statistically significant.

Gut microbiota analysis

Fecal DNA extraction

Bacteria DNA was extracted from the fecal samples using the QIAamp Fast DNA Stool Mini Kit(Qiagen, Germany) with some modified instructions. Briefly, the stool sample was centrifuged at 13,200 rpm for 10 min to remove the storage buffer and lysis using InhibitEX buffer. After homogenization, proteinase K and ethanol were added to obtain the processed supernatant. Finally,

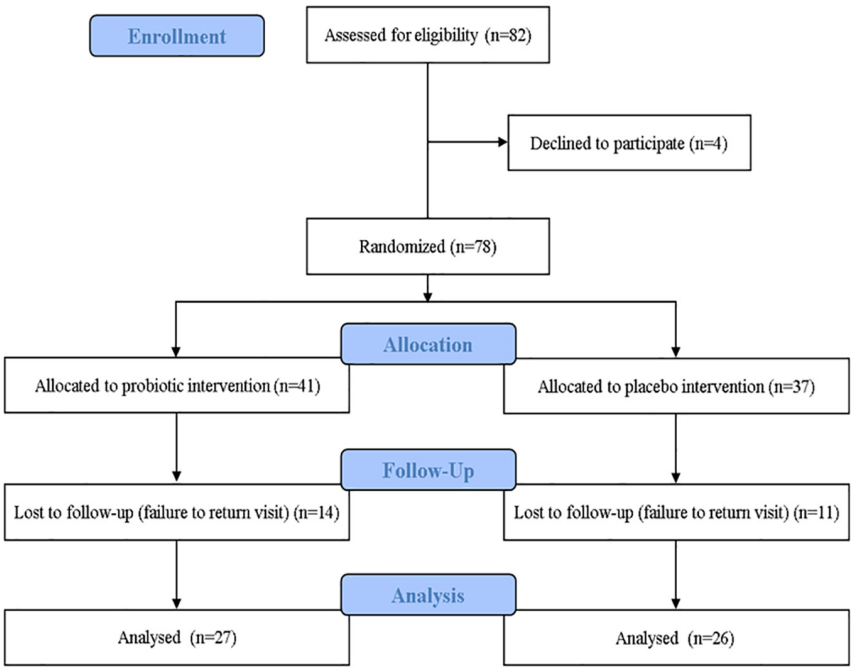

FIGURE 1
Clinical trial flowchart. A total of 82 people were admitted in the recruitment stage, and 4 people withdrew before the next stage. A total of 78 people entered the distribution stage: 41 people were allocated to the probiotic group, and 37 people were allocated to the placebo group. During the trial period, 14 people from the probiotic group withdrew, and 11 people from the placebo group withdrew. In the end, 27 people in the probiotic group and 26 people in the placebo group completed the study. Data from these 53 people were accounted for in the statistical analysis.

the supernatant was washed with a QIAamp spin column and eluted with elution buffer. The concentration was assessed by NanoDrop 2000 and a 10× dilution was then performed with elution buffer.

Next generation sequencing (NGS) analysis

The gut microbiome library was constructed with the standard V3–V4 region of the 16S rRNA gene. PCR was amplified with KAPA HiFi hotstart readymix (Roche, United States) and purified with AMPure XP magnetic beads (Beckman Coulter, United States). The amplified and quality of the PCR product was assessed using a Fragment Analyzer (Advanced Analytical, United States) and was quantified using a Qubit 3.0 Fluorometer. Then, the library was sequenced on a MiSeq (Illumina, United States) with paired-end reads (2 × 301 nt) and at least 100,000 reads of every sample.

Bioinformatics Analysis and Statistics

The raw paired-end reads were trimmed, and those that passed the quality filters were assigned to operational taxonomic units (OTU) with ≥ 97% similarity according to the GreenGene Database (v13.8). OTU taxonomic, alpha diversity, beta diversity, and heatmaps were performed with MicrobiomeAnalyst,[1] GraphPad Prism 8 (GraphPad Software, United States), and the CLC genomics workbench (Qiagen, Germany). The bacterial abundance analysis (Linear discriminant analysis Effect Size, LEfSe), which is widely used to analyze significant differences between groups, and functional analysis (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PICRUSt) were performed using the Galaxy/Hutlab website.[2] A p-value less than 0.05 was considered statistically significant. The statistical significance was further adjusted by False Discovery Rate (FDR). The FDR was performed by using the smallest Benjamini-Hochberg adjusted p-value when utilizing unpaired t-tests with a Welch's correction. An FDR-adjusted p-value (also called q-value) of 0.05 indicates that 5% of significant tests may result in false positives.

Results

The multi-strain probiotic blend reduced triacylglycerol (TG) accumulation in Caco-2 cells (**Supplementary Figure 1**)

1 https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/upload/OtuUploadView.xhtml

2 http://huttenhower.sph.harvard.edu/galaxy/

and was packaged as powder supplements. Children aged 6–18 years who met the eligibility criteria of overweight or obesity were recruited, and the trial was carried out as shown in the flowchart in Figure 1. A total of 82 people entered the distribution stage, and 53 people fully completed the trial. Data from 27 people in the probiotic group and from 26 people in the placebo group were analyzed and compared at the end of the trial. The baseline characteristics of every subject were recorded, and no significant differences were found between the placebo and probiotic groups, with the exception of systolic blood pressure (Supplementary Table 1).

Probiotic supplements reduced body mass index and body weight in obese children

After a 3-month intervention, the BMI of both groups was significantly reduced, which was probably due to regular exercise and diet. The subjects were educated regarding their diet and received exercise guidance, but no fixed meal recipes were assigned, nor was there a mandatory exercise schedule in this trial. Importantly, the reduction in the BMI level was significantly greater in the probiotic group (Table 1). The BMI level reduced by 0.5 kg/m^2 in the placebo group ($^*p = 0.015$) and by 1.2 kg/m^2 in the probiotic group ($^{***}p < 0.001$), respectively. In addition, the BMI change rate was significantly different between the probiotic and placebo groups ($^{\#}p = 0.026$). As expected for children who are around age 11, there was a significant increase in body height in both groups (Table 1). Body height increased by 1.2 cm in the placebo group ($^{***}p < 0.001$) and increased by 1.5 cm in the probiotic group ($^{***}p < 0.001$). Body weight was reduced in both groups, but a significant reduction was only observed in the probiotic group. There was a 0.5 kg body weight reduction in the placebo group ($p = 0.594$) and a significant reduction of 1.5 kg in the probiotic group ($^{**}p = 0.007$).

The number of people with acanthosis nigricans, striae, gynecomastia, and snoring was not affected by the intervention in either the placebo or the probiotic group (Supplementary Table 2). It should be noted that obesity is frequently associated with liver damage and elevated liver enzymes in serum (15). The probiotic supplement significantly reduced serum GOT and GPT (Supplementary Table 2). The additional physiological test values that were obtained before and after the intervention are compared in Table 1. The waist and hip circumference were slightly reduced in both groups, but the change rates did not reach a significant difference between the two groups. A significant difference was observed in the systolic blood pressure between the placebo and probiotic groups before the intervention, and this difference no longer existed after the intervention. The diastolic blood pressure and heartbeat were not affected in both groups. It is well known

TABLE 1 The comparison of physiological and blood biochemical values obtained before and after the intervention.

Parameter	Probiotics	Placebo	*P-value*$^{\#}$
BMI (kg/m^2)			
Baseline	29.7 ± 1.1	29.7 ± 1.0	0.790
End	28.5 ± 1.1	29.2 ± 1.0	0.533
P-value*	< 0.001***	0.015*	
Change rate (%)	95.9 ± 0.8	98.1 ± 0.9	0.026$^{\#}$
Weight (kg)			
Baseline	68.2 ± 4.4	66.5 ± 4.6	0.824
End	66.7 ± 4.2	66.0 ± 4.6	> 0.999
p-value*	0.007**	0.594	
Change rate (%)	98.0 ± 0.7	99.6 ± 0.8	0.062
Height (cm)			
Baseline	149.8 ± 2.8	147.3 ± 3.1	0.533
End	151.3 ± 2.7	148.5 ± 3.0	0.522
p-value*	< 0.001***	< 0.001***	
Change rate (%)	101.1 ± 0.3	100.9 ± 0.2	0.915
Waist circumference (cm)			
Baseline	89.9 ± 2.7	90.4 ± 2.5	0.715
End	89.3 ± 2.8	89.7 ± 2.5	0.625
p-value*	0.228	0.061	
Change rate (%)	99.3 ± 0.7	99.3 ± 0.5	0.742
Hip circumference (cm)			
Baseline	98.0 ± 2.6	97.5 ± 2.6	0.873
End	96.1 ± 2.6	96.4 ± 2.7	0.845
p-value*	0.055	0.051	
Change rate (%)	98.2 ± 0.8	98.9 ± 0.6	0.669
Systolic blood pressure (mmHg)			
Baseline	113.8 ± 3.1	124.7 ± 3.4	0.031$^{\#}$
End	115.9 ± 3.5	123.5 ± 3.1	0.157
p-value*	0.605	0.819	
Change rate (%)	102.4 ± 2.4	100.5 ± 3.2	0.522
Diastolic blood pressure (mmHg)			
Baseline	72.9 ± 2.8	76.4 ± 2.7	0.277
End	68.3 ± 2.7	72.8 ± 2.9	0.306
p-value*	0.109	0.451	
Change rate (%)	96.3 ± 4.6	98.4 ± 5.4	0.637
Heart rate (beats/min)			
Baseline	88.9 ± 2.6	87.3 ± 2.7	0.986
End	89.2 ± 2.1	84.4 ± 2.6	0.383
p-value*	0.958	0.353	
Change rate (%)	101.9 ± 3.0	98.1 ± 3.3	0.708
Glucose AC (mg/dl)			
Baseline	89.3 ± 1.2	89.7 ± 1.4	0.215
End	88.9 ± 1.4	90.2 ± 1.5	0.393
p-value*	0.178	0.849	
Change rate (%)	99.7 ± 1.6	100.8 ± 1.7	0.488
HbA1c (%)			
Baseline	5.6 ± 0.1	5.6 ± 0.0	0.548
End	5.5 ± 0.1	5.6 ± 0.0	0.100

(Continued)

TABLE 1 (Continued)

Parameter	Probiotics	Placebo	*P-value*[#]
p-value*	0.531	0.055	
Change rate (%)	99.0 ± 1.2	101.1 ± 0.5	0.378
C-peptide (ng/ml)			
Baseline	2.1 ± 0.2	2.4 ± 0.2	0.255
End	2.0 ± 0.2	2.2 ± 0.2	0.203
p-value*	0.923	0.909	
Change rate (%)	100.7 ± 7.5	99.4 ± 6.0	0.901
Insulin (μ IU/ml)			
Baseline	13.8 ± 1.2	15.9 ± 2.0	0.887
End	15.9 ± 2.2	14.2 ± 1.4	0.972
p-value*	0.486	0.485	
Change rate (%)	114.4 ± 9.3	112.2 ± 13.9	0.423
HOMA-IR			
Baseline	3.1 ± 0.3	3.5 ± 0.4	0.838
End	3.5 ± 0.5	3.2 ± 0.3	0.972
p-value*	0.548	0.424	
Change rate (%)	115.8 ± 11.0	116.0 ± 16.0	0.374

Data are presented as mean ± SEM of the results from each subject. Change rate: $value_{end}/value_{baseline} \times 100\%$ in the same subject. *The Wilcoxon signed rank test was used to compare the difference between before and after the intervention in each group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. [#]The Mann–Whitney U test was used to compare the difference between placebo and probiotic groups, $^{\#}p < 0.05$.

that obesity is highly associated with insulin sensitivity, so carbohydrate metabolism was also investigated. Neither the placebo nor probiotic interventions affected the serum levels of glucose AC, HbA1c, C-peptide, insulin, and HOMA-IR.

Probiotic supplements modulated blood lipid content and increased serum adiponectin levels in obese children

After the 3-month intervention, the body fat was analyzed by measuring the thickness of the subcutaneous tissue at four sites: biceps, triceps, subscapular, and thigh. The result showed a significant body fat reduction at two sites in the placebo group, and at all sites in the probiotic group (**Figure 2A**). In the placebo group, body fat reduced from 43.2 ± 1.9 to 38.9 ± 1.8 mm ($^{**}p = 0.001$) at the triceps and from 45.3 ± 1.3 to 42.3 ± 1.6 mm ($^{*}p = 0.032$) at the thigh. In the probiotic group, body fat reduced from 33.5 ± 1.4 to 31.0 ± 1.1 mm ($^{*}p = 0.045$), 44.1 ± 1.7 to 39.0 ± 1.7 mm ($^{**}p = 0.001$), 46.4 ± 1.6 to 42.4 ± 1.3 mm ($^{*}p = 0.012$), and from 46.8 ± 1.6 to 41.6 ± 1.3 mm ($^{***}p < 0.001$) at the biceps, triceps, subscapular, and thigh, respectively.

The blood lipid content was significantly modulated in the probiotic group (**Figures 2B,C**). The total cholesterol (TC) level was reduced from 172.3 ± 5.9 to 154.3 ± 6.8 mg/dl in the probiotic group ($^{**}p = 0.004$), and the change rate in the TC was significantly different between the probiotic and placebo groups ($^{\#}p = 0.046$). Moreover, two key components of TC, LDL and HDL, were investigated (**Figures 2B,C**). The LDL level was reduced from 119.4 ± 5.0 to 109.8 ± 4.4 mg/dl in the probiotic group ($^{**}p = 0.002$), and the change rate in the LDL was significantly different between the probiotic and placebo groups ($^{\#}p = 0.048$). The HDL level increased from 45.6 ± 1.7 to 51.3 ± 2.3 mg/dl in the probiotic group ($^{***}p < 0.001$), and the change rate in the HDL was also significantly different between the probiotic and placebo groups ($^{\#}p = 0.039$). There were a slight reduction in the triacylglycerol (TG) levels in both groups, and the difference did not reach statistical significance.

Adiponectin and leptin are cytokines that are excessively produced by adipocytes. Leptin is thought to be responsible for several cardiovascular diseases associated with obesity, while adiponectin is considered to be cardioprotective (16). The serum adiponectin level was increased from 7.1 ± 0.6 to 8.8 ± 0.7 μg/ml in the probiotic group ($^{**}p = 0.001$), and the serum adiponectin change rate was significantly different between the probiotic and placebo groups (**Figure 2D**, $^{\#}p = 0.042$). The serum leptin level was reduced from 2,552.2 ± 131.9 to 2,424.1 ± 135.1 pg/ml in the placebo group ($^{*}p = 0.040$) and was reduced from 2,792.4 ± 175.1 to 2,393.3 ± 150.8 pg/ml in the probiotic group (**Figure 2E**, $^{***}p < 0.001$). Notably, serum leptin was reduced in both groups, but the serum leptin change rate remained significantly different between the probiotic and placebo groups ($^{\#}p = 0.048$).

Obesity is associated with chronic low-grade inflammation immune conditions and is usually accompanied by elevated pro-inflammatory cytokine levels such as TNF-α (17). Interestingly, the TNF-α level was not affected in the placebo group but was significantly reduced from 27.5 ± 9.2 to 24.9 ± 8.7 pg/ml in the probiotic group (**Figure 2F**, $^{*}p = 0.015$). The TNF-α change rate was significantly different between the probiotic and placebo groups ($^{\#\#}p = 0.001$).

Probiotic supplements changed the composition of the top 10 most abundant genera in obese children

Growing evidence has linked gut dysbiosis as a potential risk factor for the pathophysiology of obesity (18). To analyze whether the probiotic supplement would change the diversity of the gut microbiome, the alpha diversity (the complexity within a community) and beta diversity (the differences between microbial communities) of the fecal samples were further investigated. No significant changes were observed in neither the alpha nor the beta diversity between the probiotic and placebo groups (**Supplementary Figure 2**). The microbial compositions of the top 10 most abundant gut bacteria were compared in obese and overweight children before and after

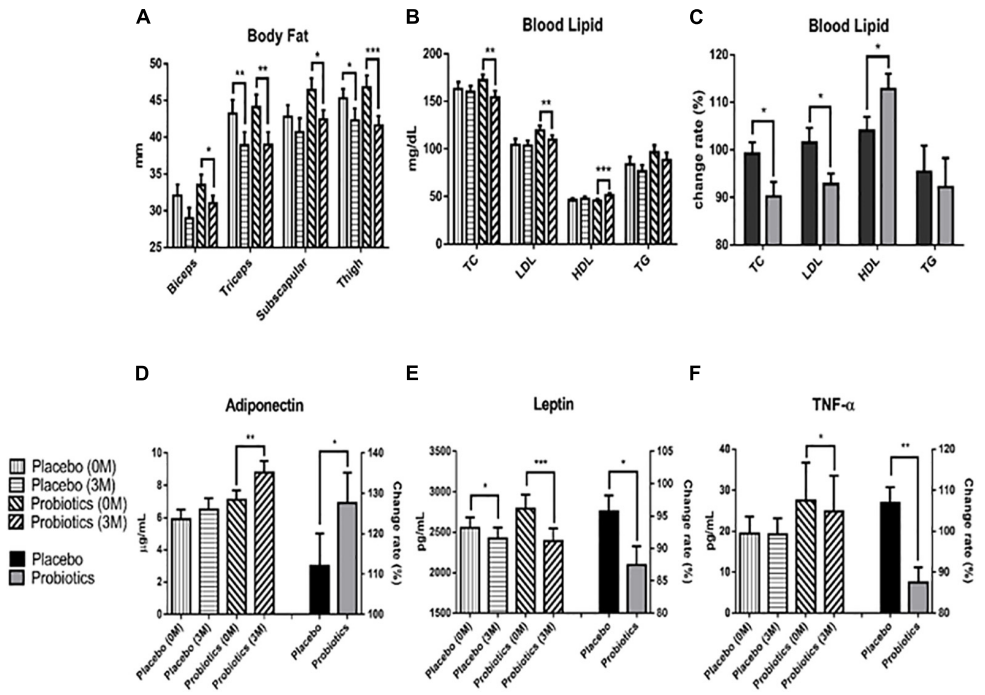

FIGURE 2
Probiotic supplements modulated blood lipids and adipokines in obese children. **(A)** The thickness of the subcutaneous tissue was measured at the biceps, triceps, subscapular, and thigh before (0 M) and after (3 M) the intervention. **(B)** TC (total cholesterol), LDL (low-density lipoprotein), HDL (high-density lipoprotein), and TG (triacylglycerol) were measured before and after the intervention. **(C)** The change rates of the TC, LDL, HDL, and TG were compared between the placebo and probiotic groups. **(D)** The adiponectin values before (0 M) and after (3 M) the intervention are plotted on the left *Y* axis, and the serum adiponectin change rate in the placebo and probiotic groups is plotted on the right *Y* axis. **(E)** The serum leptin values before and after the intervention are plotted on the left *Y* axis, and the change rate of leptin in the placebo and probiotic groups is plotted on the right *Y* axis. **(F)** The serum TNF-α values before and after the intervention are plotted on the left *Y* axis and the serum TNF-α change rate in the placebo and probiotic groups is plotted on the right *Y* axis. Data are presented as mean ± SEM. The Wilcoxon signed rank test was used to compare the differences between before and after the intervention within the group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The Mann–Whitney U test was used to compare the differences between the placebo and probiotic groups: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

the intervention (Figure 3 and Supplementary Table 3). At the phylum level, *Proteobacteria* spp. increased ($P = 0.042$; FDR adjusted $q = 0.255$) and *Bacteroidetes* spp. decreased ($P = 0.045$; FDR adjusted $q = 0.135$) after the administration of the probiotic supplement (Figure 3A and Supplementary Figure 3A). At the genus level, *Blautia* spp. ($p = 0.043$; FDR adjusted $q = 0.428$) and *Ruminoccus* spp. ($p = 0.049$; FDR adjusted $q = 0.162$) decreased in the probiotic group compared to the placebo group (Figure 3B and Supplementary Figure 3B). Instead, the abundance of *Collinsella* spp. increased ($p = 0.045$; FDR adjusted $q = 0.226$) after the administration of the probiotic supplement.

Gut microbiota were modulated differently in placebo and probiotic groups

Forty-four bacterial species were selected based on their abundance in the intestinal microbiota or according to their correlation to obesity as reported in the literature (19). In the probiotic group, *B. animalis* increased by 0.89% and *Bacteroides vulgatus* decreased by 1.37% after the intervention. In the placebo group, *Streptococcus salivarius* subsp. *thermophilus* increased by 1.07% and *B. longum* subsp. *longum* decreased by 2.00% (Figure 4A). Significant differences were observed in the change rates of seven species: *B. animalis*, *Lactococcus garvieae* subsp. *garvieae*, *Bacteroides coprocola* DSM 17136, *Collinsella stercoris*, *Lactobacillus salivarius*, *Pediococcus acidilactici*, and *Ruminococcus callidus* ATCC 27760, between probiotic and placebo groups ($p < 0.001$, 0.016, 0.026, 0.048, 0.001, 0.007, and 0.038, respectively).

To identify the core gut microbiota affected by the intervention, LEfSe [Linear discriminant analysis (LDA) Effect Size] was performed to analyze the core bacteria of the gut microbiome in obese and overweight children (Figure 4B). *B. animalis* and *B. pseudolongum* were significantly increased in the probiotic group. In contrast, *Sutterella* spp. and *Bacillus flexus* were significantly decreased in the probiotic group. Another online tool (Random forester analysis, Microbiome Analyst) was used to analyze the importance of the abundant

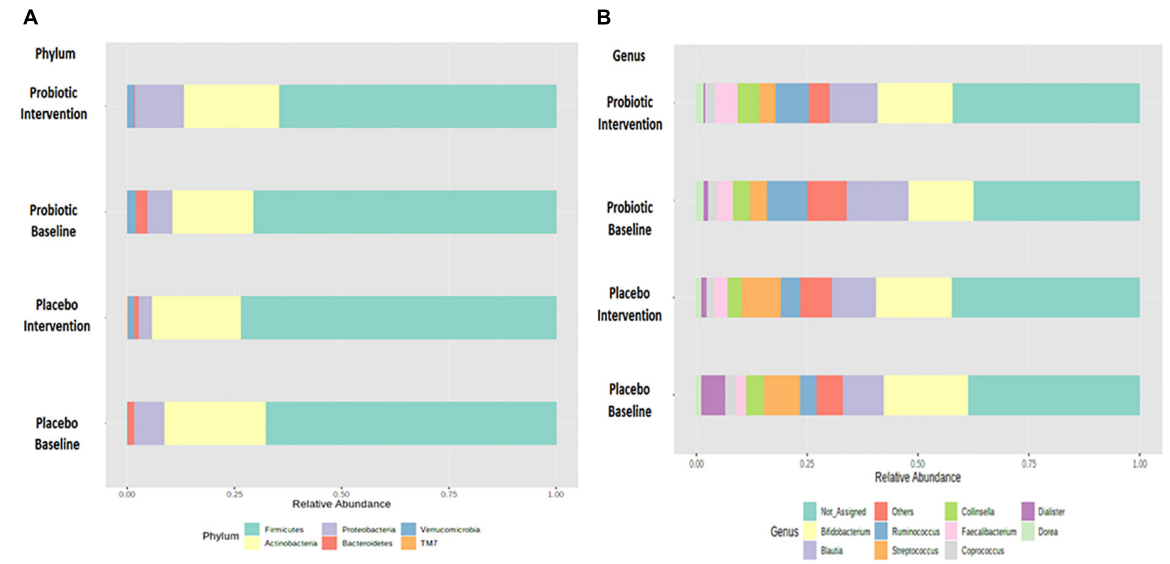

FIGURE 3
Probiotic supplements changed the composition of top 10 most abundant phyla and top 10 most abundant genera in obese children. The change in the microbial composition is presented at the **(A)** phylum and **(B)** genus levels.

bacteria from each group that was involved. *Bifidobacterium animalis* was found to be the most important abundant bacterium upon probiotic intervention (Figure 4C).

The probiotic supplement increased *Lactobacillus* spp. and *B. animalis* in obese children

Based on the microbial compositions of the OTU (Figure 3) and the core bacteria found by the LEfSe and Random forester analysis (Figure 4), 11 genera and five species were commonly abundant between the placebo and probiotic groups (Supplementary Table 4). At the genus level, *Blautia* spp., *Ruminoccus* spp., *Streptococcus* spp. *Coprococcus* spp., *Dorea* spp., and *Bacteroides spp.* were decreased in the probiotic group, but *Streptococcus spp. Coprococcus spp., Dorea spp., and Bacteroides spp.* did not reach statistical significance (Supplementary Figure 3). Notably, *B. animalis* significantly increased in the probiotic group (p = 0.002; FDR adjusted q = 0.012, Figure 5A). However, the elevation of *Lactobacillus* spp. was less certain (p = 0.027; FDR adjusted q = 0.240, Figure 5B). Consequently, the correlation between different bacteria in response to the intervention was performed by an online tool [Correlation Analysis (SparCC), Microbiome Analyst] (Figure 5C). A gut microbiome network was created to display the complicated correlations between different bacterial genera. *Bifidobacterium* spp. played a central role in the network, which was in good agreement with the core bacteria deduced from the OTU, heatmap analysis, and LEfSe analysis.

Lactobacillus spp. and *B. animalis* affected fat metabolism in different aspects

The probiotic supplement was able to reshape obesity-related dysbiosis, and these alterations were able to be linked to the modulation of functional pathways *via* the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) method. In the probiotic group, the intervention significantly decreased ether lipid metabolism compared to the probiotic baseline group (Figure 6A). Similarly, compared to the placebo group, the reduction in ether lipid metabolism was mainly attributed to probiotic supplementation (Figure 6B). It was noted that the probiotic supplement contained two *Lactobacillus* strains (*L. salivarius* AP-32 and *L. rhamnosus* bv-77) and one *Bifidobacterium* strain (*B. animalis* CP-9). Therefore, how *Lactobacillus* spp. and *B. animalis* are correlated with lipid metabolism were further investigated.

Spearman's correlation analysis was performed to analyze how *Lactobacillus* spp. and *B. animalis* were correlated with all of the blood biochemistry values in obese children (Figures 6C–F). Among the blood lipid contents, HDL displayed a positive correlation with both *B. animalis* and *Lactobacillus* spp. (Figure 6C, both p = 0.029). One of the adipokines, adiponectin, displayed a positive correlation with *B. animalis* (Figure 6D). Ether lipid metabolism was negatively correlated to the abundance of *Lactobacillus* spp. (Figure 6E, p = 0.026). How ether lipid metabolism was correlated with blood lipids was also analyzed, and the results showed that TC and LDL were

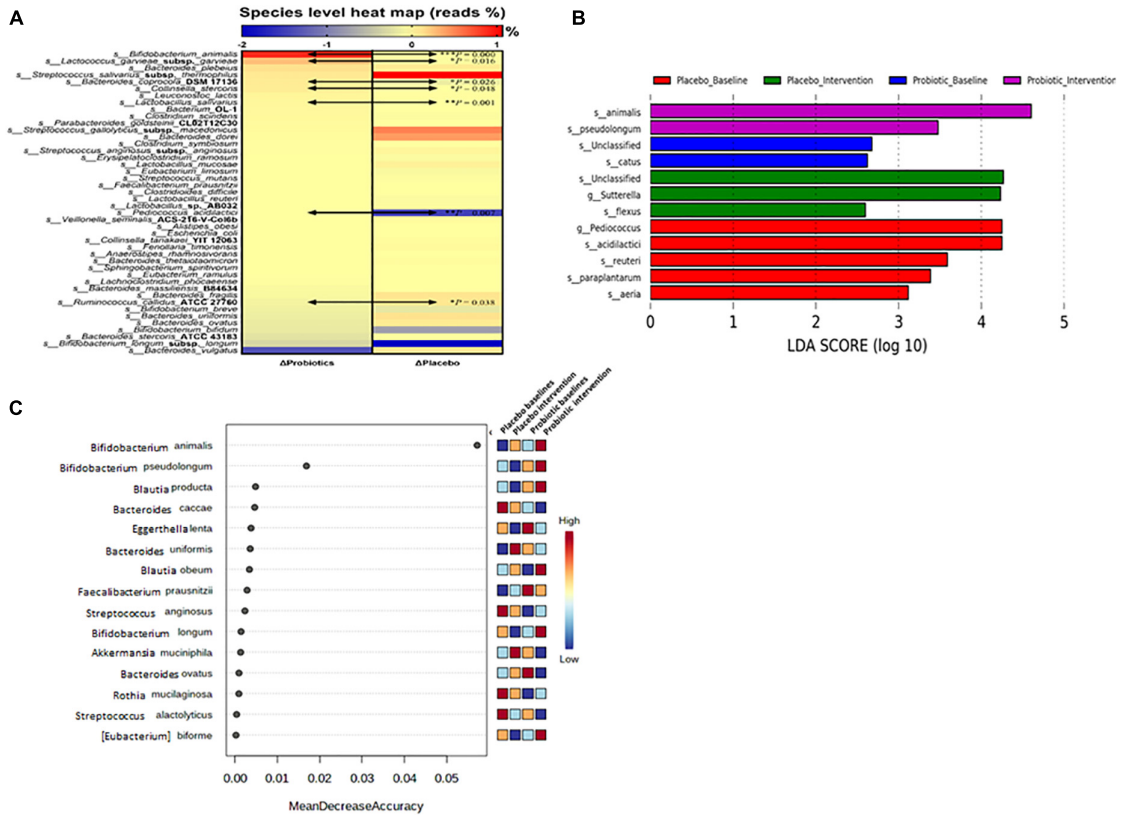

FIGURE 4
Probiotic supplements modulated fecal microbiota in obese children. **(A)** Microbial composition changes at the species level were presented as a heat map. **(B)** Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was performed in the placebo and probiotic groups before and after the intervention. **(C)** The importance of bacterial species in response to the intervention was ranked by Random forester analysis. Bacterial names are listed from top to down according to their change rates in the probiotic group. Data are presented as the mean values of the end-to-baseline ratio. The increase in the ratio is presented in red, and the decrease in the ratio is presented in blue. The Mann–Whitney U test was used to compare the continuous variables. Statistical comparisons were obtained by Student's *t*-test, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

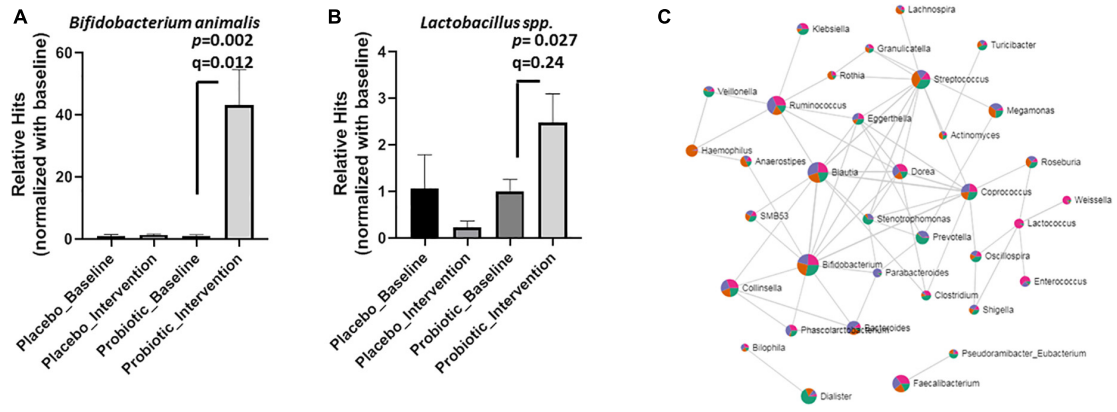

FIGURE 5
Probiotic supplements increased *Lactobacillus* spp. and *B. animalis* in obese children. The relative hits of **(A)** *B. animalis*, **(B)** *Lactobacillus* spp. were compared before and after the intervention in the placebo and probiotic groups. **(C)** The correlations between different bacteria in response to the intervention were displayed as a network. Data are presented as mean ± SEM. Statistical comparisons were obtained by Student's *t*-test.

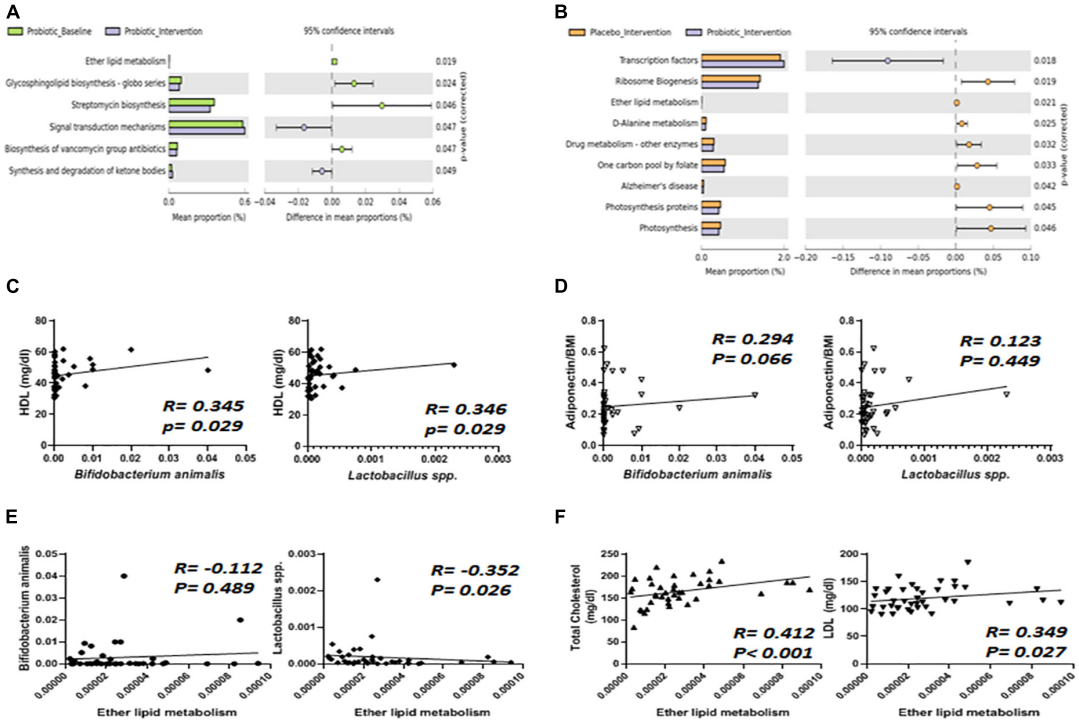

FIGURE 6
The abundance of *Bifidobacterium animalis* and *Lactobacillus* spp. displayed correlations with ether lipid metabolism, HDL, and adiponectin. **(A)** Six functional pathways were significantly affected by the intervention in the probiotic group. **(B)** Nine functional pathways were altered differently between the placebo and probiotic groups. The abundance of *B. animalis* and *Lactobacillus* spp. were correlated with **(C)** HDL, **(D)** adiponectin, and **(E)** ether lipid metabolism. **(F)** Total cholesterol (TC) and LDL were correlated with ether lipid metabolism.

positively correlated with ether lipid metabolism (Figure 6F, $p < 0.001$ and 0.027, respectively).

Discussion

It has been suggested that obese adults who use probiotic-related products can effectively control their weight and metabolic disorders related to weight (11). However, the effect of probiotics in obese children may not be the same as in adults (12, 13). In the present study, the supplementation of multi-strain probiotics, *L. salivarius* AP-32, *L. rhamnosus* bv-77, and *B. animalis* CP-9, significantly decreased BMI, TC, LDL, leptin, and TNF-α in overweight/obese children. Meanwhile, the intervention significantly increased serum HDL and adiponectin levels as well as reshaped or improved obesity-related gut dysbiosis. From the observation of the BMI profiles, the results indicate that the supplementation of functional ingredients together with probiotics was able to provide better weight control than supplementation with functional ingredients only. The BMI reduction in the placebo group was due to an increase in body height but not a decrease in body weight. The BMI reduction in the probiotic group was due to a simultaneous increase in body height and decrease in body weight. According to WHO guidelines, children should have at least 60 min of moderate-to-vigorous physical activity (PA) every day (20). However, a general survey reported that the amount of PA in grade 1 to grade 12 students in Taiwan did not meet WHO's recommendation (21). In other words, exceeding the amount of PA recommended by the WHO can be more challenging for overweight/obese children and their families. In this trial, families were educated on the importance of PA, but it was not mandatory. An animal study showed that a combination of probiotics and exercise may synergistically assist obesity management and health improvement (22). Therefore, further studies combining programmed exercise with increased strength are needed to elucidate the synergistic effect of probiotics and PA in overweight/obese children.

Notably, better effects were observed in terms of body fat and blood lipids, such as reductions in the TC and LDL, and increase in the HDL were observed in the probiotic group. In the placebo group, body fat was reduced at two of the four measured sites, and no effects on blood lipids were observed. The placebo supplement contained functional ingredients, including white kidney bean extract, Psyllium husk, and Garcinia cambogia extract, which have been reported to modulate the gut microbiota in animals and humans (23–25). Therefore, the effect on weight loss could be expected

in the placebo group (26–28). Our results indicated that the composition of the gut microbiota was altered differently in the placebo (functional ingredients) and probiotic (functional ingredients + probiotics) groups. Interestingly, the abundance of *B. animalis* was not affected in the placebo group, while it was significantly increased in the probiotic group. A high proportion (80%, 8×10^9 CFU) of *B. animalis* CP-9 and a lower proportion of *Lactobacillus* (10%, 10^9 CFU *L. salivarius* AP-32 and 10%, 10^9 CFU *L. rhamnosus* bv-77) were intentionally used to compensate obesity-related dysbiosis (29) in this trial. Therefore, the ratio (AP-32: bv-77: CP-9 = 1: 1: 8) of probiotic strains in the supplement could be related to the relative abundance of the species in the fecal samples at the end of the trial. The probiotics were taken together with functional ingredients in the probiotic group, so it remains unclear to what extent probiotic bacteria itself could improve lipid metabolism in humans. One probiotic strain may reduce BMI without affecting lipid metabolism-related parameters (30). Moreover, other studies have shown that the same fermented products have controversial effects on the serum lipid level in humans (31, 32). A clinical study showed that the supplementation of probiotic strains and functional ingredients had a positive influence on blood lipoprotein profiles (33). Taken together, supplementation with solely abiotic ingredients or biotic strains may not have the best effect due to the lipid metabolism complex in the human body. The combination of probiotics and functional ingredients may exert synergistic effects and provide a more profound outcome than either one of them.

Adipokines and adipocytokines are bioactive products that are secreted by the adipose tissue and have become important biomarkers for metabolic disorders in the current decade (34, 35). The imbalance of decreased adiponectin plasma concentrations and increased leptin levels is closely related to obesity (36, 37). Additionally, leptin upregulates tumor necrosis factor alpha (TNF-α) expression (38). TNF-α is a multifunctional cytokine, and its elevated production leads to the inflammatory nature of obesity (39). Changing the efficiency of cellular fatty acid uptake to modulate leptin expression and production could be feasible (40). In a previous study, the same multi-strain supplement used in this study, which contained AP-32, bv-77, and CP-9 showed a better capability to reduce the ketone body, and non-esterified fatty acids (NEFA), and blood lipids than a mono-strain supplement in HFD-induced obese rodents (14). In this study, adiponectin upregulation and leptin and TNF-α downregulation were demonstrated in humans. Studies have reported that supplementation with *Bifidobacterium* spp. is able to induce adiponectin expression in animal models (41, 42). With the results of the *in vitro* TG accumulation assay, the supplement of this probiotic mixture may reduce lipid absorption in the small intestine and then reduce the lipid levels in blood. A lower serum lipid level may downregulate leptin secretion and then increase adiponectin levels. Leptin downregulation consequently resulted in downregulated TNF-α expression and ameliorated chronic inflammation. Adipose tissue inflammation increases the infiltration of M1 macrophage in the liver, and TNF-α is a potent pro-inflammatory mediator that is secreted by activated M1 macrophages (43). Obesity induces TNF-α elevation in the blood and liver (44). TNF-α elevation in the liver particularly influences the progression of non-alcoholic fatty liver disease (NAFLD) and results in liver damage (45). Higher incidences of abnormal GPT levels have been ascribed to the higher degree of obesity in children (46). In this study, the serum levels of GOT and GPT were responsive to treatment in both the placebo and probiotic groups. All of the participants received dietary fiber in their supplement, demonstrating the importance of fiber intake to liver health (47–49).

Ten years ago, the novel concept of "MicrObesity" (Microbes and Obesity) was proposed to address the specific role of dysbiosis and its impact on host metabolism and energy storage (50). Since then, more and more differences in the gut microbiota have been observed between obese and lean individuals. Decreased levels of the bacteria *Bifidobacterium*, *Desulfovibrio*, and *Lactobacillus* were reported to be associated with obesity in children (51), and high levels of *Bacteroides fragilis* (*B. fragilis*) and *Escherichia coli* (*E. coli*) were found in overweight/obese children (52). In this study, *Bifidobacterium* (CP-9) and *Lactobacillus* (bv-77 and AP-32) supplementation increased their populations in the gut and elevated the abundance of *Desulfovibrio*, which was not included in the probiotic package, unexpectedly. Although the role of *Desulfovibrio* in human health remains controversial and may be age-related (51), more studies are required to verify whether the effect of *Desulfovibrio* was species-specific (53). Moreover, lower levels of *B. fragilis* and *E. coli*, whose abundances were positively correlated with obesity, were observed in the probiotic group. In short, the probiotic intervention was able to modulate the gut microbiota and reshape dysbiosis in overweight/obese children.

Many studies have suggested that the function and efficacy of multi-strain probiotic supplements should be superior to mono-strain ones, demonstrating synergy among strains (54). The multi-strain probiotic supplement used in this trial contained three species from two genera. The effects of mono- (AP-32, bv-77, or CP-9) and multi-strain (AP-32/bv-77/CP-9) probiotic supplementations have been previously investigated in obese rats (14). Consistent with the animal study, improved blood lipid profiles and gut microbiota modulation was observed in the subjects of this trial who received multi-strain probiotics. In terms of the blood lipid profile, the improvement was indistinguishable among the animals receiving mono- and multi-strains over the course of 4 weeks. However, the amelioration of weight gain and the modulation of the gut microbiota were more effective in animals receiving multi-strain probiotics. For instance, the abundance of *B. animalis* was significantly increased by the multi-strain probiotics over the course of 4 weeks, but surprisingly, the increment achieved by the mono-strain supplement (i.e., *B. animalis* CP-9) was not significant due to wide variation in the results. We speculated

that the multi-strain supplements containing *Lactobacillus spp.* (*L. salivarius* AP-32 and *L. rhamnosus* bv-77) created an optimal pH environment for probiotic colonization in the small intestine and promoted the better survival of the *B. animalis* subsp. *lactis* CP-9 in the large intestine. This hypothesis needs to be supported by further studies, such as *in vivo* bioluminescence imaging in whole animals after one oral administration (55). The mechanism of these additive effects is still far from clear and is believed to involve complicated networks of cell–cell interactions and communications known as quorum sensing (QS) (56). Although observations have found the association between the gut microbiota and metabolism, the evidence of strong linkages between specific bacteria and functional pathways remained rare. In our study, the analysis was focused on the correlation of the gut microbiota with lipid metabolism, and the results elucidated the role of *Lactobacillus* spp. and *B. animalis* in fat metabolism. *Lactobacillus* spp. was positively correlated with HDL and negatively correlated with TC and LDL. A meta-analysis revealed a significant reduction in TC using *L. plantarum* and a reduction in LDL-C using *L. plantarum* or *L. reuteri* (57). Two *Lactobacillus* strains, *L. salivarius* AP-32 and *L. rhamnosus* bv-77, were included in our multi-strain probiotic blend, and further studies will be needed to verify their specific roles in lipid profiling. Our results also showed a positive correlation between the abundance of *B. animalis* in the gut microbiota and serum HDL and adiponectin. Several beneficial effects on inflammatory and oxidative biomarkers were seen in healthy subjects and metabolic syndrome patients received *B. lactis* HN019 supplementation (58). In other words, our results indicate that *Lactobacillus* spp. had more of an impact on the blood lipid profile, while *B. animalis* had more of an impact on anti-inflammation. Our results reveal the delicate differences between different bacteria and provide insight into the synergy of probiotic strains in the host. Taken together, this clinical study demonstrated that a well-designed multi-strain probiotic supplement can exert synergistic effects and regulate metabolism from more comprehensive aspects.

Ether lipids are major polar lipids in the cell envelope and play potential roles in cellular functions, such as membrane homeostasis and membrane trafficking. Ether lipids can affect cholesterol homeostasis, and crosstalk between the two metabolic pathways was proposed (59). A group of ether-linked lipids has been reported to be elevated in morbidly obese humans (60). In this study, ether lipid metabolism was positively associated with total cholesterol and LDLs. Intriguingly, *Lactobacillus* spp. displayed a negative association with ether lipid metabolism. Elevated TC and LDL levels were associated with a higher risk of coronary heart disease, Alzheimer's disease, and mild cognitive impairment (61, 62). Therefore, supplementation with the multi-strain probiotic blend elevated *Lactobacillus* spp. in the gut microbiota and potentially reduced the risk of dyslipidemia-related diseases.

Other than the ether lipid metabolism pathway, the correlation analysis indicated the induction of signal transduction *via* intervention in the probiotic group. Compared to the placebo groups, the functional pathway of the transduction factors was also significantly induced in the probiotic group. Signal transduction is a series of molecular events by which a chemical or physical signal is transmitted through a cell and ultimately results in a cellular response. During the signal transduction process, the activity of nuclear transcription factors is carefully modulated to exert a precise cellular response (63). Energy metabolism regulation requires the activation of the corresponding signal transduction pathways. For instance, extra-cellular insulin regulates sugar metabolism by activating the cascade of intra-cellular phosphatases and substrates (64). Therefore, part of signal transduction activation may be due to fat metabolism modulation. Notably, the probiotic supplement also affected other functional pathways, such as the biosynthesis of antibiotics and drug metabolism. Moreover, lower levels of *B. fragilis* and *E. coli* were observed in the probiotic group. Based on the results, further studies investigating the immune response pathways against pathogens are recommended.

There were some key limitations including diet and exercise which were not strictly recorded during the clinical trial. They are also confounding factors that may impact the composition of gut microbiota. For instance, the ratio of *Bacteroides* and *Prevotella* can be modulated by a diet high in animal protein and saturated fat or a plant-based diet rich in fiber and simple carbohydrates (65). Exercise is supposed to reduce inflammatory infiltration but increase microbial diversity in the gut (66). Our results showed that the abundance of *Bacteroides* was reduced in both groups, implicating that the subjects of both two groups might take a more plant-based diet according to the diet guidance and no significant impact of the diet on gut microbiota was observed between them. Although TNF-α was reduced in the probiotic group, gut microbial diversity was not affected in both groups. This result might indicate that the subjects of both groups might not take enough exercise to impact their gut microbial diversity. Further studies with well-designed diet and exercise courses will be warranted to demonstrate the real effectiveness of the multi-strain probiotics against obesity. In addition, functional ingredients contained 300 mg of white kidney bean extract, 100 mg of psyllium husk, and 100 mg of garcinia cambogia extract per package used in our study. Although these ingredients were reported to modulate gut microbiota (23–25), we could not clearly conclude their impacts on gut microbiota and weight control when in combination. The amounts used in this study might be not high enough to observe the impacts on weight control and on alterations of gut microbiota such as *Prevotella* and *Streptococcus* in mice as well as *Veillonella* and *Subdoligranulum* in humans (23–25). For example, a daily supplement of 7 g psyllium husk can increase *Veillonella* but decrease *Subdoligranulum* in healthy adults (67).

On the other hand, although we evenly allocated participants according to their physical measures, the bias of different systolic blood pressures occurred between groups before the intervention. The higher systolic blood pressure may reflect a poor cardiovascular condition and a different type of microbiota dysbiosis from the probiotics group and thus may lead to a bias of the impacted alterations of gut microbiota in the placebo group when compared to the probiotic group. Although we did not observe significant differences in the baseline of several microbial biomarkers in our study (**Supplementary Figure 3**) between the two groups, the extension of the allocation criteria to cardiovascular measures will be necessary to avoid bias when including high BMI participants in further study. It is eventually noted the possibility of a pair of variables that show a significant *p*-value with no biological association due to aleatory phenomena when comparing the 16S rRNA sequencing. A stricter significance threshold for individual comparisons will be required. In our case, the elevation of *Bifidobacterium animalis* displayed a statistical significance with both *p*- and *q*-values (adjusted *p*-value by FDR) less than 0.05. However, in the case of *Lactobacillus* spp., a *p*-value less than 0.05 was presented by using a loose threshold, but a *q*-value represented higher than 0.05 using a strict threshold. Therefore, the former statistical significance can be included and interpreted.

In conclusion, the probiotic blend of *L. salivarius* AP-32, *L. rhamnosus* bv-77, and *B. animalis* CP-9 (1: 1: 8) enhanced the effect of functional ingredients and displayed a greater influence on improving lipid metabolism. The probiotic supplement was able to modulate the gut microbiota and consequently improve the blood lipid profile, alleviate low-grade inflammation, and reduce body weight in overweight/obese subjects. However, the dietary supplement demonstrated limitations in terms of weight loss, with BMI reductions of less than 5% after the 3-month intervention. Weight loss is not easy, and better outcomes can make it easier for people to maintain their motivation to lose weight. Future studies combining diet control and exercise programs are suggested to evaluate the facilitating effects of probiotic supplements on weight loss.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the CMUH105-REC2-096 – China Medical University Hospital. Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

Y-TY and H-CL contributed equally to the conception and design of the study. A-CC and T-JF contributed equally to this work. A-CC, T-JF, H-HH, J-FC, Y-WK, and Y-YH organized the database. S-YT and S-FW performed the statistical analysis. A-CC and T-JF wrote the first draft of the manuscript. H-HH, J-FC, Y-WK, and Y-YH wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Conflict of interest

H-HH, J-FC, Y-WK, Y-YH, and S-YT were employed by Glac Biotech Co., Ltd.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.922993/full#supplementary-material

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Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Franck Carbonero,
Washington State University Health Sciences Spokane, United States
Ahmad Ud Din,
Sichuan University, China

*CORRESPONDENCE
Christophe Lacroix
christophe.lacroix@hest.ethz.ch

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A proof of concept infant-microbiota associated rat model for studying the role of gut microbiota and alleviation potential of *Cutibacterium avidum* in infant colic

Vanesa Natalin Rocha Martin[1,2], Christophe Del'Homme[3], Christophe Chassard[4], Clarissa Schwab[1], Christian Braegger[2], Annick Bernalier-Donadille[3] and Christophe Lacroix[1]*

[1]Laboratory of Food Biotechnology, Department of Health Sciences and Technology, Institute of Food, Nutrition and Health, ETH-Zurich, Zurich, Switzerland, [2]Division of Gastroenterology and Nutrition, University Children's Hospital Zurich, Zurich, Switzerland, [3]INRAE UMR 454, MEDIS Unit, Clermont-Ferrand Research Centre, Saint Genes-Champanelle, France, [4]INRA UMR 545 Fromages, Aurillac, France

Establishing the relationship between gut microbiota and host health has become a main target of research in the last decade. Human gut microbiota-associated animal models represent one alternative to human research, allowing for intervention studies to investigate causality. Recent cohort and *in vitro* studies proposed an altered gut microbiota and lactate metabolism with excessive H_2 production as the main causes of infant colic. To evaluate H_2 production by infant gut microbiota and to test modulation of gut colonizer lactose- and lactate-utilizer non-H_2-producer, *Cutibacterium avidum* P279, we established and validated a gnotobiotic model using young germ-free rats inoculated with fecal slurries from infants younger than 3 months. Here, we show that infant microbiota-associated (IMA) rats inoculated with fresh feces from healthy (n = 2) and colic infants (n = 2) and fed infant formula acquired and maintained similar quantitative and qualitative fecal microbiota composition compared to the individual donor's profile. We observed that IMA rats excreted high levels of H_2, which were linked to a high abundance of lactate-utilizer H_2-producer *Veillonella*. Supplementation of *C. avidum* P279 to colic IMA rats reduced H_2 levels compared to animals receiving a placebo.

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Taken together, we report high H_2 production by infant gut microbiota, which might be a contributing factor for infant colic, and suggest the potential of *C. avidum* P279 in reducing the abdominal H_2 production, bloating, and pain associated with excessive crying in colic infants.

KEYWORDS

infant gut microbiota, infant colic, human microbiota-associated rats, gnotobiotic, *Cutibacterium* (*Propionibacterium*) *avidum*, hydrogen

Introduction

Human cohort studies often reveal associations between the gut microbiota and host health, but valid *in vitro* and *in vivo* models are needed to provide mechanistic understanding (1). Animal models allow experimental procedures otherwise limited in humans due to ethical and safety concerns or due to limited accessibility to biological samples, while providing a controlled environment and standardized genetic background. Inoculation of germ-free animals with fecal microbiota from human donors has been used successfully to study the role of gut microbiota in various pathophysiological conditions. For example, Crouzet *et al.* (2) showed that the transfer of fecal microbiota from irritable bowel syndrome patients in germ-free rats was associated with increased abdominal sensitivity, compared to animals inoculated with fecal microbiota from healthy human subjects. Increased adiposity was also observed in germ-free mice that received fecal microbiota from obese individuals compared to mice inoculated with the microbiota of lean individuals (3). In a more recent study, germ-free mice inoculated with feces from patients suffering from inflammatory bowel disease showed a dysregulated immune response compared to mice receiving feces from healthy individuals (4). However, validated gnotobiotic models to study young infant gut microbiota are still lacking.

Infants are born with low numbers of microbes in their gut, and first colonizers are mainly transferred from the mother's skin, mouth, vagina, and breast microbiota (5–7). The gut microbiota of breast- and formula-fed newborns is characterized by a high abundance of lactate-producing bacteria (LPB), mainly *Bifidobacterium, Streptococcus*, *Enterococcus,* and Enterobacteria (8–10). Lactate is an important intermediate metabolite that, if not further metabolized by lactate-utilizing bacteria (LUB), might accumulate leading to acidosis, neurotoxicity, and cardiac arrhythmia (11–13). The LUB community in infants is mainly composed of propionate-producing *Propionibacterium*/*Cutibacterium* and *Veillonella* spp., together with some species of the genus *Bacteroides* and with a lower abundance of butyrate-producing bacteria like *Anaerostipes* spp., *Anaerobutyricum hallii,* and *Eubacterium limosum,* and sulfate-reducing bacteria (SRB) like *Desulfovibrio* (8, 14–17). Hydrogen (H_2), which is formed by *Anaerostipes* spp., *A. hallii,* and *Veillonella* spp., can accumulate and serve as a substrate for H_2S formation by SRB (16, 15). Imbalances in lactate, H_2, and H_2S metabolism may induce flatulence and bloating and associated pain in colic infants (defined as infants younger than 5 months crying more than 3 h per day, for at least 3 days in a week) (16, 18–21). Several studies have identified higher breath H_2 excretion in colic infants compared to healthy controls, and positively correlated breath H_2 with crying time (22–24). Pham et al. (16) reported higher ratios of H_2-producing to H_2-utilizing LUB in crying infants compared to healthy controls at 3 months of age, and higher colonization by H_2-producer *A. hallii* in colic infants compared to healthy controls at 2 weeks of age.

Because lactate is an important substrate for H_2 production by LUB and accumulation of lactate and H_2 might be contributing factors for infant colic (IC), supplementation of non-H_2-producing LUB has been suggested as a potential strategy for IC alleviation. Pham et al. (16) demonstrated that infant isolates of non-H_2-producing LUB *E. limosum* reduced H_2 production by LUB *Veillonella ratii* when grown in co-culture in lactate-containing media. Alternatively, non-H_2-producing LUB *Cutibacterium avidum* strains of infant origin have shown to persist, reduce lactate, and compete with *Veillonella* when supplemented in a recently developed and validated continuous colonic fermentation model (PolyFermS platform), inoculated with immobilized fecal microbiota and mimicking proximal colon conditions of 2-month-old formula-fed infants (17, 25). Considering the previous observations, we hypothesize that *Cutibacterium avidum* P279, selected for its colonization ability and effects observed in *in vitro* infant colonic fermentations, can decrease H_2 excretion in a rat model of colic infants and has therefore potential for the treatment of IC. In order to test this hypothesis, we (1) developed and validated a rat model based on the inoculation of young germ-free animals with fecal slurries from healthy and colicky young infants (< 3 months) and (2) investigated the impact on gut microbiota composition and activity of supplementation of *C. avidum* P279 to colic infant microbiota-associated (IMA) rats.

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Materials and methods

Donors and sample collection

Fecal samples from two healthy infants (H1: 59 days old and H2: 92 days old) and two infants suffering from colic (C1: 71 days old and C2: 68 days old) who satisfied Rome IV criteria (20) were used for inoculation of germ-free Fischer 344 rats (**Figure 1**). Inclusion criteria were as follows: good health, a full-term delivery (gestation time 37–42 weeks), normal birth weight (female: 2.7–5.0 kg; male: 2.9– 5.2 kg), and an exclusively milk-based diet. Infants with a history of antibiotic treatment were excluded. Healthy infants (H1 and H2) did not present gastrointestinal (GIT) disorders, while colic donors (C1 and C2) complied with the following criteria: i) caregiver reported infant crying or fussing, for more than 3 per day during 3 h or more days in the last week to the nurse; ii) total 24-h crying confirmed to be 3 or more h measured prospectively by caregivers; and iii) no evidence of failure to thrive (20). Infants were born by vaginal delivery and living in Zürich (Switzerland). Healthy donors were breastfed, while colic donors received infant formula complementary to breast-feeding. Parents of donors were asked to provide a fresh fecal sample under highly controlled conditions (presented below) to a study nurse, and the data and material were subsequently anonymized. Anonymized biological material is not under the scope of the Swiss Federal Human Research Act (Art. 2 para. 2 let. b and c), which excluded this study from requiring ethical approval. Fecal samples (2 - 4 g) were collected by the parents by scraping from diapers with a sterile spatula into 50-mL sterile conical-shaped polypropylene tubes and immediately placed into closed airtight jars (Mitsubishi AnaeroPack, Thermo Fisher Diagnostics AG, Pratteln, Switzerland) containing one AnaeroGen sachet (Oxoid, Thermo Fisher Diagnostic AG). Samples were kept under cold conditions until they were picked up from the family house and transported into a Styrofoam box with frozen gel packs to INRAE - Theix (France). Fecal slurries from healthy and colic donors were inoculated in germ-free rats after 24–36 h of fecal deposition.

Bacterial strain, growth conditions, and preparation of supplementation doses

Cutibacterium avidum P279 was isolated from feces of a 13 week-old infant and selected for its colonization ability observed in *in vitro* infant colonic fermentations, and was obtained from the strain collection of the Laboratory of Food Biotechnology (ETH-Zürich) and used for supplementation of colic IMA rats (26). *C. avidum* P279 was grown in yeast extract sodium lactate medium (YEL) consisting of 1% (w/v) trypticase soy broth without dextrose (Becton Dickinson AG, Allschwil, Switzerland), 1% (w/v) yeast extract (Merck, Darmstadt, Germany), 117 mM sodium DL-lactate (60% v/v syrup, Central Drug House, New Delhi, India), 0.025% (w/v) KH_2PO_4 (VWR International AG, Dietikon, Switzerland), and 0.0005% (w/v) $MnSO_4$ (Sigma-Aldrich, Buchs, Switzerland). Glycerol stocks stored at -80°C were reactivated on agar plates and incubated in airtight jars (Mitsubishi AnaeroPack, Thermo Fisher Diagnostics) containing one AnaeroGen sachet (Oxoid, Thermo Fisher Diagnostics AG) at 37°C for 5 days. Single colonies were inoculated into liquid YEL and incubated for 48 h at 37°C. After incubation, cultures were centrifuged for 10 min at 7,000 g, and pellets were resuspended in the same volume of sterile reconstituted skim milk (10% w/v). Aliquots of 350 µL (containing approximately 10^9 CFU) were transferred into cryovials (Huber Co, Reinach, Switzerland). For supplementation of the control group, aliquots of 350 µL of sterile reconstituted skim milk (10% w/v) were prepared in cryovials (Huber Co). Cryovials were snap-frozen in liquid nitrogen and stored at -80°C for a maximum of 5 weeks until supplementation.

Animal experiments

Germ-free Fischer 344 rats bred at INRAE-Theix facilities were kept in sterile isolators with positive pressure over the entire trial period as described before (2). All experimental protocols were approved by the Local Institutional Animal Care and Use Committee (Approval reference N° 2019101512192520).

Rats were weaned at 3–4 weeks of age and inoculated with 0.2 mL of infant fecal slurries from single donors by a single intragastric gavage [100-fold dilution of fecal sample in anaerobic mineral solution (27) that was composed of 7.5% v/v mineral solution I (0.3% w/v K_2HPO_4), 7.5% v/v mineral solution II [0.3% w/v K_2HPO_4; 0.6% w/v NaCl; 0.6% w/v $(NH_4)_2SO_4$; 0.06% w/v $MgSO_4$; 0.06% w/v $CaCl_2$], 0.01% w/v resazurin, 0.4% w/v $NaHCO_3$, 0.05% w/v cysteine hydrochloride, and distilled water; all chemicals were obtained from Sigma-Aldrich]. IMA rats were placed in cages housing two animals. They were fed with gamma-irradiated infant formula (Aptamil Pre, Milupa GmbH, Germany) reconstituted in sterile water (13% w/v; approximately 80 mL per day during the first 2 weeks and afterwards 100 mL per day until the end of experimentation) mimicking infant diet. The commercial formula contained 8% (w/v) galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (ratio of 9:1). Two to three pellets of the standard diet for growing rats (A03/R03, SAFE, France) per day were also supplied to each cage (after 2 weeks from weaning for healthy IMA rats and during entire intervention period for colic IMA rats) in order to prevent bloating due to the lack of indigestible fibers and high content

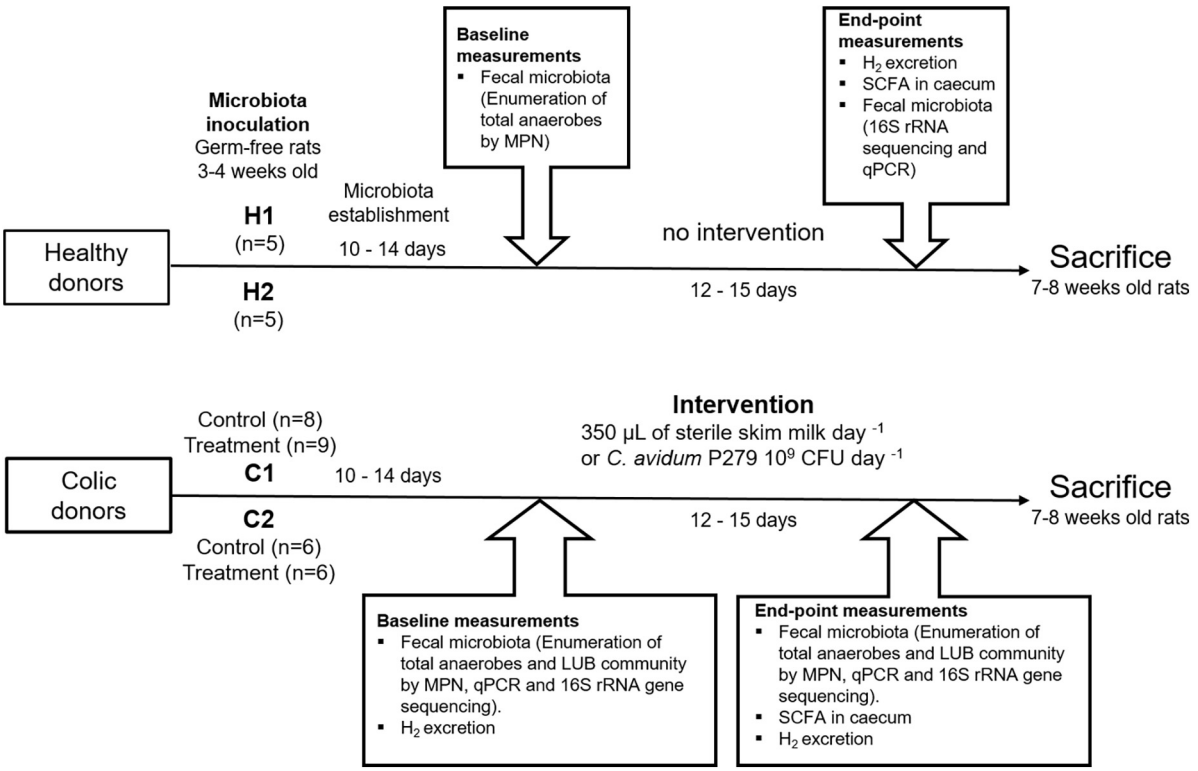

FIGURE 1
Diagram of the experimental design. Fecal samples from two healthy (H1 and H2) and two colic infants (C1 and C2) were used for inoculation of germ-free Fischer 344 rats (n = 39). The microbiota was allowed to establish for 10–14 days before baseline (T0) measurements. Rats inoculated with donors C1 and C2 were divided into treatment group (gavaged daily with 10^9 colony forming units of *Cutibacterium avidum* P279 in 350 µL of sterile skim milk) and control group (received 350 µL of sterile skim milk). End-point measurements (T1) were obtained on days 26–28. Rats were sacrificed at 7–8 weeks of age. Rats were fed a gamma-irradiated infant formula to mimic an infant diet and supplemented with 1–3 pellets per day of standard diet for growing animals. All rats were given free access to sterile water.

of lactose of the infant formula. All rats were given free access to sterile water.

Ten germ-free rats were inoculated with fecal slurries from healthy donors H1 (n = 5) and H2 (n = 5), and were allocated to two different isolators (Figure 1). Seventeen rats inoculated with fecal slurries from colic donor C1 were divided into two isolators housing a control group (n = 8) and a treatment group (n = 9). Fecal slurries from colic donor C2 were used to inoculate 12 rats housed in two isolators (control group n = 6 and treatment group n = 6).

After inoculation, the microbiota was established for a period of 10–14 days before further measurements (Figure 1).

Sample collection from infant microbiota-associated rats

Fecal samples from rats were collected directly from the anus by manual perineal stimulation and were immediately used for microbial enumeration (see below) or stored at –80°C until further processing. Feces collection and fecal microbial enumeration were done 10–14 days from inoculation (baseline measurements) and at the end of experimentation (Figure 1). After baseline measurements, rats inoculated with fecal microbiota from donors C1 and C2 were gavaged daily for 12–15 days with 10^9 CFU of *C. avidum* P279 in 350 µL of sterile skim milk (treatment groups) or with the same amount of sterile milk alone (control groups). Rats were sacrificed at 7-8 weeks of age, and the cecum contents were harvested for analyzing fermentation metabolites.

Enumeration of total bacteria and functional bacterial groups

Freshly voided feces from donors and rats were serially 10-fold diluted (wet w/v) in an anaerobic mineral solution. Total anaerobes, and non-SRB-LUB and SRB-LUB communities were enumerated by most probable number estimation (MPN) in a three-replicate design (28). Total anaerobes in all donors and rat feces were enumerated in an O_2-free CO_2 complex medium containing clarified rumen fluid (29). Oxygen-free

CO_2 basal medium with L-lactate (35 mM) as the sole energy source and Postgate E medium were used for the enumeration of non-SRB-LUB and SRB-LUB communities, respectively (30, 31). After incubation for 5 days at 37°C, lactate concentration in supernatant of culture tubes of each inoculated dilution (10^{-5} to 10^{-10}) was determined by high-performance liquid chromatography with refractive index detection (HPLC-RI) (described below). Cultures with a final lactate concentration below 25 mM (lactate consumption of at least 10 mM) were considered positive for non-SRB-LUB bacteria (32). Cultures on Postgate E medium were inoculated with 0.3 mL of fecal dilutions (10^{-2} to 10^{-5}) and were considered positive for MPN determination when a black precipitate of FeS was visually observed.

DNA extraction and quantitative PCR analysis

Genomic DNA was extracted from infant and rat stool samples (200 mg) and from MPN-positive cultures (2 mL) in Postgate E and basal medium with L-lactate, using the Fast DNA SPIN kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. The extracted DNA was then used to amplify regions of the 16S rDNA gene by qPCR using specific primers (**Supplementary Table 1**). Reactions were performed using LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland) and the SensiFAST SYBR No-ROX 2X mix (5 μL), and 500 nM primers (Biolab Scientifics Instruments SA, Chatel-St-Denis, Switzerland) in a total reaction volume of 10 μL. Thermal cycling started with an initial denaturation step at 95°C for 3 min, followed by 40 cycles of a two-step PCR consisting of a hold at 95°C for 5 s and at 60°C for 60 s. The cycle threshold (Ct) values were obtained using automatic baseline and threshold settings provided by the LightCycler 480 Software, Version 1.5. Samples were analyzed in duplicate. To generate standards, PCR amplicons were cloned into the pGEM-T Easy Vector and heterologously expressed in *E. coli* according to instructions of the supplier (Promega AG, Dübendorf, Switzerland). Standard curves were prepared from 10-fold dilutions of linearized plasmids harboring the target gene of interest. Bacterial groups abundant in the infant feces were amplified using primers listed in **Supplementary Table 1**. Melting curve analysis was conducted to confirm amplification specificity. To estimate cell counts for *Cutibacterium*, gene copies were corrected for multiple copies (n = 3) of 16S rRNA genes (33).

16S rRNA gene amplicon sequencing

The bacterial profiles in fecal samples obtained from donors and rats and in pellets from cultures in Postgate E and basal medium with lactate were determined using tag-encoded 16S rRNA gene Miseq-based (Illumina, San Diego, CA, United States) high-throughput sequencing, as presented before (17). Briefly, DNA concentration was standardized to 20 ng μL^{-1}. The V3 region of the 16S rRNA gene was amplified using primers including adapters for the Nextera Index Kit, NXt_388_F, and NXt_518_R (**Supplementary Table 1**). One MiSeq flow cell and the V2 2 × 250 bp paired-end Nextera chemistry were used, and were supplemented with 20% of PhiX. Library preparation and sequencing were performed at the Genomic Diversity Center (ETH Zurich, Zurich, Switzerland). The raw datasets containing pair-ended reads with corresponding quality scores were merged and trimmed using settings as previously described (34). The minimum length of merged reads was 200 bp. Following analysis steps were done using Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.8.0 and 1.9.0) (35). Purging the datasets from chimeric reads and constructing *de novo* operational taxonomic units (OTUs) were conducted using the UPARSE pipeline (36) and the Greengenes database as a reference (37). Taxonomic assignment of OTUs at the genus level could not differentiate between *Propionibacterium* and *Cutibacterium* because the new taxonomy (a division of former *Propionibacterium* spp. in gen. nov. *Cutibacterium* including cutaneous species and *Propionibacterium* spp. including only dairy species) has not been updated in the reference database (38). Alpha-diversity was analyzed using observed species (species richness in individual samples) and Shannon index (species richness and evenness estimator, which increase when the number of species and evenness increase). The beta-diversity (compositional diversity between two samples) was analyzed as previously described using iterative subsampling (39). Permutational multivariate analysis of variance using distance matrices (PERMANOVA) based on 999 Monte Carlo simulations was used to analyze the differences in unweighted and weighted UNIFRAC distances between infant and IMA rat fecal samples from different donors. Linear discriminant analysis effect size (LEfSe) was applied to identify genera that differed significantly between IMA rats inoculated with fecal samples from the same donor, from treatment and control groups at baseline (T0) and after the intervention period (T1). LEfSe analysis was done with default parameters and a logarithmic linear discriminant analysis score threshold of two (40).

Metabolite analysis by high-pressure liquid chromatography-refractive index detection

The concentration of acetate, propionate, butyrate, isobutyrate, isovalerate, formate, succinate, and lactate were

determined in cecum content and in the supernatant of LUB enumeration media after centrifugation for 10 min at 13,000 g. The supernatant (500 μL) was filtered through a 0.45-μm membrane (Millipore AG, Zug, Switzerland) into glass HPLC vials (Infochroma, Hitachi LaChrome, Merck, Dietikon, Switzerland) and sealed with crimp caps. An HPLC (Hitachi LaChrome) equipped with a Security Guard Cartridges Carbo-H column (4 × 3 mm; Phenomenex Inc., Torrance, CA, United States), a Rezex ROA-Organic Acid H + column (8%, 300 × 7.8 mm; Phenomenex), and a refractive index detector (HPLC-RI) was used. The column was eluted with 10 mM H_2SO_4 (Fluka, Buchs, Switzerland) as a mobile phase at a flow rate of 0.4 mL min^{-1} at 25°C. All tested metabolites were quantified using external standards using EZChrom Elite software (version 3.3.2 SP2, Agilent Technologies, CA, United States).

Rats hydrogen excretion

The amount of H_2 excreted by each rat was measured by housing a single animal in a respiratory chamber for 24 h (41). Measurements were done on days 27 and 28 for rats inoculated with feces from healthy donors (H1 and H2) and before (baseline measurements T0, 10–14 days from inoculation) and after treatment (end-point measurements T1, after 12-15 days of intervention) for rats inoculated with feces from colic donors (C1 and C2). H_2 concentration in the chamber atmosphere was determined using a gas phase chromatograph (Microanalyzer DP, Quintron Instruments, Milwaukee, WI, United States).

Statistical analysis

Statistical analyses were done using SigmaPlot (Systat Software, San Jose, CA, United States) with statistical significance set at a p-value of less than 0.05. The Student's t-test with two-tailed distribution was used, after testing for normal distribution using the Shapiro–Wilk test, to compare changes in the abundance of microbial taxa detected by qPCR (log_{10}-transformed), cecum metabolites (mmol L^{-1}) and H_2 excretion (ppm) values between baseline and after intervention for the same IMA rat group and between control and treatment IMA rat groups after the intervention period. The Mann–Whitney test was used when data were not normally distributed.

Availability of supporting data

Sequence information is available in the NCBI database, BioProject ID PRJNA590392.

Results

Fecal microbiota of infant donors

The bacterial composition in feces of infant donors was analyzed using qPCR and 16S rRNA gene amplicon sequencing. Species richness in donors ranged from 10 to 46 observed species (**Table 1**). *Bifidobacterium* [Log 9.6 gene copies g $feces^{-1}$, 70.5% relative abundance (rel. ab.)] and *Bacteroides* (Log 10.2 gene copies g $feces^{-1}$, 13.4% rel. ab.) were the most abundant genera in donor H1, while *Bifidobacterium* dominated in donor H2 (Log 10.1 gene copies g $feces^{-1}$, 96.4% rel. ab.) and C1 (Log 8.9 gene copies g $feces^{-1}$, 80.3% rel. ab.) (**Figure 2** and **Table 1**). Donor C2 fecal microbiota was dominated by Firmicutes (Log 9.1 gene copies g $feces^{-1}$, 70.7% rel. ab.), mainly represented by *Clostridium* cluster I (Log 9.0 gene copies g $feces^{-1}$). *Enterobacteriaceae* were detected in high abundance in donors H1, C1, and C2 (Log 8.0-8.8 gene copies g $feces^{-1}$ and 11.6 - 29.1% rel. ab.) but at low concentrations in donor H2 (Log 3.8 gene copies g $feces^{-1}$, < 0.0001% rel. ab.). *Veillonella* was identified in all donors at sub-dominant levels (Log 4.6-7.5 gene copies g $feces^{-1}$, 0.01 - 0.3% rel. ab.) (**Figure 2** and **Table 1**). The donor fecal samples used to inoculate IMA rats are representative of the highly variable taxonomic composition of the infant gut microbiota.

Colonization of infant fecal microbiota in germ-free rats

Viable cell numbers in donor slurries used for inoculating germ-free rats were estimated by an MPN-based cultivation approach. Slurries from healthy donors had higher viable cell numbers at inoculation (H1: Log 11.5 cells g $feces^{-1}$ and H2: Log 10.2 cells g $feces^{-1}$) than colic donors (C1: Log 9.5 cells g $feces^{-1}$ and C2: Log 9.6 cells g $feces^{-1}$). After implantation, viable cell numbers of healthy and colic IMA rats were very similar, averaging Log 10.6 ± 0.5 cells g $feces^{-1}$, while some differences were observed among donor feces using MPN (**Table 1**).

Quantification of bacterial groups in IMA rats by qPCR revealed higher levels of Firmicutes (Log 9.5–9.8 gene copies g $feces^{-1}$) in rats compared to donors (**Table 1**). The levels of other bacterial taxa were donor-dependent and generally matched the composition of the donor microbiota. *Bifidobacterium* colonized H1, H2, and C1 rats at high abundance (Log 10.0–10.4 gene copies g $feces^{-1}$) and C2 rats at a much lower level (Log 5.9 ± 0.4 gene copies g $feces^{-1}$) similar to donor C2 (Log 6.5 gene copies g $feces^{-1}$). Engraftment of *Bacteroides* in H1 rats (Log 10.7 ± 0.1 gene copies g $feces^{-1}$) and H2 rats (Log 5.1 ± 0.3 gene copies g $feces^{-1}$) also corresponded with donor fecal concentrations (H1: Log 10.2 gene copies g $feces^{-1}$ and H2: Log 5.2 gene copies g $feces^{-1}$). *Bacteroides* were enriched approximately 100-fold in C1 (Log 10.4 ± 0.1

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TABLE 1 Main bacterial communities in feces from infant donors and IMA rats at baseline and fermentative metabolites in cecal supernatant from IMA rats after euthanasia.

	Healthy microbiota				Colic microbiota			
	H1		H2		C1		C2	
	Donor	IMA rats (n = 5)	Donor	IMA rats (n = 5)	Donor	IMA rats (n = 10)	Donor	IMA rats (n = 10)
Cultivable bacteria (Log cells g feces^{-1})								
Total anaerobes	11.5	11.0 (0.1)^{A}	10.2	10.5 (0.1)^{A}	9.5	10.6 (0.5)	9.6	10.4 (0.5)
non-SRB LUB	ND	ND	ND	ND	ND	10.1 (0.3)	ND	10.4 (0.3)
SRB LUB	ND	ND	ND	ND	BDL	BDL	4.4	BDL
Fecal microbiota α -diversity (16S rRNA gene amplicon sequencing)								
observed species	46	51 (3)	10	17 (1)	32	56 (2)	24	26 (3)
Shannon Index	3.3	3.8 (0.4)	0.8	2.0 (0.2)	3.2	4.4 (0.2)	2.5	2.8 (0.3)
Abundance of bacterial taxa (Log gene copies g feces^{-1})								
Total bacteria	10.5	11.0 (0.1)	10.2	10.7 (0.2)	9.0	10.0 (0.1)	8.9	9.3 (0.2)
Firmicutes	8.6	9.8 (0.1)	8.6	9.5 (0.2)	9.0	9.6 (0.2)	9.1	9.6 (0.2)
Enterobacteriaceae	8.8	9.0 (0.4)	3.8	9.0 (0.2)	8.0	8.5 (0.6)	8.2	8.1 (0.4)
Clostridium cluster I	BDL	BDL	BDL	BDL	BDL	BDL	9.0	10.4 (0.2)
Bacteroides spp.	10.2	10.7 (0.1)	5.2	5.1 (0.3)	8.2	10.4 (0.1)	7.1	9.4 (0.4)
Bifidobacterium	9.6	10.4 (0.2)	10.1	10.3 (0.2)	8.9	10.0 (0.3)	6.5	5.9 (0.4)
Veillonella	6.1	9.8 (0.2)	5.0	3.8 (0.4)	4.6	9.7 (0.2)	7.5	8.6 (0.5)
Microbial metabolites in rat caecum								
Acetate	ND	73.2% (2.4%)	ND	67.3% (13.8%)	ND	72.5% (4.2%)^{B}	ND	43.2% (5.4%)^{B}
Propionate	ND	15.8% (6.1%)	ND	2.1% (4.7%)	ND	21.2% (5.0%)	ND	23.1% (5.7%)
Butyrate	ND	2.0% (4.1%)	ND	5.3% (1.6%)	ND	0.6% (0.5%)	ND	33.7% (6.8%)
Formate	ND	4.6% (5.5%)	ND	1.3% (2.9%)	ND	2.8% (1.8%)	ND	BDL
Lactate	ND	4.4% (1.5%)	ND	24.0% (14.4%)	ND	3.0% (5.5%)	ND	BDL

Data are shown as mean ± SD. ^{A} Enumerated in two rats from each group.^{B} Values detected in rats from control group. Quantification by qPCR in donor feces was done on the same sample used for inoculation of IMA rats. ND, not determined. Metabolites in donor samples could not be determined due to small sample amount. BDL, below detection limit; LUB, lactate-utilizing bacteria.

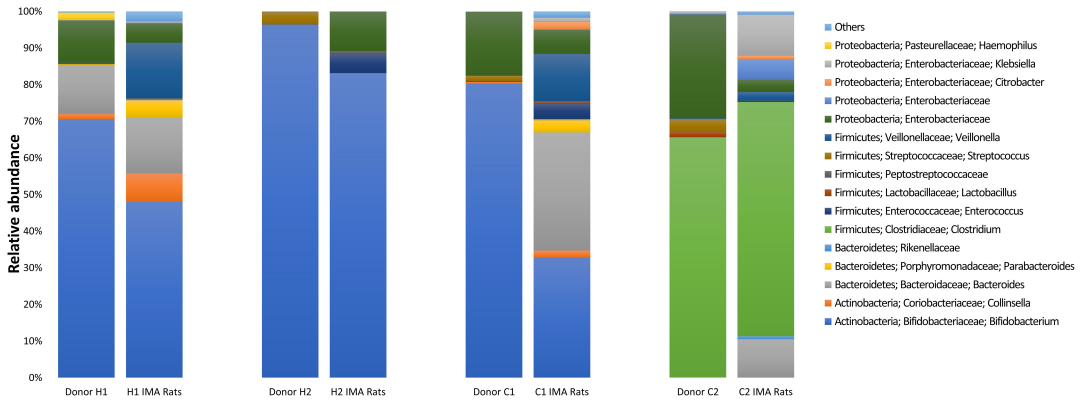

FIGURE 2
Bacterial community composition was determined by 16S rRNA gene amplicon sequencing in feces from infant donors and respective infant microbiota-associated (IMA) rats. For IMA rats, each column represents the average relative abundance. The relative abundance of taxa with an average relative abundance of >0.1% are depicted with the taxa nomenclature; lower abundant taxa (< 0.1%) are shown as Others.

gene copies g feces^{-1}) and C2 rats (Log 9.4 ± 0.4 gene copies g feces^{-1}) compared to respective donors (C1: Log 8.2 gene copies g feces^{-1} and C2: Log 7.1 gene copies g feces^{-1}). *Enterobacteriaceae* were well maintained in H1, C1, and C2 rats, but reached a much higher number in H2 rats (Log 9.0 ± 0.2 gene copies g feces^{-1}) than in donor H2 which had a very low

number of *Enterobacteriaceae* (Log 3.8 gene copies g feces^{-1}) compared to the other donors. *Veillonella* was much higher in H1 (Log 9.8 ± 0.2 gene copies g feces^{-1}), C1 (Log 9.7 ± 0.2 gene copies g feces^{-1}), and C2 (Log 8.6 ± 0.5 gene copies g feces^{-1}) rats compared to the respective donors (H1: Log 6.1 gene copies g feces^{-1}, C1: Log 4.6 gene copies g feces^{-1}, and C2: Log 7.5 gene copies g feces^{-1}) but not in H2 rats (Log 3.8 ± 0.4 gene copies g feces^{-1}) compared to donor H2 (Log 5 gene copies g feces^{-1}) (**Table 1**).

The number of observed species and Shannon indices were higher in rats compared to donors (**Table 1**). Except for C1 rats, fecal microbiota communities from IMA rats and respective donors were qualitatively similar, as indicated by the clustering of donor samples together with recipient rats when considering unweighted and weighted UniFrac distances in a principal component analysis (PCoA) plot (**Supplementary Figure 1**). When comparing groups of donors and respective recipient rats, cluster separation by donors was significant under unweighted and weighted UniFrac distances (PERMANOVA, p = 0.001) (**Supplementary Figure 1**).

Main shifts in the bacterial populations identified by gene amplicon sequencing were aligned with qPCR observations (**Figure 2**). *Bacteroidaceae* increased from 0.02% in donor C1 to 32.2% ± 14.8% in C1 rats and from 0.07% in donor C2 to 10.5% ± 8.4% in C2 rats. Colonization by *Enterobacteriaceae* was higher in feces of H2 rats (10.7% ± 3.5%) compared to donor H2 (< 0.01%) (**Figure 2**). Higher abundance of *Veillonellaceae* was also detected for H1 (15.4% ± 5.0%), C1 (12.9% ± 5.9%), and C2 rats (2.4% ± 1.6%) compared to respective donor levels (H1: 0.3%, C1: 0.1%, and C2: 0.2%) (**Figure 2**). In contrast, shifts in the relative abundance of *Bifidobacteriaceae* detected by sequencing were not aligned with qPCR observations for donors H1 and C1 and the corresponding IMA rats. In contrast to qPCR, the members of *Bifidobacteriaceae* were at a lower relative abundance in H1 rats (48.0% ± 11.4%) than in donor H1 (70.5%) and C1 rats (32.7% ± 9.1%) compared to donor C1 rats (80.3%) (**Figure 2**).

Overall, dominant microbes and microbiota profiles of infant donors were transferred and established with similar composition in H1, H2, and C2 rats as shown by qPCR and sequencing data. Microbiota engraftment in C1 rats measured with qPCR was consistent with donor microbiota composition, but differences among C1 rats were observed with sequencing, with an overall increased abundance of *Bacteroidaceae* compared to donor C1 feces.

Microbial fermentation activity in infant microbiota-associated rats

Different metabolic profiles were detected in cecal water from rats inoculated with different donors by HPLC-RI. Acetate (73.2% ± 2.4%) and propionate (15.8% ± 6.1%) were main metabolites identified in H1 rats, while acetate (67.3% ± 13.8%) and lactate (24.0% ± 14.4%) were the main metabolites detected in H2 rats. In colic rats, acetate (72.5% ± 4.2%) and propionate (21.2% ± 5.0%) were the main SCFAs identified in the cecal supernatant of C1 rats, while acetate (43.2% ± 5.4%), butyrate (33.7% ± 6.8%), and propionate (23.1% ± 5.7%) were the main SCFAs in C2 rats (**Table 1**).

After 2 weeks of inoculation, H_2 accumulation was measured in respiratory chambers after housing single IMA rats for a 24-h period. H1 rats excreted higher H_2 levels (105.2 ± 91.3 ppm) compared to H2 rats (28.6 ± 26.4 ppm; p 0.09) (**Figure 3**). However, significantly higher amounts of H_2 were measured in colic than in healthy IMA rats (C1 rats control group: 200 ± 97.3 ppm and C2 rats control group: 3,248.0 ± 1,686.9 ppm; p 0.001) (**Figure 3**). Rats inoculated with feces from donor C2 excreted significantly higher levels of H_2 than the C1 rats (p 0.002).

Lactate-utilizing bacteria abundance in colic infant microbiota-associated rats

The LUB community was enumerated in colic IMA rat feces at baseline using an MPN-based cultivation approach (**Table 1**). Non-SRB LUB colonized C1 (Log 10.1 ± 0.3 cells g feces^{-1}) and C2 rats (Log 10.4 ± 0.3 cells g feces^{-1}) at comparable and high levels. 16S rRNA gene amplicon sequencing of pellets from MPN cultures (in basal medium with L-lactate) detected mainly *Veillonella* (82.5 ± 5.6% rel. ab.) followed by *Enterobacteriaceae* (8.9 ± 3.9%) and *Bacteroides* (2.0 ± 1.8%). Acetate and propionate were the main products of lactate metabolism, which was in agreement with the bacterial populations recovered.

The SRB LUB (Log 4.4 cells g feces^{-1}) cultured in Postgate E medium from feces of C2 donor were mainly identified as *Desulfovibrio* (80.1 ± 0.0% rel. ab.). SRB LUB were below detection limit in C2 rat feces at all time points and were not detected in feces from donor C1 and corresponding C1 rats.

Impact of *Cutibacterium avidum* P279 on fecal microbiota composition and metabolic activity of colic infant microbiota-associated rats

The effect of daily supplementation of 10^9 CFU *C. avidum* P279 in sterile milk, a non-H_2-producing LUB, after 12–15 days of intervention on gut microbiota composition and metabolism was tested in colic IMA rats and compared to control groups receiving only sterile milk. The colonization of *C. avidum* P279 in IMA rats from treatment groups was tested by qPCR and 16S rRNA gene amplicon sequencing. Donor-dependent effects were observed on microbiota composition and metabolism.

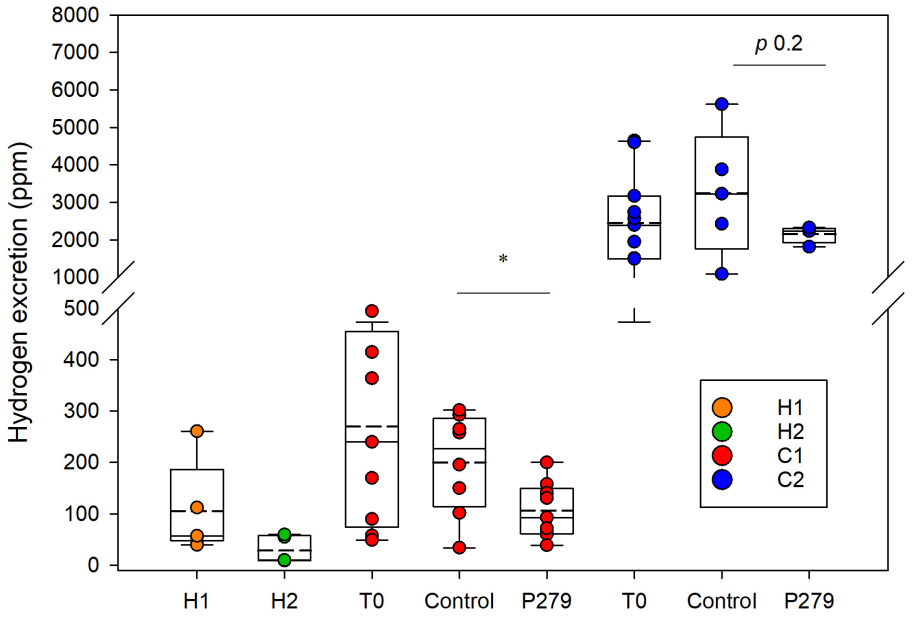

FIGURE 3
Hydrogen excretion by IMA rats. Hydrogen concentration (ppm) accumulated in the respiratory chambers after 24 h of housing single IMA rats. Measurements were done at the end of experimentation for rats inoculated with feces from healthy donors (H1 and H2) and at baseline (T0) and after intervention for rats inoculated with feces from colic donors (C1 and C2). Treatment groups (P279) were gavaged daily for 12–15 days with 10^9 CFU of *Cutibacterium avidum* P279 in 350 μL of sterile skim milk, and control groups received daily 350 μL of sterile skim milk. Values are mean (H1 n = 5; H2 n = 5; C1 T0 n = 9; C1 P279 n = 9; C1 control n = 8; C2 T0 n = 11; C2 P279 n = 4; and C2 control n = 5). Central horizontal solid lines show the median and dashed lines the mean, upper and lower box borders show the 90th and 10th centiles, respectively, and upper and lower whiskers show the 95th and 5th centiles, respectively.

Cutibacterium avidum P279 colonized C1 rats with fecal abundance of Log 7.5 ± 0.2 cells g feces $^{-1}$ (Table 2). LEfSe analysis based on 16S rRNA gene amplicon sequencing of the fecal microbiota of C1 rats also identified an increase in *Propionibacteriaceae* in the supplemented rats after the intervention period compared to baseline (Supplementary Figure 2D). When treated C1 rats were compared to the control group, LEfSe analysis identified increased *Propionibacteriaceae* in the supplemented rats, while *Veillonellaceae* abundance was higher in the control rats (Supplementary Figure 2B). In both groups, the *Enterobacteriaceae* population decreased after the intervention period, which corresponded with a significant reduction of Log 1.0 gene copies g feces^{-1} (Table 2 and Supplementary Figures 2C,D). Interestingly, formate and H_2 excretion were significantly lower in the treatment group compared to control animals (0.4 ± 0.6 mM vs. 1.7 ± 1.2 mM for formate, respectively, p 0.02; 106.3 ± 53.8 ppm vs. 200.0 ± 97.3 ppm for H_2 production, respectively, p 0.01) (Figure 3 and Table 2).

Cutibacterium avidum P279 colonized C2 rats with fecal abundance of Log 5.4 ± 0.3 cells g feces^{-1}, which was about log 2 lower than in C1 rats (Table 2). After treatment, the abundance of Firmicutes and *Clostridium* cluster I was significantly reduced by Log 0.5 ± 0.2 and Log 0.5 ± 0.2 gene copies g feces^{-1} (p < 0.01), respectively (Table 2). In agreement, LEfSe analysis identified a decrease in *Clostridiaceae,* and an increase of *Propionibacteriaceae* in treated animals. In C2 rats, *Veillonellaceae* increased after *C. avidum* P279 supplementation (Supplementary Figure 3D). The abundance of *Propionibacteriaceae* and *Veillonellaceae* was higher in the C2 treated than in the control rats, while the abundance of *Bifidobacteriaceae* and *Enterobacteriaceae* was lower (Supplementary Figure 3B).

Acetate and propionate levels, but not butyrate, were significantly higher in the C2 treatment group (11.9 ± 2.9 mM, 7.2 ± 3.3 mM, and 5.6 ± 2.2, respectively) compared to the control animals (acetate 6.4 ± 2.5 mM, p < 0.01; propionate 3.3 ± 0.8 mM, p < 0.05; and butyrate 5.1 ± 2.7 mM p > 0.05) after intervention. We measured a high although not significant reduction of the mean H_2 production in the treated C2 rats with *C. avidum* P279 compared to the rats from the control group, which is likely due to the large spread observed in the control group (2,152.5 ± 228.96 ppm vs. 3,248.0 ± 1,686.86 ppm, respectively; p 0.2). Surprisingly, much lower variability in H_2 excretion among the treated animals (1,815–2,325 ppm) was observed compared to the control rats (1,085–5,620 ppm) (Figure 3).

TABLE 2 Main bacterial communities in feces from colic IMA rats at baseline and after intervention.

	C1 IMA rats				C2 IMA rats			
	Control (n = 8)		Treatment (n = 9)		Control (n = 8)		Treatment (n = 9)	
	Baseline	End-point	Baseline	End-point	Baseline	End-point	Baseline	End-point
Cultivable bacteria (Log cells g feces^{-1})								
Total anaerobes	10.7 (0.5)	11.2 (0.1)*	10.4 (0.4)	10.5 (0.3)*	10.3 (0.6)	11.0 (0.3)*†	10.5 (0.4)	10.5 (0.3)*
non-SRB LUB	10.2 (0.3)^A	10.2 (0.5)	9.9 (0.1)^A	10.2 (0.6)	10.3 (0.2)	10.5 (0.0)†	10.5 (0.3)	10.1 (0.7)
SRB LUB	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Abundance of bacterial taxa (Log 16S rRNA gene copies g feces^{-1})								
Total bacteria	10.0 (0.2)	9.6 (0.2)*†	10.0 (0.1)	9.8 (0.1)*†	9.3 (0.1)	9.3 (0.1)	9.4 (0.2)	9.2 (0.1)
Firmicutes	9.6 (0.3)	9.2 (0.2)*†	9.6 (0.2)	9.5 (0.2)*	9.5 (0.2)	9.3 (0.2)	9.7 (0.2)	9.2 (0.2)†
Clostridium cluster I	BDL	BDL	BDL	BDL	10.3 (0.2)	10.0 (0.1)	10.5 (0.2)	10.0 (0.2)†
Enterobacteriaceae	8.3 (0.3)	7.3 (0.5)†	8.6 (0.7)	7.6 (0.6)†	8.2 (0.4)	8.1 (0.2)	7.9 (0.3)	8.1 (0.3)
Bacteroides spp.	10.5 (0.2)	10.2 (0.2)†	10.3 (0.1)	10.3 (0.2)	9.4 (0.4)	9.5 (0.1)	9.3 (0.4)	9.4 (0.2)
Bifidobacterium	9.9 (0.4)	9.9 (0.3)	10.0 (0.3)	10.0 (0.2)	6.0 (0.4)	5.7 (0.4)	5.8 (0.5)	5.7 (0.2)
Veillonella	9.6 (0.2)	9.8 (0.1)†	9.7 (0.2)	9.7 (0.2)	8.6 (0.5)	8.9 (0.2)	8.7 (0.3)	8.6 (0.5)
Cutibacterium	BDL	BDL	BDL	7.5 (0.2)	BDL	BDL	BDL	5.9 (0.3)
Microbial metabolites in rat caecum								
Acetate	ND	72.5% (4.2%)	ND	77.8% (4.9%)	ND	43.2% (5.4%)	ND	47.1% (5.6%)*
Propionate	ND	21.2% (5.0%)	ND	19.4% (4.3%)	ND	23.1% (5.7%)	ND	27.9% (8.4%)*
Butyrate	ND	0.6% (0.5%)	ND	0.5% (0.7%)	ND	33.7% (6.8%)	ND	22.3% (8.0%)
Formate	ND	2.8% (1.8%)	ND	0.5% (1.0%)*	ND	BDL	ND	1.7% (2.6%)
Lactate	ND	3.0% (5.5%)	ND	1.8% (5.1%)	ND	BDL	ND	1.0% (1.6%)

Data are shown as mean ± SD. Treatment groups were gavaged daily for 12–15 days with 10^9 CFU of *C. avidum* P279, and control groups received 350 μL of sterile skim milk. ^{A} Enumerated in two rats from each group. LUB: lactate-utilizing bacteria. ND: not determined. BDL: below detection limit. $^\dagger p < 0.05$: comparison between fecal samples at baseline and after intervention within the same IMA rat group. $^* p < 0.05$: comparison between fecal samples from control and treatment IMA rat groups from the same donor at the same intervention period.

Discussion

Human microbiota-associated animal models are valuable tools to study the effect of the resident gut microbiota on host pathobiology, and to perform invasive procedures that are not possible in humans due to ethical constraints. In this study, we focused on the establishment of a rat model for studying gut microbiota in infants younger than 3 months and tested the modulation potential of *C. avidum* P279 supplementation on H_2 excretion in colic IMA rats. In general, microbiota diversity and taxonomic composition in IMA rats and donors were similar, while inter-individual differences in the microbiota composition could be reproduced.

Infant microbiota establishment in germ-free rats

We inoculated 3- to 4- week-old germ-free rats with fresh fecal microbiota collected from healthy and colic donors ensuring high viability at inoculation by storing fresh samples immediately after deposition under anaerobic conditions and at cooling temperatures. We chose to use feces from single donors and not pooled samples for inoculation, as there is a high inter-individual variability of the gut microbiota during the first year of life (8, 14, 28). The gut microbiota of the four donors participating in this study was representative of infant profiles dominated by *Bifidobacteriaceae* and *Clostridiaceae* as reported previously (14, 28, 42). The donors gut microbiota profiles were overall highly conserved in IMA rats. The fecal microbiota profiles of C1 rats identified by 16S rRNA gene amplicon sequencing were discordant with qPCR findings for *Bifidobacterium* and *Bacteroides*. The bias can be partly explained by the occurrence of species with different numbers of 16S rRNA gene copies within the targeted taxa, and differences in the efficiency of primers to amplify different members within a taxonomic group (43). The metabolic profiles identified in the ceca of rats corresponded with the metabolic capacity of the most abundant fecal colonizers identified. Preserving donor's individuality in models used to investigate gut microbiota modulation strategies is of high relevance, as the effect of such interventions might be dependent on the individual microbiome characteristics as it has been previously shown in *in vitro* fermentation models (25). This is the first report of IMA rat model. The model will be helpful

for studying the gut microbiome of young infants with a personalized approach.

Diet and host physiology influence infant microbiota colonization in IMA rats

The individual microbiota profiles of donors, H1, H2, and C2, were preserved in IMA rats, while C1 rats showed decreased *Bifidobacteriaceae* and enriched *Bacteroides*. *Bifidobacteriaceae* and *Bacteroides* are common infant colonizers that are able to degrade HMOs (10, 44–46). Providing IMA rats with infant formula that lacked HMOs and contained FOS and GOS could be a contributing factor to the quantitative differences observed in the establishment of these taxa in C1 recipient rats. Furthermore, important differences in the morphology and physiology of the rat gastrointestinal system compared to infants, as well as individual and variable digestive and environmental conditions in infants, can also explain differences between infant donors and IMA rat fecal microbiota. A lower relative abundance of *Bifidobacterium* and higher colonization of *Bacteroides* were recently reported for IMA mice and piglets inoculated with fecal samples from a 5-month-old infant (47). The differential establishment of these two core infant taxa might be also explained by differences in the host physiology that could determine bacterial colonization ability.

Working with rats instead of mice has a clear advantage in our goal to model microbiota in young animals (3–4 weeks old). The small size of mice at that same age would not have allowed to handle and gavage the animals and obtain reliable measurements of fecal samples and respiratory activities. Moreover, rats develop slower than mice, which was an advantage for our study which involved testing a 2-week supplementation in a model of infant colic. Rats reach puberty at day 50 after birth (48), while mice reach it at 28 days (49).

We initially fed IMA rats inoculated with the microbiota of healthy donors with only infant formula, but severe bloating was observed for some animals after approximately 2 weeks, possibly due to a surplus of rapidly metabolized di- and oligosaccharides (lactose, FOS, and GOS) and a lack of fibers in the infant formula. The appearance of bloating was coincidental with previous reports of decreased lactase expression and activity in rats from day 21 of life (50). Supplementation with a standard chow diet (two to three pellets per cage per day) from the second week of experimentation improved this condition, probably due to a bulking effect of the dietary fibers included in the pellets as reported before, although some animals still showed little cecal content and bloating at euthanasia (51).

The high availability of lactose, FOS, and GOS might have supported LPB metabolism resulting in high lactate concentrations, which would explain the higher abundance of *Veillonella*, an H_2-producing LUB identified in H1, C1, and C2 rats compared to the donor levels. Strikingly, *Veillonella* remained low in rats inoculated with feces from H2 donor, suggesting limited enrichment when originally present at low abundance.

Metabolite profiles in infant microbiota-associated rats were reflective of dominant bacterial populations

Acetate and propionate or lactate are the main fermentation metabolites identified in feces of infants younger than 6 months of age (28, 52, 53). In agreement, acetate was the main microbial metabolite identified in the ceca of all rats, while propionate was also produced in the ceca of H1 and C1 rats, which could be linked to a high abundance of propionate-producing *Bacteroides* and *Veillonella* (54). Lactate was the other metabolite identified with acetate in the ceca of H2 rats in alignment with the dominant colonizer LPB *Bifidobacterium*, while butyrate detection in C2 rats might be due to the high presence of butyrate producer *C. neonatale* (64% rel. ab.) belonging to *Clostridium* cluster I, as identified in C2 donors and rats by 16S rRNA gene amplicon sequencing.

H_2 production in infant microbiota-associated rats was high and could be linked to H_2-producing species

The H_2 production varied greatly according to the IMA rat group. H_2 excretion of H2 rats was the lowest in agreement with the lowest abundance of lactate-utilizer H_2-producer *Veillonella*. The extremely high H_2 production in C2 rats might be due to *C. neonatale* which is able to ferment lactose to acetate, lactate, and butyrate while also producing H_2. *In vitro* gas production by *C. neonatale* infant isolates during milk fermentation has been reported (55).

Surprisingly, compared to previous experiments in germ-free rats inoculated with fecal microbiota from healthy adults and irritable bowel syndrome patients (12.3 $\pm$ 7.3 ppm and 33.6 $\pm$ 3.6 ppm excreted H_2, respectively) (2), H_2 released by IMA rats was up to 100-fold higher. High breath H_2 excretion values were reported for ca. 30% of non-colic and ca. 60% of colic infants due to physiologic lactose malabsorption followed by gut microbial metabolism (22–24, 56). The higher H_2 excretion detected for colic IMA rats compared to healthy IMA rats could result from a functional dysbiosis in relation to high production and accumulation of H_2, as has been proposed for colic infants previously (16, 22–24). More trials should be done with different donors, both healthy and colicky, to corroborate these findings.

Lactose- and lactate-utilizer non-H_2-producing *Cutibacterium avidum* P279 as candidate probiotic for infant colic treatment

Colic infants are diagnosed based on their crying time (more than 3 h per day, during at least 3 days in a week). Studies consider an intervention to be successful if crying time is reduced by at least 50% compared to the baseline values (20, 57, 58). The number of trials reporting the supplementation of probiotics in IC has steadily increased in recent years, but until now there is no clear evidence of beneficial effects of any strains/products on crying time reduction (20, 59, 60).

In this study, we evaluated infant gut colonizer *C. avidum* P279 as a candidate probiotic for inhibition of H_2 production for IC treatment, based on its capability to metabolize lactose and lactate to propionate and acetate without producing H_2, and on the hypothesis that imbalances in lactate and H_2 metabolism may be the cause of bloating and associated pain in colic infants (16). We observed a reduction and lower variability in H_2 excreted by colic IMA rats supplemented with *C. avidum* compared to control animals receiving the sterile milk carrier.

In an already propionigenic gut microbiota (C1), the SCFA profile in rat feces indicated a slightly lower formation of formate, but a significant reduction in excreted H_2 and *Veillonellaceae* abundance in colic C1 rats compared to the control animals. We have recently reported that the addition of *C. avidum* P279 to an *in vitro* colonic fermentation also led to a lower abundance of *Veillonella* and formate production (17).

On the other hand, in C2 IMA rats, *C. avidum* P279 supplementation shifted the SCFA profile from a butyrogenic to higher propionate concentrations with an observed high decrease in H_2 excretion, although not significant due to the high variability observed in the control rats. Interestingly, lower variability in H_2 excretion was also observed in treated compared to non-treated C2 rats. Supplementation with *C. avidum* P279 in C2 IMA rats decreased colonization of dominant *Clostridium* in treated rats after the intervention compared to baseline values. These observations might have been consequence of a possible competition for lactose between *C. avidum* P279 and H_2-producing *Cl. neonatale* (26, 55). *Cl. neonatale* has only recently been isolated and described as main colonizer in preterm infants (9, 55). *C. avidum* P279 also increased the abundance of taxa involved in lactate metabolism in C2 IMA rats, mainly LPB *Lactobacillaceae* and LUB *Veillonellaceae*. We previously reported the modulation in the lactate trophic chain by a *C. avidum* strain of infant origin, in an *in vitro* PolyFermS model mimicking infant proximal colon conditions and inoculated with fecal microbiota from a 2-month-old infant, with enhanced lactate consumption and increased LUB *Eubacterium limosum* (25).

Overall, our results indicate that *C. avidum* P279 supplementation can reduce H_2 excretion and donor-dependently modulate the gut microbiota composition and metabolic activity in colic IMA rats, with no adverse effects observed on the health status of IMA rats.

Conclusion

We showed for the first time that fecal microbiota composition and functional profiles of single infants younger than 3 months old could be transferred upon transplantation to young germ-free rats fed infant formula and supplemented with a conventional chow diet. Our results indicate that this gnotobiotic rat model is suitable for investigating the role of gut microbiota in the nutrition and health of young infants. By transferring fecal microbiota of milk-fed infants to germ-free rats, we first report an increased level of H_2 production by infant microbiota *in vivo*, compared to adult microbiota, and a very high H_2 excretion in colic IMA rats. Furthermore, a pronounced reduction in H_2 excretion was shown after supplementing *C. avidum* P279, lactose- and lactate-utilizing non-H_2-producing strain, supporting the potential of the strain to reduce abdominal H_2 production and IC-associated symptoms. Further experiments using this rat model with additional infant donors could help to identify the range of effects that the strain could have on different microbiota profiles. In order to confirm the potential efficacy of *C. avidum* P279 in colic alleviation, crying time reduction has to be evaluated in human clinical trials previous safety assessment of the strain.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Local Institutional Animal Care and Use Committee.

Author contributions

VRM, CD, CC, AB-D, and CL designed the experiments. VRM and CD performed the gnotobiotic experiments and

data analysis. VRM, CS, AB-D, and CL supported with result interpretation. VRM wrote the manuscript which was subsequently edited and revised by all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.902159/full#supplementary-material

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Sergio Perez-Burillo,
Public University of Navarre, Spain
Ankur Naqib,
Rush University, United States

*CORRESPONDENCE
Annalisa Terranegra
aterranegra@sidra.org

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

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Modulation of gut microbiota: The effects of a fruits and vegetables supplement

Arun Prasath Lakshmanan[1†], Alessandra Mingione[2†], Francesca Pivari[3†], Elena Dogliotti[4], Caterina Brasacchio[3], Selvasankar Murugesan[5], Daniele Cusi[6,7], Monica Lazzaroni[8], Laura Soldati[3‡] and Annalisa Terranegra[1*‡]

[1]Precision Nutrition, Research Department, Sidra Medicine, Doha, Qatar, [2]Department of Biomedical and Clinical Sciences L. Sacco, University of Milan, Milan, Italy, [3]Department of Health Sciences, University of Milan, Milan, Italy, [4]Fondazione Umberto Veronesi, Milan, Italy, [5]Microbiome and Host-Microbes Interactions Lab, Research Department, Sidra Medicine, Doha, Qatar, [6]Institute of Biomedical Technologies, Italian National Research Council, Milan, Italy, [7]Bio4Dreams Scientific Unit, Bio4Dreams-Business Nursery for Life Sciences, Bio4Dreams S.p.A., Milan, Italy, [8]Laboratory of Clinical Pathology, Foundation IRCCS Neurological Institute C. Besta, Milan, Italy

The consumption of an optimal amount of fruits and vegetables is known to improve physical fitness and physiological body functions. Healthy eating habits, including intake of fruits and vegetables, can modify gut microbiota. This study aimed to demonstrate the effectiveness of a formulated fruit and vegetable supplement (FVS) in modulating the antioxidant capacity and the gut microbiota composition. We enrolled 30 healthy volunteer subjects, matched for age, gender, BMI, and smoking habits, and randomized them into the FVS and the placebo (PLA) groups. Among the serum vitamins, the folic acid level was significantly higher (p = 0.001) in the FVS group than in the PLA group, whereas the vitamin B2 level was significantly higher in the PLA group than in the FVS group (p = 0.028). The antioxidant capacity, measured by using the oxygen radical absorbance capacity (ORAC) method, was also slightly higher in the FVS group than in the PLA group but did not reach statistical significance. The dietary intake, assessed by 24-h recalls, did not show any significant changes after the supplementation in both the groups. The gut microbiome composition, measured by 16S rDNA sequencing, showed no difference in both alpha and beta diversities, whereas the LEfse analysis revealed a microbial shift after the treatment, with a decreased abundance of the genus *Ruminococcus* from the Lachnospiraceae family (p = 0.009), and the unclassified genus from the family Erysipelotrichaceae (UC36, p = 0.003) in the FVS group compared with the PLA group (confirmed by SIAMCAT analysis, AUC = 74.1%). With a minor effect, the genus *Faecalibacterium* and unclassified genus and family from the order Lactobacillales (UC31) were also increased in the FVS group compared with the PLA group (p = 0.0474, p = 0.0352, respectively). SCFA measurement by gas chromatography–mass spectrometry showed an increased level of 2-methylbutyrate in the FVS group compared with the PLA group (p = 0.0385). Finally, the Spearman correlation analysis showed

that in the FVS group, the genus *Faecalibacterium* positively correlated with 2-methyl butyrate (p = 0.040). In the PLA group, none of the significant bacteria correlated with either SCFA or serum biomarkers. The network analysis confirmed the positive correlation between genus *Faecalibacterium* and 2-methyl butyrate. We can conclude that the FVS in healthy individuals modified the gut microbiota composition and metabolites, and it can potentially contribute to reduce the pro-inflammatory response along with the antioxidant capacity.

KEYWORDS

diet supplements, gut microbiota, antioxidant capacity, fruits and vegetables, SCFA

Introduction

The reports from clinical and preclinical studies suggest that consumption of an optimal and routine intake of fruits and vegetables, which are rich in vitamins, minerals, fibers, and active molecules, is beneficial for the overall physical fitness, immune functions, and normal physiological functions of the body (1–4). Fruits and vegetables contain a high level of antioxidants, which counteract the action of free radicals. Unfortunately, the continuous exposure to free radicals, present in many harmful substances, can cause cell damage, favoring the onset of various diseases. A positive correlation between the continuous intake of fruits and vegetables and the reduced risk of vascular diseases and cancers has been established (5–7). Fruit and vegetable nutrients, such as fiber and polyphenols, are known to affect the gut microbial composition (4, 8). Gut microbiome realizes mutualistic relations with the gut, influencing the maturation of the immune system, modulating the responses to epithelial cell injury, affecting energy balance, and protecting against physiologic stress (9). According to the reports from world-renowned national and international monitoring agencies that deal with diet and nutrition, consumption of an adequate amount of fruits and vegetables could change the world map of major chronic diseases (cardiovascular disease, stroke, cancer, osteoporosis, diabetes, metabolic diseases, etc.). Nowadays, the majority of the world population is exposed to Western diets, characterized by an over-intake of saturated and omega-6 fatty acids and reduced intake of omega-3 fatty acids, fruits, vegetables, and fibers (10). A Western-like dietary pattern, together with a sedentary lifestyle, leads to several inflammatory-related disorders, such as metabolic syndrome, cardiovascular disease, and neurodegenerative diseases (11). Most of these disorders are also associated with alterations in microbiota composition in humans, especially those with reduced bacterial richness and diversity (12). In this context, the Mediterranean diet (MD) is recognized as one of the healthiest diets worldwide as it contains a high proportion of fiber, antioxidants, and polyphenols, present in vegetables, fruits, pulses, and extra-virgin olive oil, which are strongly associated with a reduced risk of developing non-communicable diseases related to Western diet and lifestyle (13).

It has been estimated that the consumption of 500–800 g/day of fruits and vegetables would avoid over 5.6 and 7.8 million premature deaths annually, a 30% reduction in the relative risk for coronary heart disease, a 28% reduction in stroke and cardiovascular diseases, and a 14% reduction in total cancer risk with 500–600 g/day of fruits and vegetables (14–16). Although antioxidant intake through whole foods, as well as low- to moderate-dose nutritional supplements, is generally considered to provide health-enhancing benefits, higher dose antioxidant supplements intake is somewhat controversial (17). A recent interventional study conducted by Ren et al. used a well-balanced combination of antioxidant nutrients, which provide an increased antioxidant defense (18). Thus, the proposed study aimed to demonstrate the effectiveness of formulated fruit and vegetable supplementation in modulating the gut microbiome composition and explore the potential mechanism that involves the gut microbiome compositional change in healthy subjects.

Materials and methods

Human subjects and the study protocol

Healthy adult volunteers (both male and female, N = 30) aged 18–65 years were recruited during the first phase of the study at San Paolo Hospital, Milan, Italy. The study is randomized, double-blinded, and placebo-controlled, and the volunteers were randomly assigned to the treatment or placebo group, with 15 healthy subjects in each group. The

TABLE 1 Demographic data of the study participants.

	FVS	PLA
Females (n)	7	7
Males (n)	8	7
BMI (kg/m^2)	22.60 ± 2.80	25.09 ± 5.04
Age (years)	42.79 ± 15.36	43.3 ± 14.49
Smokers	2	3

randomization was conducted through open-source software.[1] The flow diagram of the present study is given in the **Supplementary material** (**Supplementary Figure 1**). The subjects who were given the fruit and vegetable supplement are labeled the FVS group, and the subjects who were given placebo are labeled the PLA group. The study duration was 6 weeks, the lifestyle questionnaire was used, and the measurement of BMI and waist circumference was taken before and after the supplement/placebo treatment. The assessment of food intake was carried out by using the 24-h food recall method before and after the treatment. The blood and stool samples were collected from each participant before and after the supplement/placebo treatment. The study was approved by the Ethical Committee of Interaziendale Milan Area A, with the approval number 156/ST/2014.

Diet supplements

The diet supplement contains a mix of fruits and vegetables, packed in stick packs to provide an equal amount of supplements to each subject, distributed by L'Angelica Istituto Erboristico, and named fruit and vegetable supplement (FVS). The placebo supplement was also packed in stick packs without the content of fruits and vegetables and named PLA (**Supplementary Table 1**). Each subject of both FVS and PLA groups was given two stick packs per day for a total of 6 weeks. All the subjects were recruited in the same season to avoid the effect of different seasonal foods.

Oxygen radical absorbance capacity assay

The antioxidant capacity of the plasma samples was assessed by using an oxygen radical absorbance capacity (ORAC) Antioxidant Assay Kit (Zen Bio). Antioxidants present in the samples can inhibit the peroxyl radical-mediated oxidation of fluorescein formed by the breakdown of 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) at 37°C. The fluorescence signal is measured during 60 min by determining the Ex480 nm/Em520 nm ratio. The concentration of antioxidants in the plasma sample is proportional to the fluorescence intensity and is assessed by comparing the net area under the curve to that of a known antioxidant, Trolox. The ORAC of plasma samples, before and after FVS intake, was expressed as micromolar Trolox equivalents (μMTE).

Measurement of serum levels of vitamins A, E, B2, B6, and K, and folate

Blood samples were collected before and after the start of FVS treatment. The measurement of serum levels of vitamins A, E B2, and B6 was carried out using a high-performance liquid chromatography (HPLC) system using commercial reagent kits (Chromsystems Instruments & Chemicals GmbH, Munich, Germany) according to the manufacturer's guidelines. Vitamin K was determined using the ion-selective electrode. Serum folate was measured using the standard electrochemiluminescence assay (Roche Diagnostics, Basel, Switzerland).

Dietary data analysis

The expert nutritionist collected the dietary intake of each participant, at baseline and after 6 weeks of the FVS, during a structured interview using a 24-h dietary recall to estimate subjects' food consumption and the Scotti Bassani Photographic Atlas to better estimate food portions.[2] The Diet Monitoring Solution (DMS) web platform was used to collect participants' nutritional data, dietary micronutrients, and macronutrients (19).

Bacterial DNA extraction from stool samples

Stool samples were self-collected by each participant in Norgen Stool Nucleic Acid Collection and Transport Tube (Norgen Biotek Corp., Toronto, Canada) and were stored at a −80°C freezer until further use. Bacterial DNA extraction was performed using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were checked by using NanoDrop One (Thermo Fisher Scientific,Waltham, MA, United States).

16S rDNA sequencing

The 16S rDNA library preparation and sequencing were performed according to the manufacturer's instructions (MiSeq

1 http://www.jerrydallal.com/random/randomize.htm

2 https://www.scottibassani.it/atlante-fotografico-delle-porzioni-degli-alimenti/

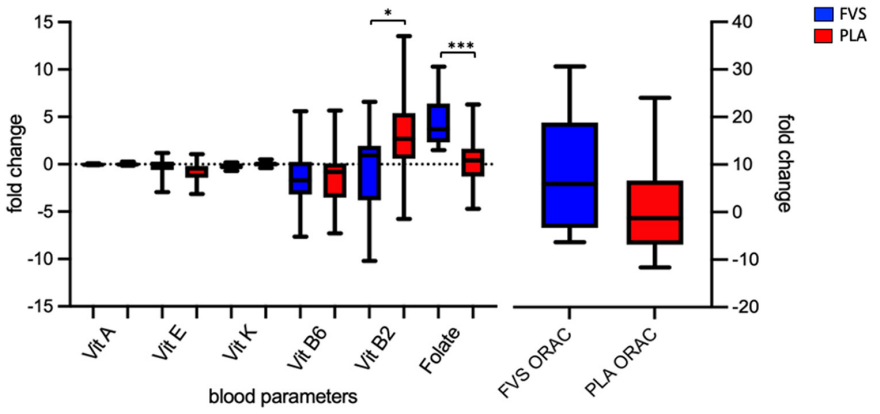

FIGURE 1
Serum biomarkers of the antioxidant capacity. Serum levels of vitamins A, E, K, B6, and B2 and folate, and antioxidant capacity in the FVS group (blue bars) and PLA group (red bars). The median and interquartile (IQR) ranges are showed in the box plots. FVS, $N = 15$, PLA, $N = 14$. $^{*}p < 0.05$ and $^{***}p < 0.001$ when compared with the PLA group using the unpaired t-test.

system, Illumina, San Diego, CA, United States), as described previously (20). In brief, the extracted genomic DNA was amplified using the primers that target the v3-v4 regions of the 16S rDNA gene. The amplified product was then cleaned up using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, United States), and the product was indexed using the Nextera XT primer (Illumina, San Diego, CA, United States). Again, the product was cleaned up using magnetic beads. The size and quantity of the prepared library were estimated using an Agilent High Sensitivity Kit and a Qubit dsDNA HS Assay Kit, respectively. The pooled library and Phix control were denatured using 0.2 N NaOH as per the manufacturer's protocol. Finally, the sample was sequenced using the MiSeq Reagent v3 (600 cycles) Kit (Illumina, San Diego, CA, United States) according to the manufacturer's instructions. Base calling was directly carried out on MiSeq. The raw data were demultiplexed using MiSeq Reporter on Illumina MiSeq. The PEAR tool was used to merge both forward and reverse end sequences for each sample (21), and the reads with a high-quality score of 30 and above were selected using the Trimmomatic tool (22). FASTQ files were converted into FASTA files using QIIME v1.9.0 (Quantitative Insights Into Microbial Ecology) pipeline (23). Operational taxonomic units (OTUs) were obtained by aligning the sequence against the Greengenes database (gg_13_08) with a confidence threshold of 97% (24).

Short-chain fatty acid analysis by liquid chromatography–tandem mass spectrometry

The analysis of short-chain fatty acid (SCFA) was performed by adaptation of the method published by Han et al. (25). In brief, the collected stool sample was homogenized using a spatula, weighed, and diluted with 50% aqueous acetonitrile (Fluka, Thermo Fisher Scientific, Waltham, MA, United States). A portion of the supernatant was taken for further analysis, along with mixed standard calibration solutions representing a range of concentrations for each fatty acid. All SCFAs from C2 to C6 along with any iso- and anteiso-methyl branched chain fatty acids were tested. The samples and standards were derivatized with 3-nitrophenylhydrazine (Sigma Aldrich, St. Louis, MO, United States) and then diluted by a factor of 10 with 10% aqueous acetonitrile. An internal standard (a mixture of SCFA derivatized as above with $^{13}C_6$-3-nitrophenylhydrazine (IsoSciences, PA, United States) was added. To test if the stool matrix influenced the recovery, controls were prepared by spiking isotopically labeled straight-chain SCFA derivatized with $^{13}C_6$-3-nitrophenylhydrazine to a mixture of stool sample supernatants from this study. These were analyzed along with the same mixture of isotopically labeled SCFA prepared in 50% aqueous acetonitrile, and a comparison was made. All samples were analyzed using a liquid chromatography–triple quadrupole mass spectrometer operated in negative ion scheduled MRM mode. A C18 column allowed the chromatographic separation of all derivatized SCFAs. The peak area for all chromatographic peaks was calculated and used for generating calibration curves and for calculating unknown concentrations of SCFA in stool.

Computational analysis of gut microbiome

Taxa summary

We used a paired-end read merger (PEAR, v0.9.8) tool to merge both forward and reverse end sequences for each

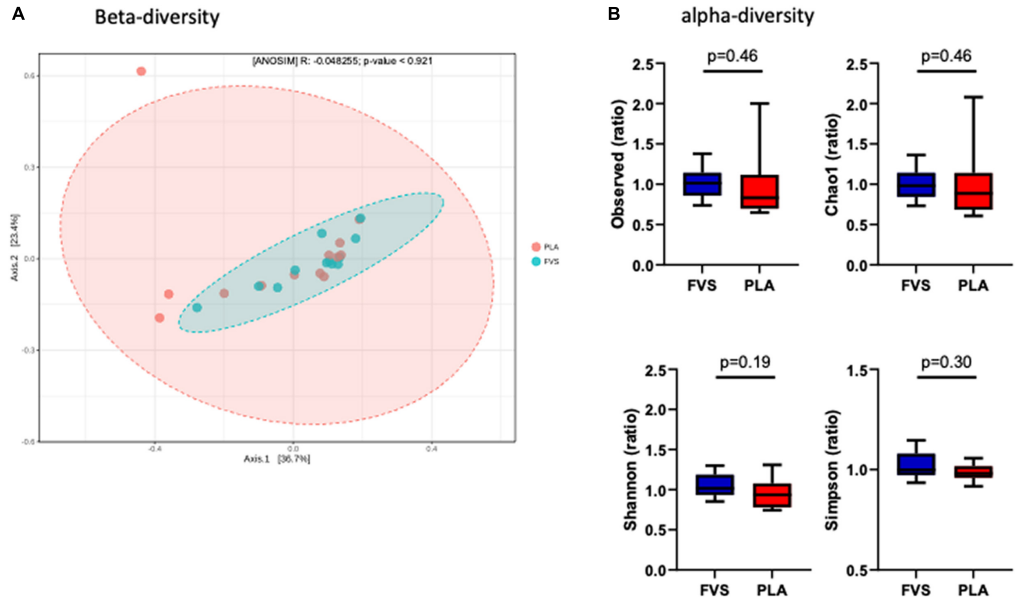

FIGURE 2
Gut microbial diversity in the FVS and PLA groups. **(A)** Beta diversity index was measured by using the Bray–Curtis method using principle coordinate analysis (PCoA) using the relative abundance of OTUs. The two variances explained by Axis.1 and Axis.2 are 36.7 and 23.4%, respectively. ANOSIM, analysis of similarity was not significant (p = 0.921). **(B)** Alpha diversity was measured by the four commonly used methods, such as observed, Chao1, Shannon, and Simpson methods. The box plots show interquartile (IQR) ranges with the median and whiskers. FVS, N = 15, PLA, N = 14. $p < 0.05$ considered statistically significant using Student's t-test.

sample (21), and the reads with a high-quality score of 30 and above (≥30) were selected using the Trimmomatic (v0.36) tool (22). FASTQ files were converted into FASTA files using Quantitative Insights Into Microbial Ecology (QIIME, v1.9.0) pipeline (23). Operational taxonomic units (OTUs) were obtained by aligning the sequence against the Greengenes database (gg_13_08) with a confidence threshold of 97% (24).

Diversity indices

Alpha diversity refers to the estimation of both richness and abundance of species in a habitat or specific area or sample. Species richness refers to the number of species present in a sample, and species abundance means the number of individuals per species. Alpha diversity was estimated using observed (species richness) and Chao1 (rare species richness), and Shannon and Simpson methods (species abundance) by using the R package (Phyloseq and ggplot2), as described previously (26). Beta diversity refers to the measurement of difference in the microbial composition between two or more groups of samples, and it was presented as a principal coordinate analysis as proposed in QIIME v1.9.0, as described previously (26).

Identification of gut microbial markers

Linear discriminant analysis effect size (LEfSe) was used to obtain the gut microbial markers for each group, and it uses the non-parametric factorial Kruskal–Wallis (KW) sum-rank test to identify features with significant differential abundance with regard to different groups, followed by linear discriminant analysis (LDA) to calculate the effect size of each differentially abundant microbial features. Features are significant if the LDA value is > 2.0 and the p-value is < 0.01 (27). Differentially abundant microbial taxa were identified also using the analysis of composition of microbiomes (ANCOM), as previously described (28).

Association analysis of microbial markers and host phenotypes

We also performed an association analysis between microbial markers and host phenotypes using a Statistical Inference of Associations between Microbial Communities and host phenoTypes (SIAMCAT) machine learning tool, followed by "lasso" regression analysis according to the previously reported method by Wirbel et al. (29).

Prediction of functional pathway

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis is a bioinformatics software package designed to predict the metagenome functional content from marker gene surveys and full genomes, and it was performed according to the literature review of Langille et al. (30).

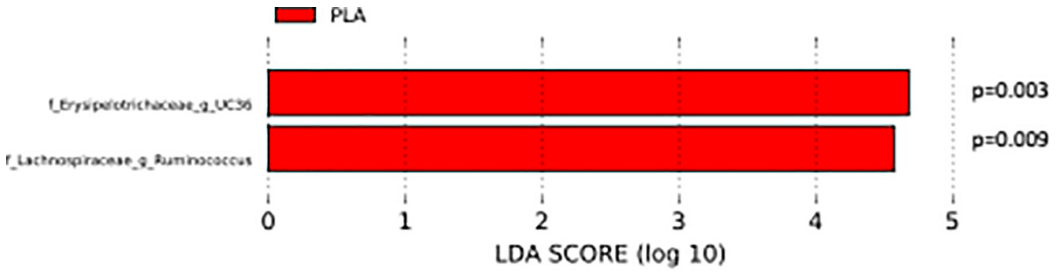

FIGURE 3
Gut microbial composition and microbial markers in the FVS and PLA groups. Gut microbial markers were measured by using the LEfSe analytical tool with a cutoff value of LDA > 2.0) in both the FVS and PLA groups. "g_UC" and "f_UC" represent unclassified bacteria at the genus level and family level, respectively.

Network analysis

We performed microbial, diet, and SCFA correlation network analysis using a MetagenoNets webtool, as described previously by Nagpal et al. (31).

Statistical analysis

Comparisons between the groups were performed by parametric (unpaired *t*-test) or non-parametric analyses (Mann–Whitney test) according to data distribution. Correlation analysis of microbial data with diet data and antioxidant blood parameters was performed using Spearman's correlation method. All analyses were run on Prism Software version 9 (GraphPad, CA, United States). A value of $p < 0.05$ was considered statistically significant.

Results

Supplementation effect on serum vitamins, antioxidant capacity, and dietary intake levels

We enrolled 30 healthy volunteers and randomized them into two groups (15 in the FVS group and 14 in the PLA group, with the dropout of 1 subject). The two groups did not differ in gender, age, BMI, and the smoking habits (**Table 1**). We measured the serum levels of vitamins A, B2, B6, E, and K, and folic acid in blood of the PLA and FVS groups, and we computed the changes in the levels after the supplementation for each subject. The level of folic acid was significantly higher in the FVS group than in the PLA group (unpaired *t*-test, $p = 0.001$). Interestingly, the level of serum vitamin B2 was significantly higher in the PLA group than in the FVS group (unpaired *t*-test, $p = 0.028$). There was no significant difference in the level of other vitamins. The level of ORAC was slightly higher in the FVS group but did not reach a statistical significance (**Figure 1**). We assessed the dietary intake of micronutrients and macronutrients in order to determine changes in the food habits, and the study participants in both the groups did not show any significant change in their dietary intake during the treatment (**Supplementary Figure 2**).

Modifications in the gut microbial composition after supplementation

To determine the effect of the FVS on gut microbial composition, we performed 16S rDNA sequencing, and the results showed that there was no significant shift in the microbial diversity, which was evident from alpha and beta diversity estimation. Bray–Curtis-based principal coordinate analysis (PCoA) was performed to visually explore the similarity and variations between the microbial composition of the samples, and it showed that there was no visual separation between the PLA and FVS groups (**Figure 2A**). Alpha diversity analysis calculated using observed, Chao1, Shannon, and Simpson methods showed that there was no significant difference between the FVS and PLA groups after treatment (**Figure 2B** and **Supplementary Figure 3**).

The analysis of the taxonomic composition revealed a shift of the microbial composition after the treatment. Applying the LefSe linear discriminant analysis, we found that the genus L-*Ruminococcus* ($p = 0.009$) and the unclassified genus from *Erysipelotrichaceae* family ($p = 0.003$) are enriched in the PLA group in comparison to the FVS group (**Figure 3**). The analysis of the composition of microbiomes (ANCOM) revealed that mostly L-*Ruminococcus* is the differential abundance bacteria between the FVS and PLA groups (**Supplementary Figure 4**). Also, the association analysis by using the SIAMCAT machine learning tool confirmed that L-*Ruminococcus* and unclassified bacteria from *Erysipelotrichaceae_UC36* family are significantly associated with the PLA group, with the robustness values of 97 and 98%, respectively. Furthermore, the mean overall prediction area under the curve (AUC) and mean

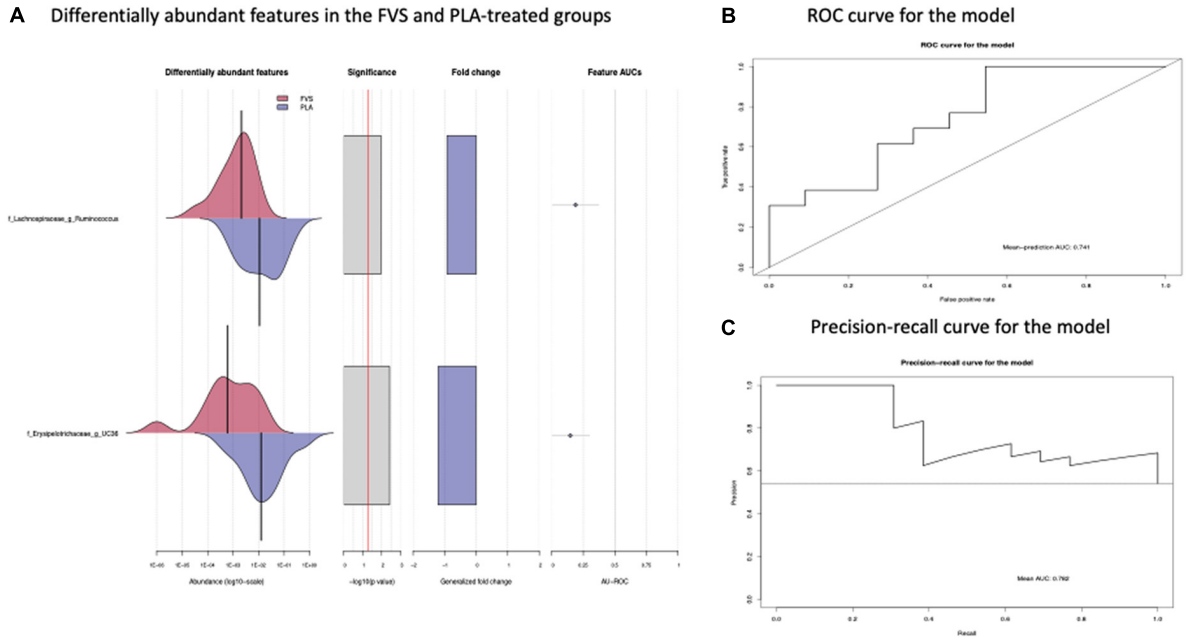

FIGURE 4
Validation of gut microbial markers using a machine learning tool—SIAMCAT analysis. **(A)** Gut microbial markers from LEfSe analysis were further validated using the SIAMCAT tool, which showed L-*Ruminococcus* and *Erysipelotrichaceae* family bacteria are significantly enriched in the PLA-treated group. **(B)** ROC curve for model, which displayed the cross-validation error as a receiver operating characteristic (ROC) curve with the 95% confidence interval. The area under the ROC (AUROC = 0.741) is given below the curve. The *x*-axis and *y*-axis represent false-positive and true-positive rates, respectively, for the tested markers, and **(C)** precision–recall curve for the model, which displays the mean AUC value of 0.782. An AUROC value of more than 0.7 is considered fairly good in terms of the discriminative ability of the test.

AUC value are 74.1% and 78.2%, respectively (Figure 4). Comparison of the relative abundance at the phyla level and the top 30 genera from the two groups did not show statistical difference, except for the genus *Ruminococcus* and the *Erysipelotrichaceae_UC36*. Furthermore, the comparison at the genus level by using Mann–Whitney test showed a slightly significant increase in the relative abundance of the genus *Faecalibacterium* and unclassified genus and family from the order of *Lactobacillales* (UC31) in the FVS group compared with the PLA group (p = 0.0474 and p = 0.0352, respectively); whereas a significant increase in the relative abundance of genus L-*Ruminococcus*, *Lachnobacterium*, and unclassified genus from the family *Erysipelotrichaceae* (UC36) were found in the PLA group compared with the FVS group (p = 0.0003, p = 0.0010, and p = 0.0059, respectively) (Supplementary Figures 5, 6).

Correlation network analysis of the gut microbial communities in the fruit and vegetable supplement and placebo groups

The network-based approach is widely used in microbiome analysis as it provides greater information about the co-occurrence nature and interaction of the microbial communities (32). Here, we observed that the genus L-*Ruminococcus* positively correlated with the genus *Lachnobacterium* (r = 0.454) and unclassified genus from the Erysipelotrichaceae family (r = 0.553) (Figure 5), indicating that the genus L-*Ruminococcus* co-existed with the other two bacteria, and the FVS that modifies the abundance of L-*Ruminococcus* could positively affect the abundance of other two bacteria.

Changes in the bacterial metabolic pathways and metabolites after supplementation

The metabolic pathways predicted by PICRUSt revealed that the FVS group has a higher level of fructose and mannose metabolism and pentose phosphate pathways than the PLA group (Figure 6A). The measurement of various SCFAs clearly indicated that only the 2-methyl butyrate level was significantly (unpaired t-test, p = 0.0385) higher in the FVS group than in the PLA group (Figure 6B). The total amount of SCFA was higher in the FVS group but did not reach a statistical difference since the rest of the SCFAs, from acetate to hexanoate, were not significantly altered between the two groups (Figure 6C).

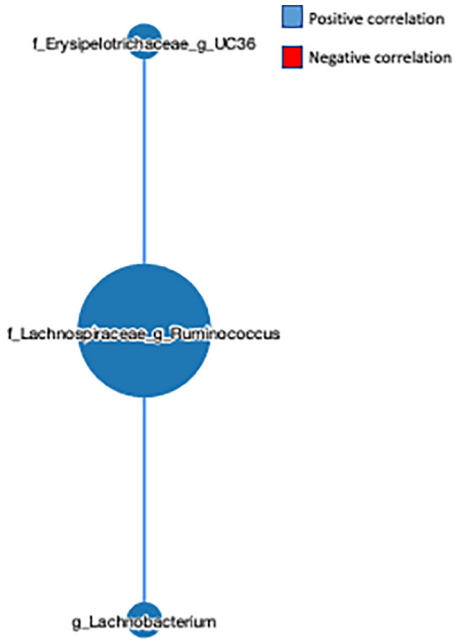

FIGURE 5
Microbial network analysis performed using a MetagenoNets webtool. The analysis revealed that L-*Ruminococcus* positively regulates the unclassified bacteria from the Erysipelotrichaceae family and the genus *Lachnobacterium*. Blue color indicates a positive correlation, whereas red color indicates a negative correlation.

Correlation analysis between the microbiome, SFCA, and serum biomarkers

To explore the effect of microbiota on SCFA and serum biomarkers, we performed a Spearman correlation analysis including the significant bacteria for each group, all the SCFAs, and the serum levels of vitamins and ORAC. In the FVS group, the genus *Faecalibacterium* positively correlated with 2-methyl butyrate (p = 0.040) and negatively correlated with the serum level of vitamin B6 (p = 0.022). 2-Methyl butyrate positively correlated with isobutyrate and isopentanoate (p = 0.006 and $p = 4.175e^{-009}$, respectively), and pentanoate with hexanoate (p = 0.005). Finally, vitamin B2 negatively correlated with acetate and hexanoate (p = 0.038, p = 0.011, respectively) (Figure 7A). In the PLA group, none of the significant bacteria correlated with either SCFA or serum biomarkers. Among the SCFAs, propionate positively correlated with butyrate and pentanoate (p = 0.003 and p = 0.027, respectively), isobutyrate with 2-methyl butyrate and isopentanoate ($p = 5.858e^{-007}$ and $p = 6.430e^{-007}$, respectively), isopentanoate with the 2-methyl butyrate ($p = 8.351e^{-009}$). Finally, vitamin K negatively correlated with vitamin E (p = 0.007), and vitamin B6 positively correlated with ORAC (p = 0.006). Intriguingly, the correlation between vitamin B2 and acetate is inverse in the FVS and PLA groups; a positive correlation was found in the PLA group (p = 0.009) and a negative correlation in the FVS group (p = 0.038) (Figure 7B). The network-based analysis of the interaction microbiota, diet, and SCFA confirmed the positive correlation of 2-methyl butyrate with the genus *Faecalibacterium* (Figure 8).

Discussion

The diet is well-known to modulate the gut microbiota composition and metabolism (33). In this study, we tested the antioxidant capacity, gut microbiota composition, and metabolic pathways in a group of healthy adult volunteers supplemented with a fruit and vegetable supplement for 6 weeks compared with a matched control group supplemented with a placebo. Typically, in the microbiome context, their health condition can be estimated by the gut microbial compositional homeostasis as the gut microbiome is proven to play a substantial role in the vicious cycle of diet-controlled pathophysiology, where the gut microbiome regulates physiology, and it is impacted by pathophysiology. Thus, any changes in the gut microbial composition clearly reflect the health or disease nature of humans. The measurement of gut microbial richness and abundance could be a vital indicator of human health. The literature review is strongly in favor of the diet as one of the factors that affect the gut microbial composition in terms of bacterial richness and abundance (34–37).

Interestingly, in this study, we observed that formulated fruit and vegetable supplementation contributes to modulate the gut microbial composition, compared with the placebo. The FVS showed a reduction in the relative abundance of genera from the Lachnospiraceae family, such as *Ruminococcus* and *Lachnobacterium*, and unclassified bacteria from the Erysipelotrichaceae family. Also, to a lesser extent, the genera *Faecalibacterium* and unclassified bacteria from the Lactobacillales order were affected (increased). A multiethnic study conducted by Frankenfeld et al. reported that the consumption of fruits and vegetables caused a gut microbial shift including the genera from Lachnospiraceae and Ruminococcaceae families (38). Results from different studies are controversial. The Medika study conducted by De Ioria et al. reported that the consumption of animal proteins showed a marked reduction in the relative abundance of *Lachnospiraceae* spp., and whether the intake of vegetable proteins, fiber, and potassium impacted the relative abundance of *Lachnospiraceae* spp. positively in the chronic kidney disease subjects (39). On the other hand, De Fillippis et al. demonstrated that the genus *Ruminococcus* from the Lachnospiraceae family (L-*Ruminococcus*) was positively affected by the animal-based diet and negatively by the vegetables–based diet (40). Thus,

a complete vegetable and fruit-based diet or a formulated supplementation, like in our study, predominantly target the genera from the Lachnospiraceae family, especially *Ruminococcus* and *Lachnobacterium*.

The genus *Ruminococcus* is assigned to both Lachnospiraceae and Ruminococcaceae families, it is greatly affected by the dietary components (41), and it can play multiple biological and pathological roles, which are still not fully understood. For example, Zheng et al. conducted an interesting study in which they estimated the dietary inflammatory potential *via* the literature-derived index (DII) with the gut microbiota composition, and they reported that the species *R. torques* is associated with the pro-inflammatory diet group (42). Also, Smith-Brown et al. demonstrated that fruit intake, especially apple and pears, drastically reduced the relative abundance of *R. gnavus* (43). In addition, in one of our recent studies, we found that *Ruminococcus callidus* and other unclassified bacteria (Ruminococcaceae family) could protect from cardiovascular diseases in obese Qatari subjects, whereas species from the genus of L-*Ruminococcus* such as *Ruminococcus gnavus* and *Ruminococcus torques* did not involve in the protection from CVD (44). Furthermore, a study conducted by Singh et al. reported that vitamin D supplementation in the healthy Qatari subjects showed a greater increase in the abundance of *Ruminococcus bromii* (45). The different *Ruminococcus* species can probably explain the different behavior. Unfortunately, we were not able to discriminate the species in our sample. Therefore, further studies are needed to clarify the role of the *Ruminococcus* spp. in health and diseases.

In addition to this, we identified a decrease in an unclassified bacterium from the *Erysipelotrichaceae* family due to the FVS. The rationale behind this decrease might be the folic acid consumption as the blood folate level was significantly higher in the FVS-supplemented group than in the PLA group. In support of this notion, Cheng et al. demonstrated a high-methionine low-folate (HMLF) diet considerably increased the relative abundance of bacteria from the *Erysipelotrichaceae* family in diet-induced HHcy mice (46). In addition, folate has been demonstrated to alter the inflammatory responses *via* DNA methylation and DNA synthesis (47), and Moein et al. demonstrated that the serum folate level is associated with inflammatory markers and disease clinical activity, and its optimal presence would help reduce the inflammatory response in IBD patients (48). Of note, many studies reported that *Erysipelotrichaceae* family bacteria are abundant in colorectal and colon cancer, but the results are not consistent (49). Strikingly, *Erysipelotrichaceae* has been demonstrated to mediate the TNF-alpha-mediated Crohn's disease (50). Moreover, the co-network analysis revealed that both L-*Ruminococcus* and unclassified bacteria from the *Erysipelotrichaceae* family positively regulate each other, with L-*Ruminococcus* having a dominant regulatory role in the *Erysipelotrichaceae* family bacteria. Thus, there is a strong possibility that the FVS could provide a long-term health benefit by managing the genus L-*Ruminococcus* abundance level.

The pro-inflammatory response is closely connected to oxidative stress as these two entities promote response of each other (51). Thus, in this study, the PLA-supplemented subjects were expected to have a low antioxidant capacity. It is a well-accepted fact that the consumption of fruits and vegetables increases the antioxidant capacity (52), and in this study, the antioxidant capacity measured by ORAC assay was higher (but not statistically significant) in the FVS-supplemented subjects than in the PLA group. Regarding the direct role of the gut microbiota in the antioxidant function, some *Lactobacillus* strains are known to exert beneficial effects due to their antioxidant activities. Probiotics, containing *Lactobacillus* strains, can produce various metabolites with antioxidant activity, such as glutathione (GSH), butyrate, and folate (53). Different mechanisms of antioxidant activity have been identified in the *Lactobacillus* strains: production of metabolites and antioxidant enzymes for reactive oxygen species (ROS) scavenging, upregulation of host antioxidant enzyme activities, downregulation of enzyme activities related to the production of ROS in the host, and regulation of pathways signaling related to host antioxidants and host intestinal flora (54–56). The ORAC assay is one of the best methods used to assess the antioxidant activities of different *Lactobacillus* strains (54). In our study, we observed an increase in plasmatic levels of folate, an increase of an unclassified genus from the order Lactobacillales and, consequently, we observed an increasing trend in the plasma antioxidant capacity of the subjects supplemented with the FVS.

The microbial metabolites, such as SCFAs, are demonstrated to be a key factor in the modulation of the inflammatory pathway, binding to specific receptors that regulate the immune function pathways (57–59). Among the measured SCFAs, we observed a nominal increase in the total SCFA levels, and a significant increase in 2-methylbutyrate positively correlated with the relative abundance of *Faecalibacterium*. 2-Methyl butyrate is a branched chain fatty acid derived from the intestinal metabolism of isoleucine, an essential branched amino acid (BCAA) (60). Gut microbiota is able to both produce and metabolize BCAA, and its levels are associated with insulin resistance (61), osteoporosis (62), and aging (63) *via* mechanisms involving the microbiota. A randomized controlled trial confirmed that the probiotic administration improves the serum amino acid concentration, including BCAA, in the subjects eating plant proteins (64). The microbiota shift that we observed in the FVS group can explain the increase in the 2-methylbutyrate level by enhancing the BCAA metabolism. Moreover, in our study, we observed an increase in the 2-methyl butyrate level and ORAC (increasing trend) in the subjects supplemented with the FVS compared with the placebo. We can speculate on a potential underlying mechanism that involves

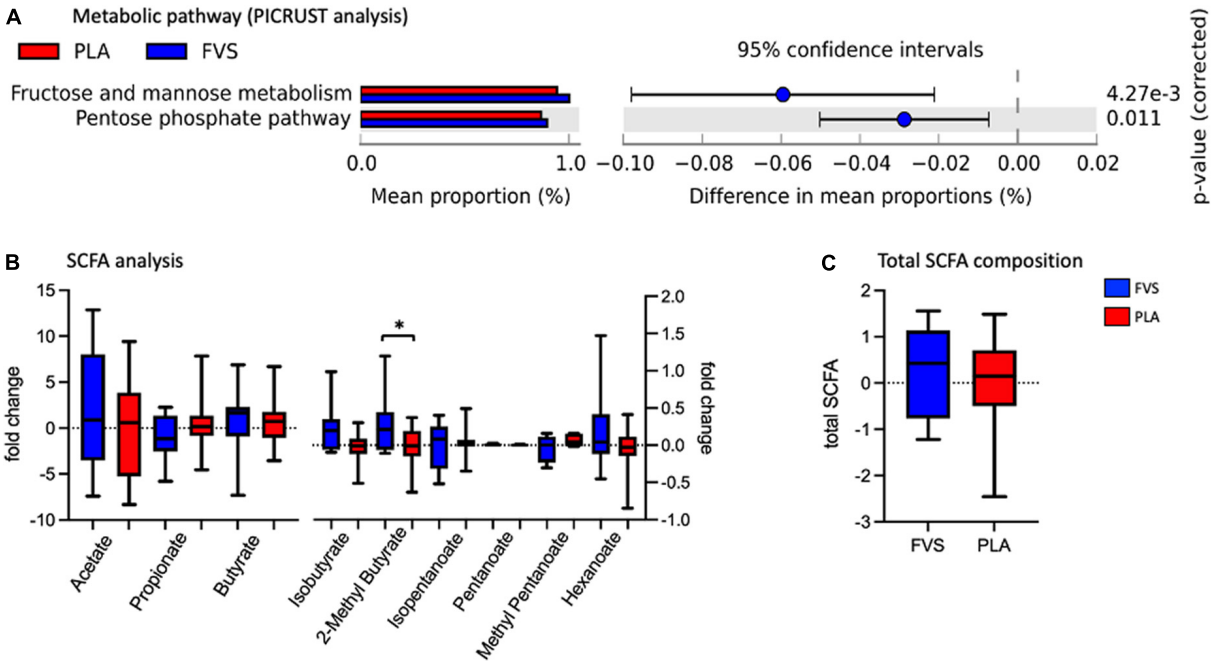

FIGURE 6
Predicted functional pathways and metabolite composition in the FVS and PLA groups. **(A)** Predicted functional pathways are obtained using the PICRUSt tool based on the bacterial abundance measured as mean proportion in the FVS and PLA groups. **(B,C)** SCFA composition was measured using liquid chromatography–tandem mass spectrometry. Single SCFAs are shown in panel b, and the total of the SCFA in panel c. The median and interquartile (IQR) ranges are showed in box plots. FVS, $N = 15$, PLA, $N = 14$. $^*p < 0.05$ when compared with the PLA group using Student's t-test.

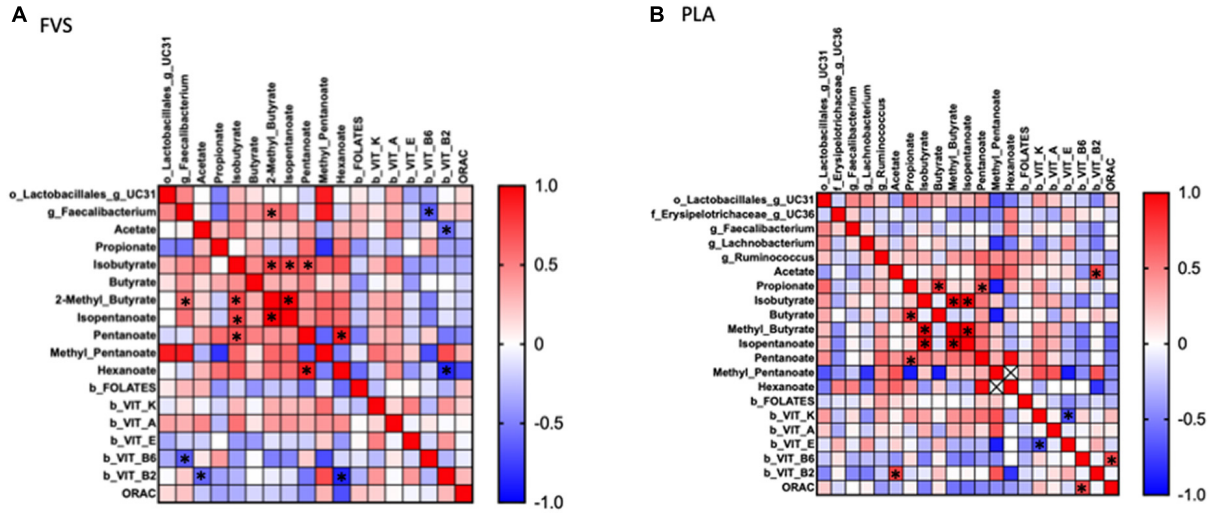

FIGURE 7
Correlation analysis between gut microbiome, SCFA, and serum biomarkers in the FVS and PLA groups. Spearman correlation was performed to analyze the interaction between significant microbial genus, SCFA, and serum biomarkers in both the FVS group **(A)** and PLA group **(B)**. Red to blue color scale indicates a positive to a negative correlation, respectively. FVS, $N = 15$, PLA, $N = 14$. $^*p < 0.05$ when compared with the PLA group.

the gut microbiota and SCFA in regulating the antioxidant capacity activated by fruit and vegetable supplementation. Our hypothesis is supported by an observational study that explored the potential anti-inflammatory capacity of the SCFA in patients under hemodialysis, and it showed a negative association of 2-methyl butyrate with bone morphogenetic protein 6 (BMP-6), a

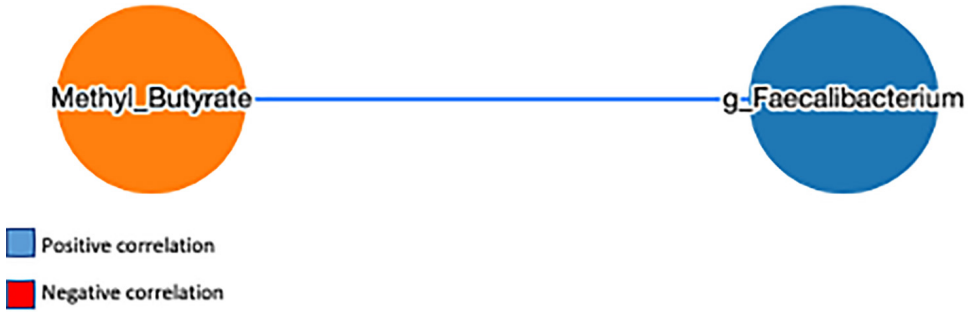

FIGURE 8
Network analysis performed using a MetagenoNets webtool. Analysis revealed that only 2-methyl butyrate positively correlated with the genus *Faecalibacterium* among all significant bacteria, diet, and SCFA. Blue color indicates a positive correlation, whereas red color indicates a negative correlation.

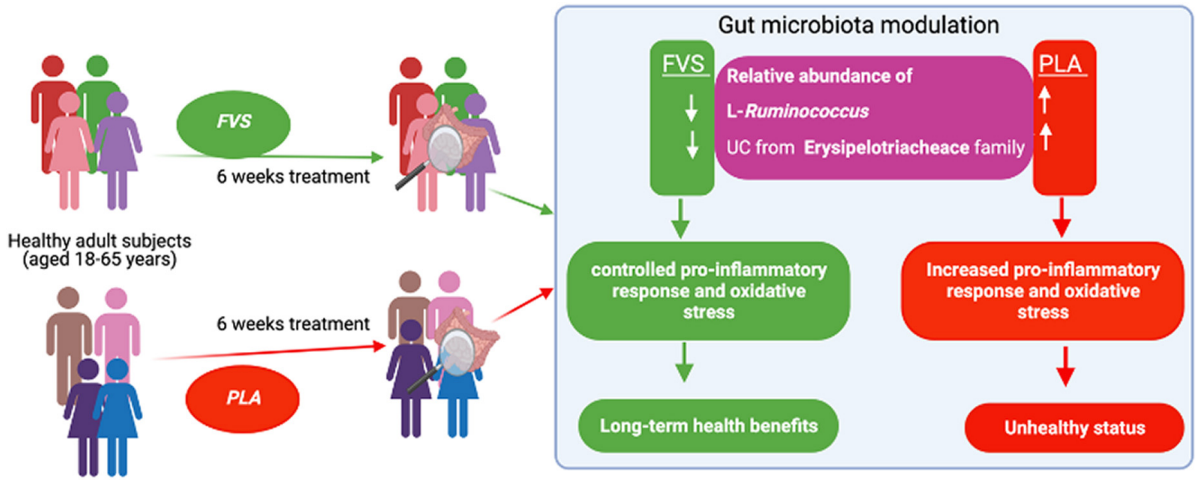

FIGURE 9
Schematic representation of the gut microbiota modulation by the FVS on healthy subjects.

biomarker of vascular calcification, and hypothesized the role of 2-methyl butyrate and other SCFAs in reducing cardiovascular risk in hemodialysis (65).

The pathway analysis showed an increase in the fructose and mannose metabolism and the pentose phosphate pathway in the subjects supplemented with the FVS compared with the placebo. Recent animal studies reported similar findings with increased fecal levels of mannose and decreased levels of fructose, among others, in rats supplemented with 1.5% of green tea polyphenols (66). The pentose phosphate pathway is also known to be affected by the microbial composition and prebiotics, as demonstrated in high-fat diet-fed mice treated with Huangjinya black tea, where a reduced lipid metabolism corresponded with many increased metabolic pathways, including the pentose phosphate pathway (67).

Our study has strengths and limitations. The strengths of this study are a randomized double-blinded study design, the supplement provided in stick packs to ensure an equal amount of nutrients to each subject, the complete set of microbial composition and metabolite analyses, the measurement of the ORAC, and finally, the exclusion of any diet effect. The main limitation of this study is the small sample size, which requires the confirmation of the findings from larger cohorts and the validation in inflammatory disease settings.

Conclusion

In conclusion, we propose that the FVS can provide long-term health benefits by controlling the relative abundance of bacteria such as L-*Ruminococcus* and unclassified bacteria from the Erysipelotrichaceae family, possibly through the reduction of pro-inflammatory response (**Figure 9**). Further studies are required to confirm these findings and explore potential molecular mechanisms.

Data availability statement

The data presented in this study are deposited in the NIH NCBI repository, accession number PRJNA833767.

Ethics statement

The study was approved by the Ethical Committee Interaziendale Milan Area A and the approval number is 156/ST/2014. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LS and DC designed the study. ED, FP, and CB were involved in the subject recruitment process, diet data, and sample collection process. AM and ML performed blood tests. AT performed the data analysis for the diet and blood tests. AL processed stool samples and performed the gut microbiome data analysis. SM contributed to the gut microbiome data analysis. AT, AL, ML, and FP wrote the manuscript. All authors reviewed the manuscript.

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Conflict of interest

The FVS and placebo products were provided by L'Angelica Istituto Erboristico, Italy, with the only purpose to perform the research study. L'Angelica Istituto Erboristico, Italy, did not interfere in the scope and the conduction of the research project. DC was employed by Bio4Dreams S.p.A.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.930883/full#supplementary-material

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Iman Zarei,
University of Eastern Finland, Finland
Kazuyuki Kasahara,
Nanyang Technological University, Singapore

*CORRESPONDENCE
Paul J. Arciero
parciero@skidmore.edu
Alex E. Mohr
aemohr@asu.edu

†This author share senior authorship

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Exploratory analysis of one versus two-day intermittent fasting protocols on the gut microbiome and plasma metabolome in adults with overweight/obesity

Alex E. Mohr[1]*, Paniz Jasbi[1,2], Devin A. Bowes[3], Blake Dirks[3], Corrie M. Whisner[1,3], Karen M. Arciero[4], Michelle Poe[4], Haiwei Gu[1,5], Eric Gumpricht[6], Karen L. Sweazea[1,7] and Paul J. Arciero[4]*†

[1]College of Health Solutions, Arizona State University, Phoenix, AZ, United States, [2]School of Molecular Sciences, Arizona State University, Tempe, AZ, United States, [3]Center for Health Through Microbiomes, The Biodesign Institute, Arizona State University, Tempe, AZ, United States, [4]Human Nutrition and Metabolism Laboratory, Department of Health and Human Physiological Sciences, Skidmore College, Saratoga Springs, NY, United States, [5]Center for Translational Science, Florida International University, Port St. Lucie, FL, United States, [6]Isagenix International, LLC, Gilbert, AZ, United States, [7]School of Life Sciences, Arizona State University, Tempe, AZ, United States

Nutritional interventions are a promising therapeutic option for addressing obesity and cardiometabolic dysfunction. One such option, intermittent fasting (IF), has emerged as a viable alternative to daily caloric restriction and may beneficially modulate body weight regulation and alter the gut microbiome (GM) and plasma metabolome. This secondary analysis of a larger, registered trial (ClinicalTrials.gov ID: NCT04327141) examined the effect of a four-week intervention comparing one vs. two-consecutive days of IF in combination with protein pacing (IF-P; 4-5 meals/day, >30% protein/day) on the GM, the plasma metabolome, and associated clinical outcomes in overweight and obese adults. Participants (n = 20) were randomly assigned to either a diet consisting of one fasting day (total of 36 h) and six low-calorie P days per week (IF1-P, n = 10) or two fasting days (60 h total) and five low-calorie P days per week (IF2-P, n = 10). The fecal microbiome, clinical outcomes, and plasma metabolome were analyzed at baseline (week 0) and after four weeks. There were no significant time or interaction effects for alpha diversity; however, baseline alpha diversity was negatively correlated with percent body fat change after the four-week intervention (p = 0.030). In addition, beta-diversity for both IF groups was altered significantly by time (p = 0.001), with no significant differences between groups. The IF1-P group had a significant increase in abundance of Ruminococcaceae *Incertae Sedis* and *Eubacterium fissicatena group* ($q \leq 0.007$), while the IF2-P group had a significant increase in abundance of Ruminococcaceae *Incertae Sedis*

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and a decrease in *Eubacterium ventriosum group* ($q \leq 0.005$). The plasma metabolite profile of IF2-P participants displayed significant increases in serine, trimethylamine oxide (TMAO), levulinic acid, 3-aminobutyric acid, citrate, isocitrate, and glucuronic acid ($q \leq 0.049$) compared to IF1-P. Fecal short-chain fatty acid concentrations did not differ significantly by time or between groups ($p \geq 0.126$). Interestingly, gastrointestinal symptoms were significantly reduced for the IF2-P group but not for the IF1-P group. Our results demonstrate that short-term IF modestly influenced the GM community structure and the plasma metabolome, suggesting these protocols could be viable for certain nutritional intervention strategies.

KEYWORDS

gut microbiome, intermittent fasting, metabolome, caloric restriction, obesity, weight loss, gastrointestinal symptoms, protein pacing

Introduction

Obesity continues to be highly prevalent in the United States. In 40% of the adult population, increased adiposity is tightly intertwined with cardiometabolic perturbation and is a primary comorbidity of major chronic disease (1). Non-invasive, nutrition-based approaches are the most widely utilized, feasible, and effective options to reduce body fat and support healthy lifestyle changes (2). To promote energy deficit, a daily caloric restriction of 10–40% is a common intervention in weight loss research and clinical practice (2, 3). However, such a dietary prescription may not be sustainable for most individuals in relation to long term adherence due to behavioral, psychosocial, and environmental factors (4). As a dietary regimen growing in scientific, clinical, and public interest, intermittent fasting (IF) is an alternative approach that holds promise for promoting healthy body weight and metabolic functioning (5). Intermittent fasting encompasses eating patterns in which individuals go extended periods (e.g., 16–60 h) with little or no energy intake and intervening periods of normal food intake (6). A common and well-tolerated IF regimen that has been implemented in long-term weight loss and weight maintenance interventions is modified fasting regimens, which allows the consumption of 20–25% of energy needs on scheduled fasting days. For example, normal feeding for five to six days and fasting for one to two days weekly (5).

In comparison to complete fasting, nutritionally supported fasts have been reported to improve glycemic control and reduce hunger ratings (7). Such nutritional regimens have offered a new frontier for research and pose a potential dietary framework for treating obesity and metabolic disease. Indeed, encouraging results have been reported for improved body composition, energy expenditure, and cardiometabolic markers (e.g., fasting plasma lipids, insulin, and glucose) (8–10). The lack of attention to the non-fasting days/period in previous investigations is a significant oversight and may impact the effectiveness of the IF. Previous research has consistently shown a higher protein diet (>30%), evenly distributed throughout the day (4–5 meals/day), moderate-carbohydrate (<40%) and low-glycemic index (<50), known as protein pacing (P) during feeding days, with and without caloric restriction, significantly enhances body composition, energy expenditure, and cardiometabolic health (8–10) and should be considered in the overall weight loss regimen.

Another emerging research area is the influence of nutrition on the composition and function of the microbes harbored in the gastrointestinal (GI) tract, known as the gut microbiome (GM). This research suggests significant shifts in microbial composition, function, and metabolic output in response to dietary changes (11, 12). Accordingly, GM dysfunction has been linked to obesity (13, 14). Diets that drive increased adiposity are generally high in energy and fat while low in fiber and diet quality. Obesogenic diets may promote luminal mucus degradation and pathogen encroachment (15), decrease community diversity and beneficial taxa (16), and malign GM function and metabolic output (17, 18). As a dense microbial bioreactor, gut microorganisms have tremendous functional capacity, producing an array of metabolites that have varying effects on host health (19). Analogously, the metabolome is defined as the complete suite of small molecules present in a biological system and is also modified by similar host-associated characteristics, including the GM (20). Therefore, any potential interactions between the GM and metabolome may be of significant interest in obesity and obesity-related conditions.

Recent, well-performed clinical trials implementing daily caloric restriction have focused on the effects of weight loss on the GM (21–23). However, evidence also demonstrates that IF significantly impacts the GM composition

and function (24). Much clinical research has employed time-restricted eating regimens in various populations, revealing significant dissimilarity compared to control but no differences in taxa abundance (25–28). More recent work utilizing IF has shown shifts in GM community metrics and gut-related metabolites compared to control in patients diagnosed with metabolic syndrome (29, 30). However, the control group participants were asked to maintain a routine diet without specific dietary instructions. Intermittent fasting research could greatly benefit by better establishing well-controlled comparison groups and carefully considering nutritional quality during feeding periods. Moreover, the evaluation of different fasting durations is sparse in the literature.

To the best of our knowledge, the direct comparison of different IF durations emphasizing dietary control and quality of nutrition consumed during both fasting and feeding days in a randomized study design assessing the GM has not yet been conducted. Therefore, as part of a larger clinical weight-loss trial, this exploratory analysis compared the effect of a four-week intervention of one-day (IF1-P) versus two-day (IF2-P) fasting with protein pacing on the GM and the plasma metabolome of overweight and obese adults. As a secondary aim, we examined self-reported GI symptomology between groups. We hypothesized that there would be a significant shift in GM community metrics and the plasma metabolome between IF1-P and IF2-P.

Materials and methods

Participant characteristics and study design

Participant samples and data used in the present analysis were procured from a larger, registered clinical trial (ClinicalTrials.gov ID: NCT04327141). Study design, clinical outcomes, and participant characteristics have been reported previously (31). Briefly, participants were healthy, non-smoking, sedentary/lightly active, males and females with overweight/obesity. Participants taking antibiotics, antifungals, or probiotics within the previous two months were excluded. This study was approved by the Institutional Review Boards of Skidmore College, NY, USA, and Arizona State University, AZ, USA, and all participants provided written informed consent before study enrollment. A total of 20 participants were enrolled and completed a one-week run-in period maintaining stable body weight and physical activity level. Following this period, participants were then randomly assigned to a modified fasting regimen consisting of one fasting day (total of 36 h) and six P feeding days per week (IF1-P, n = 10) or two fasting days (60 h total) and five P feeding days per week (IF2-P, n = 10) (**Figure 1**). This dietary regimen has been previously shown to be effective for weight loss and has high compliance rates (9, 10, 31). Detailed guidelines were provided to participants at weekly meetings with a registered dietitian nutritionist (RDN). On the fasting days, participants were supplied nutritional support providing approximately 400-500 kcals per day and comprised of various supplements and snacks, as previously described (31). On P feeding days in the IF1-P group, females consumed four meals daily, providing 1,350 kcals, and males consumed five meals daily, providing 1,700 kcals [for a full description of both fasting and feeding days, see (31)]. The nutritional profile was 35% carbohydrate, 35% protein, and 30% fat, consisting of two liquid meal replacements (Isagenix International, LLC, Gilbert, AZ, USA), one whole food dinner, and one or two snacks (female and male, respectively). To ensure an equivocal macronutrient profile and weekly energy intake as IF1-P (~8,500 kcals), IF2-P followed a similar dietary regimen consisting of four meals a day for females providing 1,500 kcals and five meals a day for males providing 1,850 kcals. The same nutritional profile of 35% protein, 35% carbohydrate, and 30% fat was used and consisted of two liquid meal replacements, one whole food dinner, and one or two snacks (female and male, respectively). Throughout the study, the RDN and investigators ensured adherence to the IF-P regimens via weekly meetings, detailed written instructions, and daily communication (e.g., email, text, and mobile phone). Moreover, two-day food diary analyses were conducted, as well as weekly inspection of dietary intake, distribution of weekly meal/supplement containers, and return of empty packets and containers.

Gastrointestinal symptom rating scale

Participants completed the 15-question gastrointestinal symptom rating scale (GSRS) (32) at baseline and the end of the four-week intervention. Briefly, each question is rated on a 7-point Likert scale (1 = absent; 2 = minor; 3 = mild; 4 = moderate; 5 = moderately severe; 6 = severe and 7 = very severe) and recalled from the previous week. Questions include symptoms related to upper abdominal pain, heartburn, regurgitation (acid reflux), empty feeling in the stomach, nausea, abdominal rumbling, bloating, belching, flatulence, and questions on defecation. The GSRS questionnaire provides explanations of each symptom, is understandable, and has reproducibility for measuring the presence of GI symptoms (33). In our analysis, a score of ≥2 (minor) was defined as symptom presence, and a score ≥4 (moderate) was defined as moderate symptom presence. Furthermore, to better categorize symptom location, bloating, flatulence, constipation, diarrhea, and defecation urgency were classified as lower GI symptoms, and nausea, heartburn, regurgitation, upper abdominal pain, empty feeling in the stomach, and belching were classified as upper GI

symptoms. Total scores were also generated for both overall symptom and moderate symptom presence.

Fecal sample collection and processing

Participants were instructed to provide stool samples at baseline and after week four of the intervention. The entire bowel movement was collected and transported within 24 h of defecation to the Skidmore College laboratory using a cooler and ice packs, and frozen at −80°C. Samples were then sent to ASU (Phoenix, AZ, USA) overnight on dry ice for analysis, where they were thawed at 4°C and processed. Wet weight was recorded to the nearest 0.01 g after subtracting the weight of fecal collection materials. Stool samples were then rated according to the Bristol Stool Scale (BSS) (34), homogenized in a stomacher bag, and the pH was measured (Symphony SB70P, VWR International, LLC., Radnor, PA, USA). DNA extraction was performed using the DNeasy PowerSoil Pro Kit (Cat. No. 47016, Qiagen, Germanton, MD, USA), per the manufacturer's instructions. DNA concentration and quality were quantified using the NanoDrop^{TM} OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific^{TM}, Waltham, MA, USA) according to manufacturer instructions. The OD_{260}/OD_{280} ratio of all samples was ≥1.80 (demonstrating DNA purity).

Fecal short-chain fatty acid analysis

High-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) was used to quantify short-chain fatty acid (SCFA) concentrations in fecal samples, as reported previously with slight modifications (35). Briefly, for each sample 300 mg fecal matter was added to 6 mL of 18Ω deionized water in a 15 mL falcon tube and vortexed at 3,200 rpm for 10 min at 24°C. This mixture was then centrifuged at 4,000 rpm for 10 min at 24°C and then filtered/sterilized through a 0.22 μm syringe filter. One mL of the resulting supernatant was then used as the analytical sample for the HPLC analysis. The organic acids were separated and identified using a Bio-Rad column (Aminex HPX-87H) with an Agilent diode array detector at 65°C, 5 mM H_2SO_4 as mobile phase, 210 nm measurement wavelength, and a 0.6 mL/min flow rate that was increased to 0.8 mL/min at 60 min.

Fecal microbiome analysis

The GM was assessed from the DNA extracted from the fecal collections at the Biodesign Institute at ASU (Tempe, AZ, USA). Amplification of the 16S rRNA gene sequence was completed in triplicate polymerase chain reactions (PCRs) using 96-well plates for GM composition. Barcoded universal forward 515F primers and 806R reverse primers containing Illumina adapter sequences, which target the highly conserved V4 region, were used to amplify microbial DNA (36, 37). These primers were selected as recommended by the Earth Microbiome Project (36, 37) and the National Institutes of Health Human Microbiome Project (38) to enhance reproducibility and comparability to other studies while obtaining broad coverage of Bacteria. PCR, amplicon cleaning, and quantification were performed as previously outlined (37). Equimolar ratios of amplicons from individual samples were pooled together before sequencing on the Illumina platform (Illumina MiSeq instrument, Illumina, Inc., San Diego, CA). Raw Illumina microbial data were cleaned by removing short and long sequences, sequences with primer mismatches, uncorrectable barcodes, and ambiguous bases using the Quantitative Insights into Microbial Ecology 2 (QIIME2) software, version 2021.8 (39).

16S rRNA gene sequencing produced 2,610,204 reads with a median of 50,609 per sample (range: 9,512 – 470,848). Paired-end, demultiplexed data were imported and analyzed using QIIME2 software. Upon examination of sequence quality plots, base pairs were trimmed at position 20 and truncated at position 240 and were run through DADA2 to remove low-quality regions and construct a feature table using amplicon sequence variants (ASVs.). All singleton reads were also removed from the dataset. Next, the ASV feature table was passed through the feature-classifier plugin (40), which was implemented using a naive Bayes machine-learning classifier, pre-trained to discern taxonomy mapped to the latest version of the rRNA database SILVA (138.1; 99% ASVs. from 515F/806R region of sequences) (41). A phylogenic tree was then constructed using the fragment-insertion plugin with the SILVA database. Based on the assessment of alpha rarefaction, a threshold of 6,407 sequences/sample was established and used to normalize samples for uneven sequencing depth for the subsequent diversity analyses (42). Alpha diversity was measured using Shannon (abundance and evenness of taxa present) and Faith's phylogenetic difference (PD) (incorporates phylogenetic difference between taxa) indexes. Beta diversity was measured using the Bray-Curtis dissimilarity index. Predicted functional potential of the overall bacterial community was surveyed via the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) algorithm (v2.4.2) (43). Pathway abundances were inferred based on structured pathway mappings of Enzyme Commission gene families to the MetaCyc database (44).

To provide an estimate of total bacterial biomass per sample (16S rRNA gene copies per gram of wet stool), DNA extracted from the fecal collections was assessed via quantitative polymerase chain reaction (qPCR) based on previously published methods (45, 46). Briefly, all 20 μL qPCR reactions contained 10 uL of 2X SYBR *Premix Ex Taq*^{TM} (Tli Rnase H Plus) (Takara Bio USA,

Inc., San Jose, CA, USA), 0.3 μM (0.6 μL) of each primer (926F: AAACTCAAAKGAATTGACGG; 1062R: CTCACRRCACGAGCTGAC), 2 μL DNA template (or PCR-grade water as negative control), and 6.8 μL nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA). PCR thermal cycling conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 15 s, 61.5°C for 15 s, and 72°C for 20 s, then hold at 72°C for 5 min, along with a melt curve of 95°C for 15 s, 60°C for 1 min, then 95°C for 1 s. Quantification was performed using a QuantStudio3 Real-Time PCR System by Applied Biosystems with QuantStudio Design and Analysis Software 1.2 from Thermo Fisher Scientific (Waltham, MA, USA). All samples were analyzed in technical replicates. For quality assurance and quality control, molecular negative template controls (NTC) consisting of PCR-grade water (Invitrogen, Waltham, MA, USA) along with positive controls created by linearized plasmids were run on every qPCR plate. Standard curves were run in triplicate and used for sample quantification, ranging from 10^7 to 10^1 copies/μL with a cycle threshold (CT) detection limit cutoff of 33. Reaction efficiency was approximately 101%, with a slope of -3.29 and $R^2 \geq 0.99$.

Targeted plasma metabolomic analysis

For the plasma metabolomic analysis, a 12-h fasted venous blood sample (∼20 mL) was collected into EDTA-coated vacutainer tubes and centrifuged (Hettich Rotina 46R5) for 15 min at 2,500 RPM at 4°C. After separation, 2 mL of plasma was aliquoted and stored at −80°C. Samples were then sent to the Arizona Metabolomics Laboratory at ASU (Phoenix, AZ, USA) overnight on dry ice for analysis, where they were thawed at 4°C and processed. Briefly, 50 μL of plasma from each sample was processed to precipitate proteins and extract metabolites by adding 500 μL MeOH and 50 μL internal standard solution (containing 1,810.5 μM $^{13}C_3$-lactate and 142 μM $^{13}C_5$-glutamic acid). The mixture was vortexed (10 s) and stored for 30 min at –20°C, then centrifuged at 14,000 RPM for 10 min at 4°C. Supernatants (450 μL) were extracted and transferred to new Eppendorf vials and dried (CentriVap Concentrator; Labconco, Fort Scott, KS, USA). Samples were then reconstituted in 150μL of 40% phosphate-buffered saline (PBS)/60% acetonitrile (ACN) and centrifuged again at 14,000 RPM at 4°C for 10 min. Supernatants (100 μL) were transferred to an LC autosampler vial for subsequent analysis. Internal quality-control (QC) was performed by creating a pooled sample from all plasma samples and injected once every ten experimental samples to monitor system performance.

The highly reproducible targeted LC-MS/MS method used in the current investigation was modeled after previous studies (47–49). The specific metabolites included in our targeted detection panel are representative of more than 35 biological pathways most essential to central carbon metabolism, and have been successfully leveraged for the sensitive and broad detection of effects related to diet (50), disease (51), drug treatment (52), environmental contamination (53), and lifestyle factors (54). Briefly, LC-MS/MS experiments were performed on an Agilent 1290 UPLC-6490 QQQ-MS system (Santa Clara, CA, USA). Each sample was injected twice for analysis, 10 μL using negative ionization mode and 4 μL using positive ionization mode. Chromatographic separations were performed in hydrophilic interaction chromatography (HILIC) mode on a Waters Xbridge BEH Amide column (150 × 2.1 mm, 2.5 μm particle size, Waters Corporation, Milford, MA, USA). The flow rate was 0.3 mL/min, the auto-sampler temperature was maintained at 4°C, and the column compartment was set at 40°C. The mobile phase system was composed of Solvents A (10 mM ammonium acetate, 10 mM ammonium hydroxide in 95% H_2O/5% ACN) and B (10 mM ammonium acetate, 10 mM ammonium hydroxide in 95% ACN/5% H2O). After the initial 1 min isocratic elution of 90% Solvent B, the percentage of Solvent B decreased to 40% at t = 11 min. The composition of Solvent B was maintained at 40% for 4 min (t = 15 min), and then the percentage of Solvent B gradually went back to 90% to prepare for the next injection.

The mass spectrometer was equipped with an electrospray ionization (ESI) source. Targeted data acquisition was performed in multiple-reaction-monitoring (MRM) mode. The LC-MS system was controlled by Agilent MassHunter Workstation software (Santa Clara, CA, USA), and extracted MRM peaks were integrated using Agilent MassHunter Quantitative Data Analysis software (Santa Clara, CA, USA).

Statistical analysis

Gastrointestinal (GI) symptom scores were on the low end of the GSRS scale and not normally distributed; therefore, non-parametric statistical tests were applied. Individual scores between groups were assessed using a Mann-Whitney U test. Symptom prevalence (number of scores ≥2) and moderate symptom prevalence (≥4) for total, upper, and lower GI GSRS clusters were analyzed using contingency tables. Specifically, differences between IF1-P and IF2-P GI symptoms at baseline were compared using a Fisher's Exact test, whereas pre-post values were compared with McNemar's test. Stool weight, BSS, fecal pH, and SCFAs were assessed for normality with Q-Q plots and Shapiro-Wilk tests and log-transformed where appropriate. These were then tested for time and interaction (group × time) effects using linear-mixed effect (LME) models, with each participant included as a random effect. All tests were performed with a significance level of $p < 0.05$. Statistical analyses were performed using SPSS 27.0 (SPSS Inc., Chicago, IL, USA).

For analysis and visualization of the microbiome data, artifacts generated in QIIME2 were imported into the R environment (v4.1.2) using the *phyloseq* package (v1.38.0)

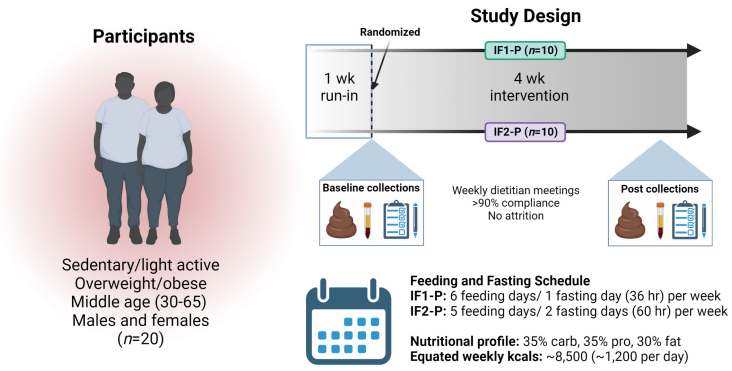

FIGURE 1
Overview of study design. After enrollment, a one-week run-in period, and randomization, 20 overweight and obese males and females followed an intermittent fasting, protein pacing-based weight loss diet consisting of one (IF1-P, n = 10) or two (IF2-P, n = 10) fasting days per week for four weeks equated for weekly energy and macronutrient intake. Clinical data and fecal and blood samples were collected at baseline and after week four. "Created with BioRender.com".

(55). Before conducting downstream analyses, sequences were filtered to remove all non-bacterial sequences, including archaea, mitochondria, and chloroplasts. For beta diversity, a permutational analysis of variance (PERMANOVA) was conducted on Bray-Curtis dissimilarities using the Adonis test in the *vegan* package (v2.6.2) with 999 permutations. The PERMANOVA model incorporated the factors of time, individual, and interaction (group × time). A permutation test for homogeneity in multivariate dispersion (PERMDISP) was conducted using the "betadisper" function in the *vegan* package to compare dispersion. To support the Adonis analysis, the first principal coordinate of a principal coordinate analysis on the Bray-Curtis dissimilarity matrix was calculated and rank-transformed. A LME model was constructed on these values, using the *nLME* package (v3.1.153), testing the interaction effect of group and time with individual as a random effect. Beta diversity first-distances were also compared between groups, as previously described (56), by calculating the within-subject distance for paired samples (baseline vs. week 4) and testing for group distances (Wilcoxon rank-sum test). After assessing normality (Shapiro-Wilk's tests), LME models were used to test the effect of time and the interaction of group and time with each participant included as a random effect on the alpha diversity metrics using the *nLME* package. Associations between baseline GM and adiposity were assessed with multiple regression models to explore potential differential diet responses using GM as a determinant. These associations were calculated using baseline alpha diversity metrics and change in percent body fat (post – pre values), accounting for age, sex, and baseline body mass index (BMI) covariates.

Estimates of bacterial biomass (qPCR data) were assessed for normality and entered into a LME model, as described above. With taxonomy, a ratio was calculated for two of the most predominant phyla, Firmicutes/Bacteroidota (FB ratio), log-transformed, and assessed via LME analysis. For differential abundance testing, analysis of compositions of microbiomes with bias correction (ANCOM-BC) was employed on GM taxa and PICRUSt2 output using the R package *ANCOMBC* (v1.4.0) (57). First, raw counts from the ASV table were filtered for any sequence not present in at least 30% of all samples, and a detection limit for the ANCOM-BC models was established at a value of 0.7 (tested at each phylogenic level). The same approach was followed on the predicted functional pathways from the PICRUST2 data. Based on the sample size, parallel-group design, and zero-inflation common to microbiome data, ANCOM-BC was implemented for each group separately, assessing the effect of time. Changes were calculated as the log2 fold change (log2FC) of abundance at week 4 versus baseline. Differentially abundance genera were also assessed for potential associations with adiposity by running Spearman rank correlation tests between change in centered log-ratio transformed taxa (post – pre abundance) and change in percent body fat (post – pre values). Where appropriate, false discovery rate (FDR) corrections were used to adjust for multiple hypothesis testing with a significance level of $q < 0.05$.

Univariate and multivariate analyses of plasma metabolites and metabolic ontology analysis were performed, and results were visualized using the MetaboAnalystR 5.0 (58). Human metabolomic data were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) human pathway library to analyze predicted states (59). The data were $\log_{10}$-transformed, and Pareto scaled to approximate normality prior to all analyses. A general linear model (GLM) was constructed with age, sex, time, and baseline BMI covariates to determine significantly affected metabolites by group intervention. Levene's test was performed to detect significant homogeneity. Spearman rank correlation tests were performed using change in percent body fat and change in metabolites (post – pre values). Corrections were performed as indicated. An FDR correction was adjusted

TABLE 1 Baseline characteristics of study participants.

Variable	IF1-P (n = 10)	IF2-P (n = 10)	Total (n = 20)
Age	47.3 ± 10.0	52.0 ± 8.6	49.7 ± 9.3
Sex,% (n)			
Male	30 (3)	30 (3)	30 (6)
Female	70 (7)	70 (7)	70 (14)
Race/ethnicity,% (n)			
White	90 (9)	90 (9)	90 (18)
Asian	10 (1)	10 (1)	10 (2)
Height (cm)	166.4 ± 12.7	172.8 ± 10.0	169.6 ± 11.6
Weight (kg)	86.9 ± 18.5	99.4 ± 25.6	93.2 ± 22.7
Body fat (%)	38.2 ± 7.4	42.0 ± 8.2	40.1 ± 7.8
Body mass index (kg/m^2)	31.3 ± 5.1	33.6 ± 9.7	32.4 ± 7.6
Waist circumference (cm)	98.0 ± 9.8	108.9 ± 17.8	103.4 ± 15.1
Physical Activity (kcals/day)	322 ± 274	289 ± 188	305.6 ± 229.9
Dietary intake			
Kcals	2452 ± 526	2483 ± 473	2467 ± 487
Carbohydrates (g)	256.9 ± 75.5	268.3 ± 82.4	262.7 ± 77.1
Sugar (g)	99.6 ± 54.6	106.9 ± 46.1	103.3 ± 49.4
Fiber (g)	20.1 ± 8.9	19.3 ± 8.0	19.6 ± 8.3
Protein (g)	93.7 ± 25.3	105.3 ± 33.4	99.5 ± 29.5
Fat (g)	103.9 ± 26.2	109.6 ± 30.6	106.8 ± 27.8
Sodium (mg)	3456.5 ± 1088.9	3196.9 ± 1392.8	3326.7 ± 1224.1

Reported as mean ± SD unless stated otherwise.

for multiple hypothesis testing with a significance level of $q < 0.05$.

Results

Participant characteristics

Baseline characteristics of the study participants of the IF1-P and IF2-P groups are displayed in Table 1. Age, sex, BMI, and physical activity level did not differ by group. The overall mean percentage of kilocalories from carbohydrate, protein and fat at baseline was 43.6 ± 12.8%, 16.5 ± 4.9%, and 39.9 ± 10.4%, respectively. Both carbohydrate and fat consumption were outside the acceptable macronutrient distribution range (AMDR) of 45–65% and 20–35%, respectively, whereas mean protein consumption was on the lower end of the AMDR range of 10–35% (60). The mean self-reported daily intake of sugar consumed was 103.3 ± 49.4 g/d. Mean daily consumption of dietary fiber for males (n = 6) and females (n = 14) was 25.9 ± 10.9 g/d and 17.0 ± 5.4 g/d, respectively, for which both fell below the AMDR for males (38 g/d) and females (25–26 g/d) (60). As previously described, both IF1-P and IF2-P similarly and significantly altered participants' dietary energy and macronutrient intake (31). Briefly, total energy intake decreased by ∼40% (∼1,000 kcals/d) with no significant difference between groups. This reduction was due to significant decreases in dietary fat (-50 to 60 g/d) and carbohydrate (-138 to 152 g/d) intake. Protein intake increased significantly (21–25 g/d) in both groups. These macronutrient changes resulted in a distribution of 32–37% carbohydrate, 34–35% protein, and 28–34% fat. Moreover, dietary fiber intake significantly increased (8–12 g/d), whereas sugar (-57 to 77 g/d) and sodium (-1,500 to 2,000 mg/d) significantly decreased. As previously reported, both groups maintained similar physical activity and energy expenditure throughout the weight loss period (31).

Gastrointestinal symptoms reduced in IF2-P participants

A baseline assessment of individual GSRS scores revealed no difference between IF1-P and IF2-P ($p > 0.05$; Supplementary Table 1). Upon summing GSRS scores, 39% of IF1-P and 45% of IF2-P participants reported at least one symptom (score ≥ 2) for total GI symptoms, and 17% of IF1 and 12% of IF2-P reported at least one moderate symptom (score ≥ 4; Table 2). There were no significant differences between groups at baseline for any GI symptom clusters ($p \geq 0.567$). However, after comparing baseline to post-intervention symptom prevalence, significant reductions in total and moderate GI symptom presence were found in IF2-P ($p < 0.001$ and $p = 0.017$, respectively). Similar findings were found in IF2-P for reductions in total upper and lower symptom presence ($p = 0.031$ and $p = 0.013$, respectfully), though no significant reductions were noted for moderate upper and lower symptom presence ($p \geq 0.146$). In comparison, there were no significant changes for total or moderate upper, lower, and overall symptom presence in IF1-P ($p \geq 0.125$). For stool characteristics, there were no significant changes from baseline or between groups for stool weight, BSS, or stool pH ($p \geq 0.146$; Table 2). Stool weights were categorized as low for Western populations (i.e., 80–120 g/day) (61), whereas values for BSS were generally within the range of an ideal stool, indicating normal colonic transit time and ease of defecating while not containing excess liquid. Stool pH was within a healthy range (reference range: 6.5–7.5). Similarly, detected concentrations of SCFAs, including acetate, propionate, isobutyrate, and butyrate, were within normal ranges but did not differ significantly by time or interaction ($p \geq 0.126$; Supplementary Table 2).

Structure of the gut microbiome altered after short-term fasting

Both IF groups' baseline microbial community structures were significantly altered after the four-week dietary intervention as assessed by the Bray-Curtis beta diversity

TABLE 2 Self-reported gastrointestinal (GI) symptoms and stool characteristics between IF1-P and IF2-P at baseline and week 4.

Variable	Baseline		Week 4	
	IF1-P(n = 10)	IF2-P(n = 10)	IF1-P(n = 10)	IF2-P(n = 10)
Total GI scores ≥ 2	43 (39%)	49 (45%)	37 (34%)	30 (27%)*
Total GI scores ≥ 4	19 (17%)	13 (12%)	8 (7%)	4 (4%)*
Total upper GI scores ≥ 2	21 (35%)	26 (43%)	16 (27%)	17 (28%)*
Total upper GI scores ≥ 4	12 (20%)	3 (5%)	1 (2%)	0 (0%)
Total lower GI scores ≥ 2	22 (44%)	23 (38%)	21 (35%)	13 (22%)*
Total lower GI scores ≥ 4	7 (14%)	10 (20%)	7 (14%)	4 (8%)
Stool weight (g)	103.20 ± 92.65	78.01 ± 40.31	65.98 ± 30.11	106.33 ± 64.49
BSS	3.50 ± 1.08	3.20 ± 1.39	2.80 ± 1.31	3.30 ± 1.41
Stool pH	6.97 ± 0.81	6.94 ± 0.32	6.76 ± 0.34	6.95 ± 0.35

GI scores are displayed as the sum of GI symptoms and the percent of participants reporting ≥ 1 symptom per category. Stool characteristic data are reported as mean ± SD. *Significant decrease from baseline values, $p < 0.05$.

metric and visualized by non-metric multidimensional scaling (NMDS) ordination (R^2 = 0.042; p < 0.001; Figure 2A; Supplementary Table 3), though no significant differences between IF1-P and IF2-P were noted (R^2 = 0.009, p = 0.823). Homogeneity of dispersion tests did not reveal any significant differences ($p \geq 0.191$), increasing our confidence that the significant compositional differences were not an artifact of variance in group dispersion. Comparison of the first distances of Bray-Curtis dissimilarity between groups was non-significant (p = 0.579; Figure 2B), indicating no difference in the change in dissimilarity from baseline between IF1-P and IF2-P. Results from the PERMANOVA analysis were paralleled with the LME model, which identified the overall IF-P intervention as a significant factor in the participant's GM composition (time effect, p = 0.013), with no differences between groups detected (group × time effect, p = 0.473; Figure 2C). Null findings were observed for Shannon diversity and Faith's PD, with no significant effects for time ($p \geq 0.155$) or interaction ($p \geq 0.341$; Figures 2D,E). Overall, these data show alpha diversity was unaffected by this short-term intervention. However, there was a significant negative correlation between baseline Shannon diversity and percent body fat change after the four-week intervention (R^2 = 0.287, p = 0.030; Figure 2F). Therefore, individuals with increased baseline Shannon diversity had the greatest reduction in body fat percentage. This finding was not paralleled with Faith's PD, though it was trending toward significance (R^2 = 0.358, p = 0.084; Figure 2G).

Alterations in gut microbiome composition and predicted function

For the qPCR analysis, no significant time (p = 0.603) or interaction effects (p = 0.653) in total bacterial numbers were detected, indicating the estimated number of microbes remained relatively constant throughout the study and across groups (Figure 3A). With high throughput 16S amplicon sequencing, we identified 115 ASVs after filtering, represented by five phyla and 67 genera (Class: 8; Order: 17; Family: 26). At baseline, the composition of the gut microbiome for both groups was dominated by Firmicutes (IF1-P: 85.1% vs. IF2-P: 81.1%), followed by Actinobacteriota (6.9% vs. 15.2%), Bacteroidota (7.2% vs. 3.5%), Proteobacteria (0.8% vs. 0.2%), and Desulfobacterota (0.1% vs. < 0.1%). The GM at the genus level displayed much greater variation by individual. No taxa above the genus level differed significantly in their abundances from baseline to week four, nor was there a significant time or interaction effect for the Firmicutes/Bacteroidota ratio ($p \geq 0.527$). The abundances of phyla and genera (at an individual level) at baseline and week four for each group with greater than 1% mean relative abundance are shown in Figures 3B,C, respectfully.

Differential abundance testing identified three genera that significantly changed from baseline to week four in the IF1-P group, including a decrease in *Sellimonas* (log2FC = −0.997, q < 0.001) and an increase in Ruminococcaceae *Incertae Sedis* (log2FC = 2.289, q = 0.007) and *Eubacterium fissicatena group* (log2FC = 2.215, q < 0.001; Figure 4A). In comparison, two genera significantly changed in the IF2-P group, including an increase in Ruminococcaceae *Incertae Sedis* (log2FC = 2.435, q = 0.005) and a decrease in *Eubacterium ventriosum group* (log2FC = −1.990, q = 0.001) (Figure 4B). Analysis between the change in these differently abundance genera with change in percent body did not reveal any significant associations, though *Sellimonas* was trending toward a negative correlation (R^2 = −0.274, q = 0.072). Predicted functional composition of microbial communities was assessed via PICRUSt2, identifying 265 MetaCyc pathways after filtering. Upon differential abundance testing, IF1-P had a significant decrease in the predicted function of peptidoglycan biosynthesis II (log2FC = −1.674; q < 0.001) and chorismate biosynthesis II (log2FC = −1.509, q < 0.001), whereas IF2-P had a significant

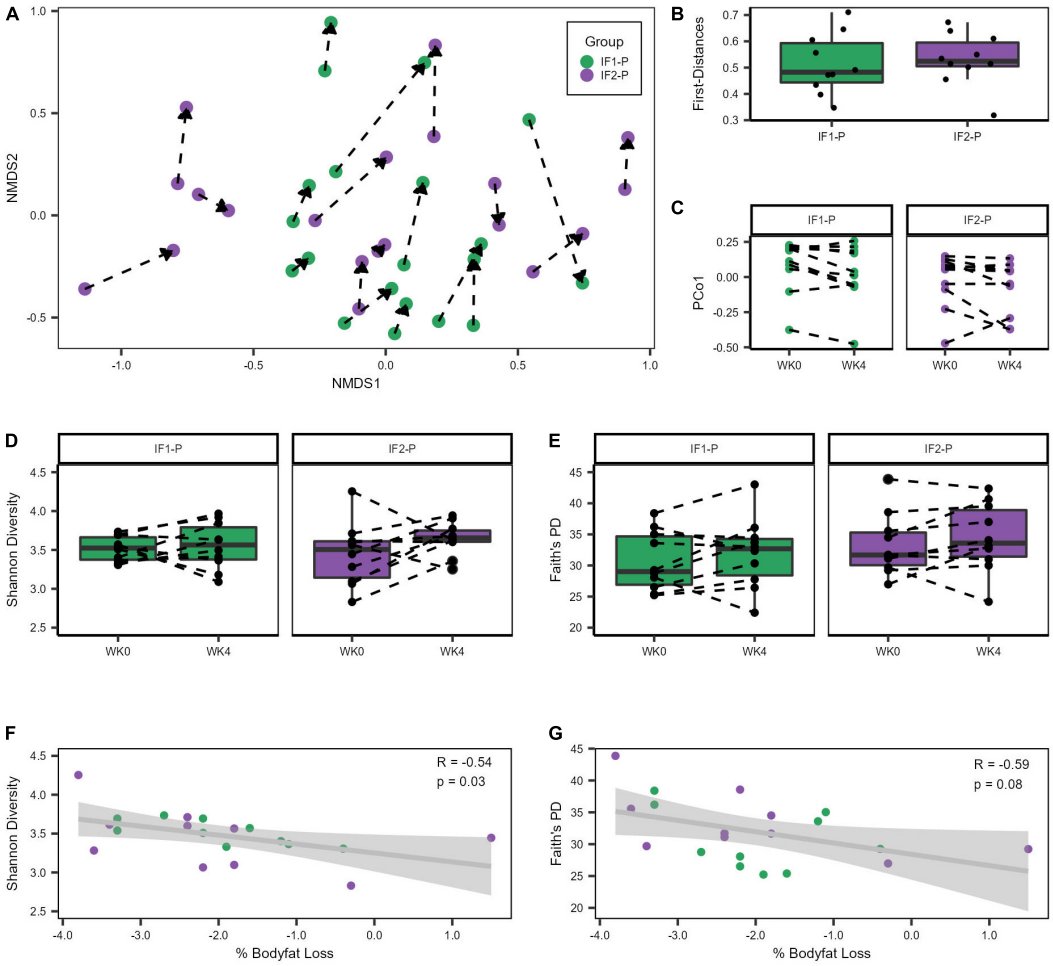

FIGURE 2
Variation in gut microbiome diversity metrics at baseline and week four of IF1-P and IF2-P participants. **(A)** Non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarity matrix. The GM of both IF1-P (n = 10) and IF2-P (n = 10) groups shifted significantly from baseline to week four (R^2 = 0.042, $p < 0.001$), but there was no difference between groups by time (R^2 = 0.009, p = 0.823). The same participant is connected by a dotted line, starting at baseline and the arrow pointing to the end of the intervention period. **(B)** The first distances of the Bray-Curtis dissimilarities between IF1-P and IF2-P were not significant (p = 0.579). Boxes denote the interquartile range (IQR) between the first and third quartiles, and the horizontal line defines the median. **(C)** First principal coordinate (PCo1) values differed by time (p = 0.013), with no differences between groups over time detected (p = 0.473). **(D)** Shannon diversity index did not change significantly over time for IF1-P and IF2-P groups ($p \geq 0.341$). **(E)** Faith's PD diversity index did not change significantly over time for IF1-P and IF2-P groups (p = 0.653). Boxes denote the IQR between the first and third quartiles, and the horizontal line defines the median. A dotted line connects the same participant. **(F)** Correlation of baseline Shannon Diversity with percent body fat loss from the four-week IF intervention. **(G)** Correlation of baseline Faith's phylogenetic diversity (PD) with percent body fat loss from the four-week IF intervention. Both groups were combined for the correlation analyses and are displayed in different colors. The gray cloud around the regression line indicates the 95% confidence interval.

increase in the predicted function of adenosine nucleotides degradation IV (log2FC = 0.719, $q < 0.001$; Figures 4C–E).

Alterations in the plasma metabolome between groups after short-term fasting

A total of 138 aqueous metabolites were reliably detected across 40 samples (i.e., QC CV < 20% and relative abundance > 1,000 in 80% of samples). Data were $\log_{10}$-transformed, and Pareto scaled (mean-centered and divided by the square root of the standard deviation of each variable) prior to all subsequent analyses and visualizations (Supplementary Figure 1). Following normalization, Levene's test of homogeneity showed equal variance between study groups ($p > 0.05$). Outlier analysis was performed via random forest (RF) and principal component analysis (PCA). RF performed with 500 decision trees indicated five potential outliers according to the greatest outlying measure (Supplementary Figure 2A), although no sample fell outside of the 95% CI as indicated by two-dimensional

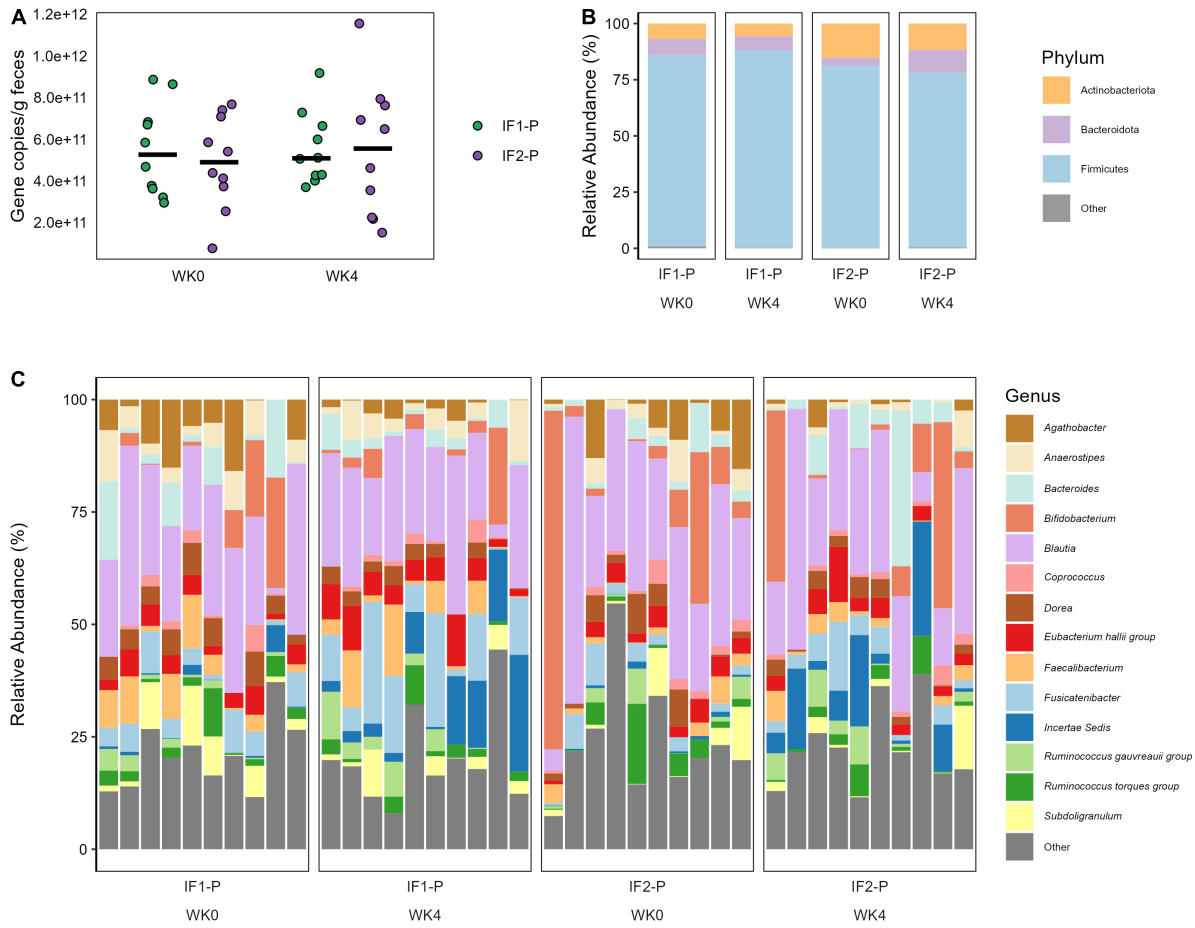

FIGURE 3
(A) Total bacterial number observed in both IF1-P (n = 10) and IF2-P (n = 10) groups at baseline and week four. No significant effects of time or interaction ($p \geq 0.603$) were noted. Total bacterial numbers were calculated as average copies of 16S rRNA gene/g wet feces via qPCR. Group means at each time point are displayed as black bars. **(B)** Average relative abundance of the most prevalent gut microbiome phyla among study participants for IF1-P (n = 10) and IF2-P (n = 10) at baseline and week four by 16S rRNA sequencing. Phyla with a median relative abundance of less than 1% are collapsed into the category "Other". **(C)** Average relative abundance of the most prevalent gut microbiome genera among study participants for IF1-P (n = 10) and IF2-P (n = 10) at baseline and week four by 16S rRNA sequencing. Genera with a median relative abundance of less than 1% are collapsed into the category "Other".

PCA (Supplementary Figure 2B). As such, no samples were confirmed as outliers, and all data were retained for subsequent analysis.

Next, we performed ANOVA-simultaneous component analysis (ASCA) to determine the significant main effects of time and group interactions. As can be seen in Supplementary Figure 3A, little separation was observed in the three-dimensional PCA, showing no observable clustering due to time or group. However, the ASCA did reveal a significant effect of time ($p < 0.01$; Supplementary Figure 3B), but no significant effect of group ($p = 0.17$, Supplementary Figure 3C) or any significant interactions between time and group ($p = 0.78$; Supplementary Figure 3D). Given the significant effect of time and our interest in the effects of the IF-P group, we prepared a GLM with age, sex, BMI, and time as covariates and corrected for FDR (Figure 5A). When controlling for these relevant covariates, we observed significant effects of IF-P on seven metabolites (Figure 5B, Supplementary Table 4), which may be considered candidate markers of intermittent fasting: serine ($q = 0.003$), TMAO ($q = 0.012$), levulinic acid ($q = 0.017$), 3-aminobutyric acid ($q = 0.029$), citrate ($q = 0.033$), isocitrate ($q = 0.033$), and glucuronic acid ($q = 0.049$).

Fold change (FC) and receiver operating characteristic (ROC) analyses assessed the magnitude of change between IF-P groups and the univariate classification performance of the candidate fasting markers, respectively. For FC analysis, groups were analyzed as IF2-P/IF1-P. While all seven significant metabolites were increased in the IF2-P group, the magnitude of change was consistent; FC ranged from 1.14 to 1.60 (see Supplementary Table 4 for significance, FC, and AUC details of metabolite markers). The area under the curve (AUC) estimates for individual candidate markers ranged from 0.63 to 0.74.

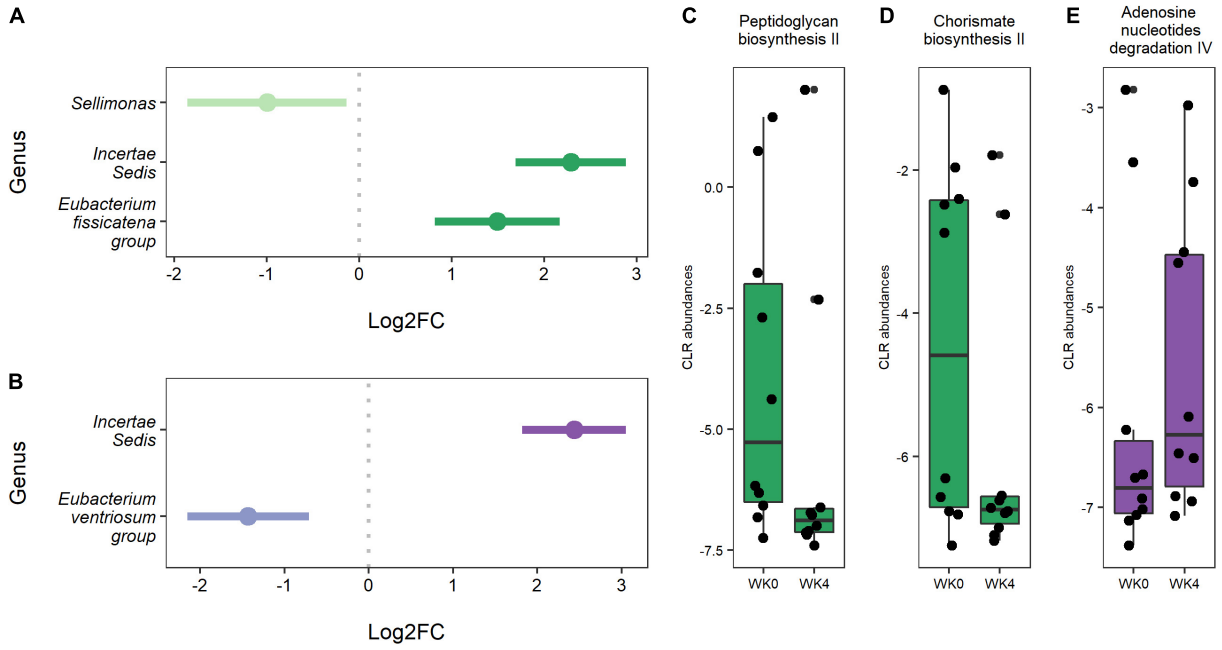

FIGURE 4
(A) Genera were differentially abundant between baseline and week four for IF1-P participants (n = 10). **(B)** Genera that were differentially abundant between baseline and week four for IF2-P participants (n = 10). Points represent each genera's log2 fold change (log2FC; effect size). A positive value indicates that a feature increased in abundance at week four (right), and a negative value indicates a decrease in abundance at week four (left). Bars represent 95% confidence intervals derived from the ANCOM-BC model. Note, the genus *Incertae Sedis* is from the Ruminococcaceae family. Boxplots displaying statistically significant differences in **(C)** peptidoglycan biosynthesis II and **(D)** chorismate biosynthesis II from baseline to week four of predicted metabolic pathways for IF1-P participants. Boxplot displaying a statistically significant difference in **(E)** adenosine nucleotides degradation IV from baseline to week four of the predicted metabolic pathway for IF2-P participants. Pathways are displayed as centered log-ratio (CLR) transformed abundances. Boxes denote the interquartile range (IQR) between the first and third quartiles, and the horizontal line defines the median.

Although no significant correlations were observed between changes in metabolite levels and percent body fat, positive associations were observed between citrate and glucuronic acid (R^2 = 0.282, p = 0.019), glucuronic acid and 3-aminobutyric acid (R^2 = 0.311, p = 0.013), and between 3-aminobutyric acid and serine (R^2 = 0.271, p = 0.022).

To increase the predictive accuracy of the candidate marker panel, we constructed a multivariate orthogonal partial least squares-discriminant analysis (OPLS-DA) model using the seven significant metabolites identified by GLM. Analysis of the OPLS-DA scores plot revealed the model to account for more variance than an orthogonal data matrix of equal dimension (Figure 6A). While the OPLS-DA model showed low-to-moderate predictive and explanatory capacity (R^2X = 0.351, R^2Y = 0.237, Q^2 = 0.202) (Figure 6B), permutation testing showed good fit to data and, notably, did not indicate model overfitting (Perm R^2Y p < 0.01, Perm Q^2 p < 0.01) (Figure 6C). Following model construction and validation, we performed ROC analysis to assess the classification performance of the multivariate OPLS-DA model. ROC analysis showed good accuracy of the model for assessing the duration of the IF-P intervention; AUC was observed to be 0.83 (95% CI: 0.70-0.94) when sensitivity and specificity were set to 0.80 (Figure 6D). Importantly, the OPLS-DA model provided greater classification accuracy than any significant metabolite individually. A box plot of model-implied Y-values derived from the OPLS-DA model is visualized between IF1-P and IF2-P groups in Figure 6E.

Next, we performed debiased sparse partial correlation (DSPC) networking modeling and metabolite ontology analysis to evaluate pathway-informed correlations and metabolite localization, respectively. The DSPC analysis was based on a least absolute shrinkage and selective operator (LASSO) algorithm, and revealed significant functional correlations in valine, leucine, and isoleucine biosynthesis (p = 8.59E-9) and degradation (p = 7.91E-5), aminoacyl-tRNA biosynthesis (p = 1.94E-4), fatty acid biosynthesis (p = 0.018), and phenylalanine, tyrosine, and tryptophan biosynthesis (p = 0.048) (Supplementary Figure 4). Qualitative metabolite ontology analysis was performed to identify organ, tissue, cellular, and subcellular localizations of study metabolites. Although metabolome coverage is admittedly limited given our targeted MS approach, network visualization of ontology results showed our experimental metabolites were derived, in order of most to

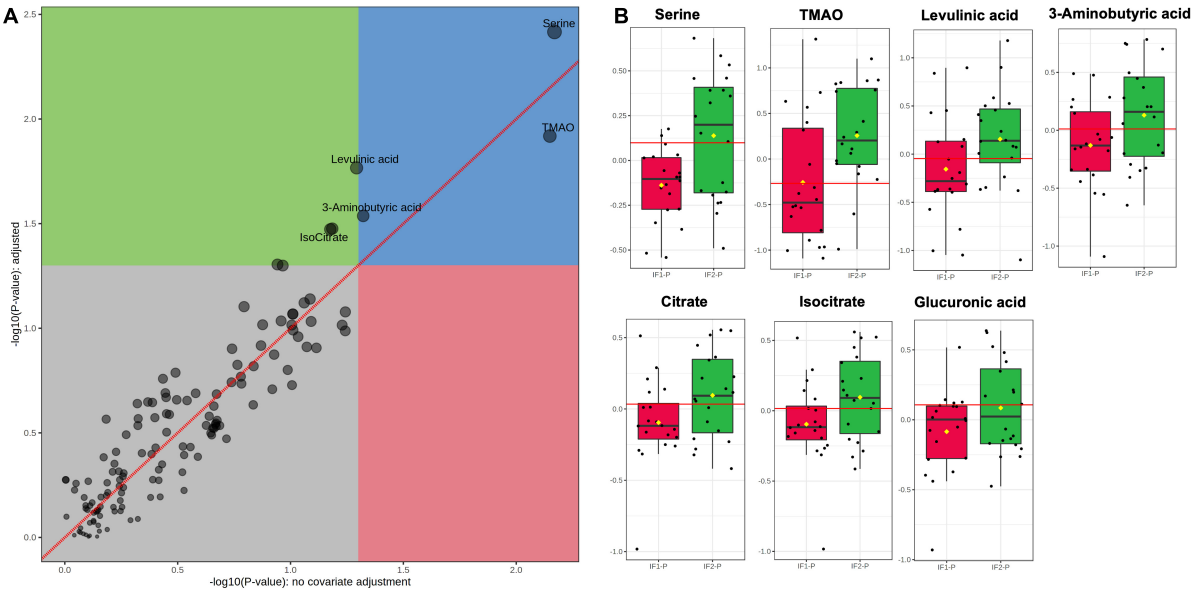

FIGURE 5
(A) GLM adjusted for age, sex, BMI, and time, with FDR-correction, and **(B)** box plots of significant metabolites as indicated by GLM: serine (q = 0.003), TMAO (q = 0.012), levulinic acid (q = 0.017), 3-aminobutyric acid (q = 0.029), citrate (q = 0.033), isocitrate (q = 0.033), and glucuronic acid (q = 0.049). Red lines in box plots denote optimal cutoff values as calculated by the Youden method, black lines indicate median values, and yellow diamonds show group averages.

least, from the central nervous system, brain, neuron, prostate, and mitochondria (**Supplementary Figure 5**).

Discussion

The GM is tightly intertwined with host health, which has raised considerable interest in how the resident GI microbes respond to dietary intervention. Advances in understanding how the GM influences and is modified by body weight will allow for a greater understanding of this relationship. Moreover, changes in the GM and associated metabolites may provide optimal dietary input for GM modulation and, ultimately, host health. Therefore, in this exploratory analysis, we sought to examine the GM and plasma metabolome of a subgroup of adult participants with overweight/obesity who were randomized into an IF-P regimen of one versus two days. Over four weeks, participants in both groups lost a significant amount of body fat with associated metabolic improvements. In contrast, the community structure of the GM was minimally impacted, with beta-diversity shifting approximately 4%. Similarly, differential abundance analysis revealed some shifts at the genus level, including increased *Incertae sedis* (from the Ruminococcaceae family) abundance in both groups. Analysis of the plasma metabolome revealed a significant increase in seven out of 138 validated metabolites in the IF2-P group. Overall, these results support that controlled, short-term IF-P regimens modestly impact the GM. Perhaps more importantly, the GM displayed resiliency and marked inter-individuality despite the significant weight loss and cardiometabolic improvement in these overweight/obese participants.

To our knowledge, this study is the first to directly compare two IF regimens on longitudinal GM changes. While a direct comparison to similar work is limited, our results generally align with the results from short-term inventions. For example, in an energy-restricted IF intervention using a modified fasting regimen of 5:2 (5 days normal feeding:2 days fasting), 12 weeks of energy restriction elicited approximately a 5% reduction in weight (62). While weight loss was associated with significant metabolic improvements, comparatively, there was less change in the GM and targeted plasma metabolome. However, the GM composition at baseline was moderately predictive of weight loss. Similarly, we noted baseline alpha diversity was significantly correlated with loss in percentage body fat, suggesting that the initial community dynamics of the GM may be an important determinant in response to periods of fasting and nutritional interventions. These findings add to previous reports that the GM of participants entering weight-loss interventions may greatly influence the host's response to these dietary regimens (63, 64). In terms of the baseline profile of the GM, increased diversity has been associated with enhanced response to a dietary weight loss intervention (65). Other calorie restriction interventions have noted increased alpha diversity (Shannon index) over short-term periods by implementing a high protein diet (30% of daily kcal) (21). Participants in the

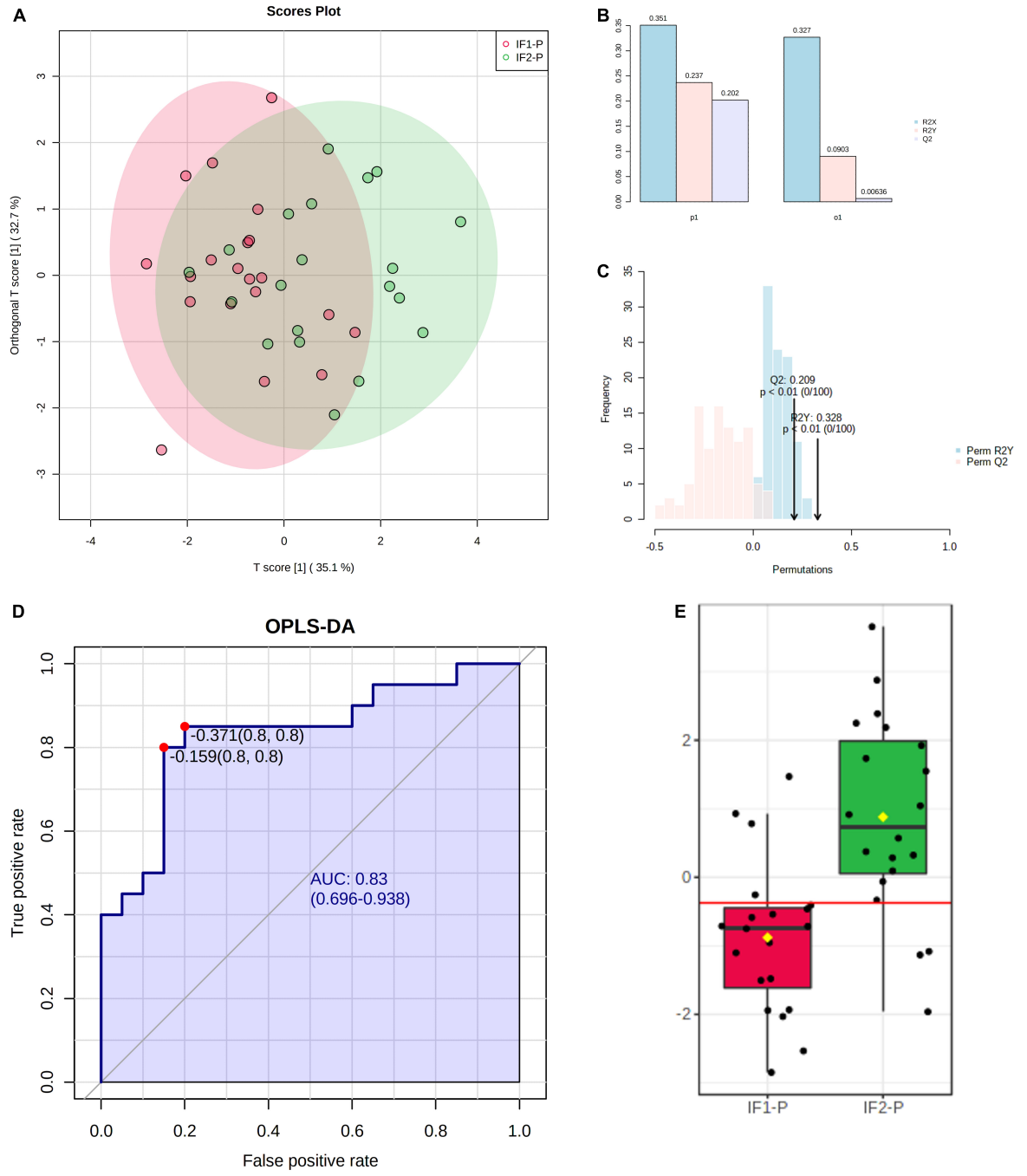

FIGURE 6
(A) Scores plot of OPLS-DA model constructed using seven significant metabolites identified by GLM, showing percent variance accounted for by experimental and orthogonal data. **(B)** OPLS-DA model overview showing predictive and explanatory capacity (R^2X = 0.351, R^2Y = 0.237, Q^2 = 0.202); *y*-axis represents proportion of total variance. **(C)** Permutation test with 100 iterations showing model fit distributions (Perm R^2Y $p < 0.01$, Perm Q^2 $p < 0.01$). **(D)** ROC analysis of OPLS-DA model for assessing IF-P (AUC = 0.83, 95% CI: 0.70-0.94, sensitivity = 0.8, specificity = 0.8). **(E)** Box plot of OPLS-DA predictive values; the red line in the box plot denotes the optimal cutoff value, while yellow diamonds show group averages, and black lines illustrate group medians.

current study consumed 35% of their total energy intake from protein, though we only reported results after four weeks, whereas the other study had a longer intervention (eight weeks). In another high-protein CR intervention, protein supplementation had little effect on microbial diversity and relative abundance (22). However, decreased body weight

and fat mass were significantly correlated with increased microbial diversity.

Differential abundance analysis revealed increases in several genera, including *Incertae sedis* and *Eubacterium fissicatena group*, and a decrease in *Sellimonas* in IF1-P. *Incertae sedis* is an unclassified genus from the Ruminococceacae family, a group of strict anaerobes normally present in healthy individuals (66). Overall, the family Ruminococcaceae plays an important role in maintaining gut health through the potential to produce butyrate and other SCFAs. *Eubacterium* is phylogenetically diverse and forms the core genera of health-associated human GM (67). Indeed, multiple species of this genus are currently regarded as promising targets for microbial therapeutics (67), though *Eubacterium fissicatena group* remains a poorly described genus, with few clinical studies detecting its presence (68). *Sellimonas* is also a genus less characterized. Under the Lachnospiraceae family, *Sellimonas* contains certain species that have been proposed as an indicator of GI homeostasis (69). *Incertae sedis* was also increased in IF2-P, while *Eubacterium ventriosum group* decreased. The decrease in *Eubacterium ventriosum group* was of interest as this microbe was previously found to be enriched in high vs. low-BMI twin-dyads (70). Similar findings have been reported in a broader examination of unrelated obese vs. non-obese study participants (71). It may be that these butyrate producers provide substrate to the host as energy harvesters, which could contribute to a positive energy balance. Conversely, reduced abundance of this genus is well noted in colorectal cancer patients (72–75) and has been proposed as a biomarker for lower disease risk (67). Such findings display the complexity of *Eubacterium*, highlighting the importance of better mapping function and host-health implications at the genus and species level.

Less affected were the predicted functional pathways of the GM. IF1-P resulted in a significant decrease in two predicted functions. This included peptidoglycan biosynthesis II, which is a step in forming cell wall components of Gram-positive bacteria, including pathogenic species (like *Staphylococcus aureus*), and plays a role in GM-derived inflammation in the host (76). Previously, IF in a diabetic mice model reduced plasma peptidoglycan, a surrogate marker for gut barrier function (77). The other pathway, chorismate biosynthesis II, contributes to chorismate synthesis and is a precursor to tryptophan and subsequent serotonin production in the GI tract. IF2-P resulted in a significant increase in adenosine nucleotides degradation IV, which may signal increased microbe and energy turnover.

Regarding the plasma metabolome, we identified an increase in several metabolites that were significant over time and increased in the IF2-P group. The elevation in these specific metabolites was intriguing as some are linked to healthy states, while others are linked to disease. For example, serine plays an important role in glutathione production (78), citrate and its isomer, isocitrate, are involved in energy metabolism, and glucuronic acid is involved in detoxifying xenobiotic compounds and has been proposed as a biomarker of longevity (79). In contrast, TMAO is a compound found in high protein foods (e.g., fish) and can be generated from precursors like choline and carnitine (e.g., found in eggs and beef) and has been associated with heart disease (80, 81). Concentrations of TMAO in circulation appear reliant on dietary intake (82, 83). Like participants in the present study, healthy individuals with a high relative abundance of Firmicutes have been reported to have greater circulating levels of TMAO (82). While increased levels of this metabolite have been associated with metabolic disease, participants in the present study displayed significant improvements in cardiometabolic outcomes, including lipid profiles. In relation to diet, a notable component of both the IF1-P and IF2-P diet was resistant starch, which has been reported to be associated with higher circulating TMAO levels when overall carbohydrates are reduced, and protein levels increased (84). We have used a similar dietary intervention, noting substantial improvements in postprandial metabolism, including reduced glucose and insulin response (85, 86). Therefore, it may be that circulating concentrations of TMAO were reflective of differences in the GM community and dietary intake, rather than indicating a role of TMAO in negative cardiometabolic outcomes in the present study. More work exploring the impact of increased resistant starch and relative protein during caloric restriction is warranted, particularly concerning the GM and different metabolomic assessments (e.g., contents of the GI tract at different sections). Relatedly, while fecal metabolites might be more reflective of the direct metabolic output of the microbiota, blood metabolites provide a window into which of these compounds make it into circulation (18, 79). Indeed, the plasma metabolome is connected and partially reflective of the GM as it can present a person-specific signature and is largely predicted by host-associated characteristics (87–89).

Regarding metabolomic pathway analysis, our DSPC model detected several negative correlations in accordance with calorie restriction, including biosynthesis of the amino acids valine, leucine, isoleucine, phenylalanine, tyrosine, and tryptophan. While decreases in plasma branched-chain and aromatic amino acids are reportedly associated with weight loss and improvement in insulin resistance in obese individuals (90, 91), the implications of reduced tryptophan synthesis are less clear. Decreased circulating levels of tryptophan may influence the serotoninergic system and mood. Reductions have been observed in other short-term weight loss trials with suggestive susceptibility to food cravings (92). In contrast, these behaviors were not reflected in participants from the present study as hunger ratings significantly improved over baseline levels (31). The other notable negative correlation was fatty acid biosynthesis. Interestingly, the current study showed significant loss of body weight, total and abdominal fat loss, despite not detecting a significant association with fat oxidation. Although, alterations in fatty acid synthesis have displayed a greater

magnitude of change over fatty acid oxidation in preclinical models of calorie restriction and appears to be an important metabolic adaptation to reduced energy states (93, 94). In particular, decreased saturated fatty acid synthesis such as palmitic acid, myristic acid, and capric acid appears to be associated with cell longevity and protective against cancerous traits, perhaps mediated through the *p53* tumor suppressor gene (95). These results are notable considering the short-term intervention period and warrant future exploration with expanded omic surveys and longer time durations.

Finally, we captured self-reported GI symptoms and stool characteristics (pH, SCFAs, and biomass estimate). While routine, these assessments are generally lacking in diet-focused GM research, and adverse GI symptoms remain highly reported in Western populations (96, 97). We noted a similar trend in the participants in the present study, with nearly half reporting GI symptom presence at baseline. After the four-week intervention, participants in the IF2-P group reported significantly decreased incidence of minor and moderate symptoms, while IF1-P did not. These findings are noteworthy as restrictive diets have been positively associated with self-reported GI issues (98). Differences may have been due to the longer periods of supported fasting, providing "gut rest" (24). We did not find significant changes in stool pH after IF, which is interesting because prolonged fasting (+18 h) has been reported to produce a higher gut pH than constant feeding (99). In relation, SCFA concentrations did not change significantly despite the significant reduction in energy and periods of IF. Presumably, during periods of dietary restraint, the host would more readily absorb these SCFA, resulting in decreased detection in the stool (100). While speculatory, this presumed reduction in SCFAs was bolstered by the significant increase in dietary fiber by approximately 10 grams in the dietary intervention. Much of this increase was supplied by isomaltooligosaccharide, a non-digestible fiber metabolized by the GM (101). Regardless, SCFAs concentrations were within range of those found in stool samples of healthy humans (102, 103). Overall, our findings support the notion that short-term dietary intervention impacts the microbial ecosystem of the human gut, showcasing the resilience of the GM community (104). Indeed, this paralleled the biomass estimates provided by our qPCR analysis, where we did not detect a significant decrease over time.

The present study has several strengths, including a tightly controlled design and well-matched comparator groups regarding participant characteristics and nutrient intake during the intervention. Moreover, we provided a simultaneous investigation of the changes in structure and predicted function of the gut microbiota, plasma metabolome, and host-associated features. However, there are several limitations. First and foremost, the sample size was small. A greater sample and a more robust design protocol, such as a cross-over design, for GM research may better account for interpersonal variabilities. Second, we employed 16S rRNA gene sequencing on fecal samples to assess the GM which constrained our taxonomic resolution and survey of microbial gene content and function. In fasting, where many important microbial changes in more proximal sections of the GI tract are suggested, we were limited by our sample collection. Third, the fecal metabolome was not assessed, which would have better reconciled the apparent gap between the GM and the plasma metabolome. Fourth, we did not collect samples directly after fasting and fed periods to make these important comparisons. Such investigations will likely require time-series and in-patient designs, features that were not within the scope of this current exploratory work. Finally, our participants were overweight/mildly obese individuals with baseline cardiometabolic parameters frequently characterized in the literature as "healthy obese" (31). Generally, host-microbe metabolic associations are more apt to be disrupted in individuals experiencing more severe obesity (i.e., BMI $\geq$ 35) relative to individuals with a normal weight (BMI $<$ 25) (105). The group BMI means of IF1-P and IF2-P in the current study could be considered only slightly obese (i.e., 31.3 $\pm$ 5.1 and 33.6 $\pm$ 9.7 kg/m^2, respectively) and thus may not have been as sensitive to change during the short-term intervention. Indeed, a health-associated GM appears to display resilience to change, including dietary intervention (104). These factors may have contributed to some of the current study's findings.

Conclusion

In the current study, we observed that short-term IF-P induced modest shifts in the GM and plasma metabolome, despite conferring significant body weight and fat reduction. These results indicate that IF may promote minor increases in health-associated taxa and alterations in microbial community structure and plasma metabolite profile. However, when controlled for overall energy intake and nutritional profile, fasting for one vs. two days did not promote significant differential changes in this short-term, calorically restricted protein pacing intervention. Importantly, we show that the GM in overweight and obese individuals appears to have great resiliency despite significant energy restriction and fat loss. Moreover, the baseline composition of the GM may be an important variable in weight loss, though larger and longer duration studies are needed to better characterize IF modifications of microbial and metabolic factors.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA847971.

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Boards of Skidmore College, NY, United States and Arizona State University, AZ, United States. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AM, EG, KS, and PA: conceptualization. KA, MP, and PA: study implementation and sample collection. AM, PJ, DB, BD, CW, HG, KS, and PA: laboratory methodology and analysis. AM, PJ, and DB: statistical analysis. AM, PJ, DB, EG, KS, and PA: original draft preparation and writing. AM, PJ, DB, BD, CW, KA, MP, HG, EG, KS, and PA: review and editing. AM and PJ: visualization. KS and PA: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

This study received funding from Isagenix International, LLC and they were not involved in the study design, collection, analysis, interpretation of data, and the writing of this article or the decision to submit it for publication. Authors AM and EG were employed by Isagenix International, LLC and contributed to the study as noted above. AM was a doctoral candidate at Arizona State University, and this work was in partial fulfillment of his dissertation. The study was conducted at Skidmore College and AM was blinded throughout data collection and analyses. Author PA is a member of the scientific advisory board at Isagenix International, LLC and the International Protein Board. Author CW is on the scientific advisory board for the Wheat Foods Council and the Hass Avocado Board's Avocado Nutrition Science Advisory.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1036080/full#supplementary-material

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Qinglong Wu,
Baylor College of Medicine,
United States
Vanessa Leone,
University of Wisconsin-Madison,
United States

*CORRESPONDENCE
Dun Deng
dengdun2008@126.com
Jinping Deng
dengjinping@scau.edu.cn

†These authors have contributed equally to this work

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Brevibacillus laterosporus BL1, a promising probiotic, prevents obesity and modulates gut microbiota in mice fed a high-fat diet

Guangying Weng[1,2†], Jian Huang[3†], Xianyong Ma[2], Min Song[2], Yulong Yin[1], Dun Deng[2*] and Jinping Deng[1*]

[1]Guangdong Provincial Key Laboratory of Animal Nutrition Regulation, South China Agricultural University, Guangzhou, Guangdong, China, [2]State Key Laboratory of Livestock and Poultry Breeding, Key Laboratory of Animal Nutrition and Feed Science in South China, Ministry of Agriculture and Rural Affairs, Guangdong Provincial Key Laboratory of Animal Breeding and Nutrition, Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangdong Engineering Technology Research Center of Animal Meat Quality and Safety Control and Evaluation, Guangzhou, China, [3]Institute for Quality & Safety and Standards of Agricultural Products Research, Jiangxi Academy of Agricultural Sciences, Nanchang, Jiangxi, China

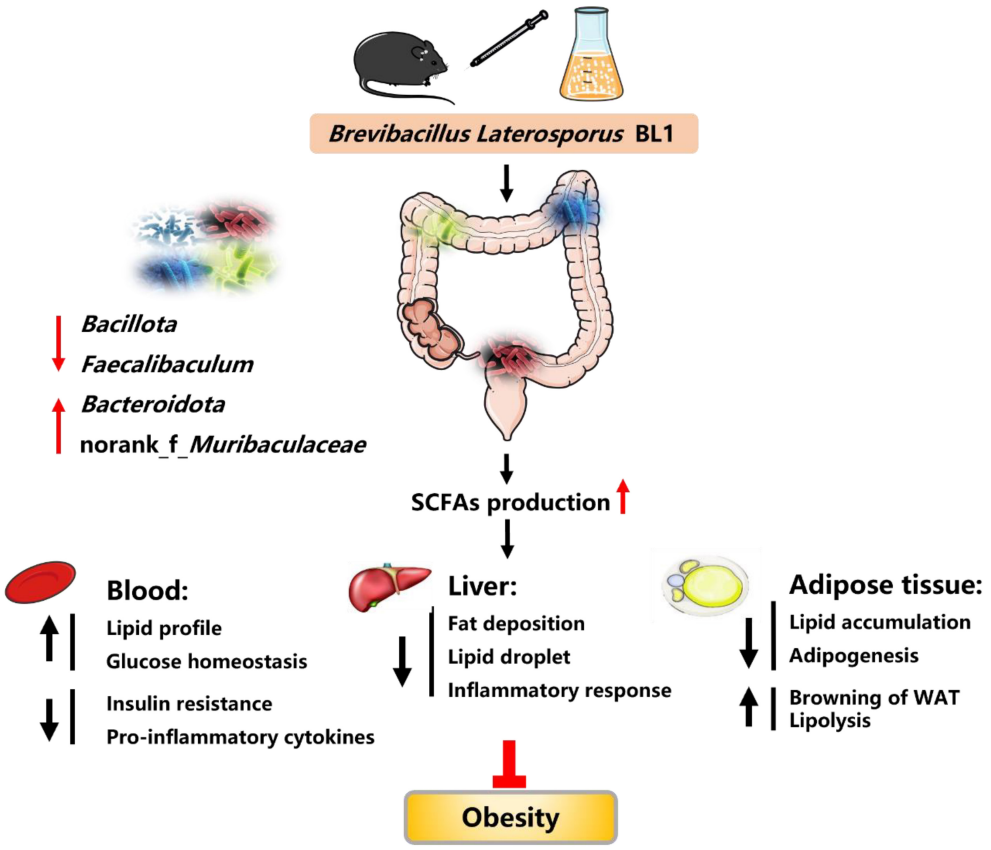
Scope: Probiotics are a potential preventive strategy for obesity. However, with discrete efficacy and limited species of probiotics, there is a demand for novel strains with excellent anti-obesity properties. This study aimed to investigate the effects of Brevibacillus laterosporus BL1 on preventing obesity in high-fat diet (HFD)-fed mice.

Methods and results: C57BL/6 male mice were randomly assigned to four groups (n = 10) and fed a control diet, HFD, HFD plus *B. laterosporus* BL1, and HFD plus supernatant of *B. laterosporus* BL1, respectively for 8 weeks. The results showed that prophylactic *B. laterosporus* BL1 treatment reduced body weight gain by 41.26% in comparison to the HFD group, and this difference was accompanied by a reduction in body fat mass and the weight of inguinal white adipose tissues and epididymal white adipose tissue (−33.39%, −39.07%, and −43.75%, respectively). Moreover, the *B. laterosporus* BL1-mediated improvements in lipid profile, insulin resistance, and chronic inflammation were associated with the regulation of gene expression related to lipid metabolism and enhancement of brown adipose tissue thermogenesis. Particularly, *B. laterosporus* BL1 intervention significantly improved HFD-induced gut flora dysbiosis, as evidenced by a reverse in the relative abundance of Bacillota and Bacteroidota, as well as an increase in the relative abundance of bacteria that produce short-chain fatty acids (SCFAs), which in turn increased SCFAs levels.

Conclusion: Our findings found for the first time that *B. laterosporus* BL1 may be a promising probiotic for prevention of obesity associated with the regulation of gut microbiota.

KEYWORDS

***Brevibacillus laterosporus*, obesity, gut microbiota, lipid metabolism, high-fat diet**



GRAPHICAL ABSTRACT

Brevibacillus laterosporus BL1 treatment effectively reduced fat accumulation, proinflammatory cytokines, and insulin resistance, alleviated BAT whitening, and improved glucose tolerance and lipid profiles in HFD-induced obese mice. *B. laterosporus* BL1 intervention also modulated the HFD-induced structural and componential alteration of gut microbiota, as evidenced by a decrease in the relative abundance of Bacillota and *Faecalibaculum* and an increase in the relative abundance of Bacteroidota and norank_f_*Muribaculaceae*, and this alteration was accompanied by an elevation on short-chain fatty acids (SCFAs) production. The alleviation of obesity parameters with *B. laterosporus* BL1 treatment was associated with the modulation of gut microbiota, especially the increase in SCFA levels.

Introduction

The incidence of obesity has risen alarmingly in recent years, and it is starting to overtake malnutrition and infectious disease as the major threat to global public health (1, 2). Obesity is characterized by an excessive or abnormal ectopic lipid accumulation and results from a serious imbalance between energy intake and energy consumption (3). There is a wealth of data demonstrating that obesity is responsible for the increased risk of numerous chronic diseases, such as cardiovascular disease, atherosclerosis, and diabetes (4, 5). Several factors such as genes, environment, dietary patterns, and lifestyle have been identified to regulate obesity and its related metabolic diseases (6). In this context, several strategies and therapies have been applied to prevent and treat obesity, including pharmacotherapy, surgery, and lifestyle modification. However, with high risk and recurrence rates, it is difficult to achieve long-term goals in clinical applications using traditional therapies (7, 8). Therefore, much research has been devoted to developing effective bioactive substances from natural biological sources for obesity prevention and control.

Probiotics are active microorganisms that help the host's health after adequate administration (9). As the most widely available probiotics, *Lactobacillus* and *Bifidobacteria* have been proven to exhibit favorable effects on lipid metabolic disorders including obesity and NAFLD through regulation of energy metabolism, inflammatory response, and intestinal barrier function (10–12). For example, administration of *Limosilactobacillus* (*Lactobacillus*) *fermentum* CECT5716 (5×10^8 CFU/d, 11 weeks) resulted in an obvious anti-obesity effect *via* amelioration of endothelial and intestinal barrier dysfunction in HFD-induced obese mice (13). Likewise, intervention with *Bifidobacteria breve* B-3 (10^8 or 10^9 CFU/d, 8 weeks) in HFD-feeding mice dose-dependently suppressed body weight gain and fat accumulation (14). Despite these positive outcomes, a recent systematic study showed that the species of probiotics that

are effective in the prevention or treatment of adiposis are scarce and limited probiotics produce a discrete benefit in clinical applications (15). For this reason, there is an urgent demand to identify novel strains with anti-obesity properties and the functional mechanisms involved in their actions.

Over time, evidence has accumulated in support of the important role that gut microbiota plays in host energy metabolism and the development of obesity (3, 16, 17). A transformation from a low-fat diet to an HFD changed gut microbial communities within 24–48 h in mice and humans, leading to obesity and its related metabolic disorders (18–20). Probiotics have been shown to improve gut microbiota composition and diversity, as manifested by enriching specific health-promoting bacteria *via* competitive exclusion of pathogens and producing antimicrobial substances, thus improving gut dysbiosis and host metabolic homeostasis (21, 22). In addition, probiotics may elevate short-chain fatty acids (SCFAs) production, which functions not only as energy sources to enhance the integrity of the intestinal epithelial barrier but as signaling molecules to enter the systemic circulation and directly regulate lipid dysmetabolism (23, 24). Therefore, the modulation of gut microbiota with probiotics should get more specific attention, as these may represent a preventive or therapeutic strategy for obesity and its related metabolic syndromes.

Brevibacillus laterosporus, a pathogen of invertebrates, produces various antimicrobial substances such as polyketides, non-ribosomal peptides, antibiotics, and chitinase, resulting in broad-spectrum antimicrobial activity (25, 26). In 2013, the Ministry of Agriculture and Rural Affairs of the People's Republic of China published a notification to include *B. laterosporus* in the catalog of feed additives. However, to our knowledge, few studies have explored the beneficial effects of *B. laterosporus* in livestock and poultry (27). Our pre-experiment performed in a porcine model implied that *B. laterosporus* has the potential to improve abnormal fat deposition, but the underlying mechanism remained unclear. Moreover, considering its excellent antimicrobial activity, we hypothesized that *B. laterosporus* could regulate gut microbiota dysbiosis and ameliorate excessive fat deposition. To confirm this hypothesis, we isolated a novel *B. laterosporus* strain BL1 from a healthy earthworm intestine and investigated the effects of *B. laterosporus* BL1 on obesity in HFD-fed mice for the first time, with particular emphasis on the potential role of intestinal flora modulation as a novel preventive approach. We also evaluated its effects on fat deposition, serum lipids, insulin resistance, chronic inflammation, intestinal flora composition, and colonic SCFAs in HFD-fed mice and further explored the correlation between key gut microbial taxa and SCFAs/obesity-related parameters.

Materials and methods

Bacterial strain and culture

Brevibacillus laterosporus BL1 strain was originally isolated from a healthy earthworm intestine and stored in Guangdong Microbial Culture Collection Center, China (GDMCC 62699). The neighbor-joining phylogenetic tree of *B. laterosporus* BL1 was added in **Supplementary Figure 1**. The strain was incubated aerobically in Luria–Bertani media for 24 h at 37°C while being vigorously shaken at 180 r/min. For administration to mice, pure bacterial cells were collected after being centrifuged (6,000 *g*, 10 min, 4°C) and cleansed twice with sterile saline, and adjusted to 5×10^9 CFU/mL. When the bacterial concentration reached 5×10^9 CFU/mL, the culture supernatant was centrifuged (6,000 *g*, 10 min, 4°C) and filtered through 0.22 μm filters to prepare cell-free supernatant.

Animals and diets

The South China Agricultural University's Animal Care and Use Committee approved the use of animals in the experiments, which were carried out following the acknowledged guidelines for animal care (Authorization Number: 2021C097).

Forty 5-week-old male C57BL/6 mice were purchased from (Zhuhai BesTest Bio-Tech Co., Ltd., Zhuhai, China) and individually kept in a controlled environment (24 ± 2°C, 45–60% humidity and 12/12 h light/dark cycle) with free access to drinking water and food throughout the experiment. Corncob granules obtained from (Slac Laboratory Animal Co., Ltd., Shanghai, China) were used as bedding for mice. Both the control diet (XTCON50J) and the HFD (XTHF60) were purchased from (Xietong Organism Co., Led., Nanjing, China) and the composition and nutrient levels of diets are listed in **Supplementary Table 1**. After 1 week of adaptation, the mice were divided randomly into four groups (n = 10 per group) as follows: CON group (control diet, 10% kcal from fat), HFD group (high-fat diet, 60% kcal from fat), BL group (HFD along with *B. laterosporus* BL1, 1×10^9 CFU/day per mouse), and BLs group (HFD along with supernatant of *B. laterosporus* BL1, 200 μL/day per mouse). The mice in CON and HFD groups were treated with 200 μL sterile saline *via* oral gavage per day at 8:00 a.m. Body weight (BW) and food intake were recorded weekly. At the end of the 8-week treatment, body fat mass was measured using a QMR body composition analyzer (Shanghai Electronic Technology CO., LTD., Shanghai, China). The infra-red thermal images and temperature of brown adipose tissue (BAT) were measured by an infra-red detector (FLIR Systems, Inc.). Blood samples were obtained through orbital blooding. Liver, inguinal white adipose tissues (iWAT), and epididymal white adipose tissue (eWAT) were weighed. Subsequently, liver,

iWAT, BAT, and colonic digesta samples were collected for further analysis (28, 29).

Intraperitoneal glucose and insulin tolerance tests

At week 7, intraperitoneal glucose tolerance tests (IGTT) and insulin tolerance tests (ITT) were performed. A glucometer (Sinocare Biological Transmission Co., Ltd., Changsha, China) was used to detect the blood glucose levels in the tail vein blood. For IGTT analysis, mice fasted overnight were intraperitoneally injected with glucose of 2 g/kg BW, followed by measuring blood glucose levels at 0, 15, 30, 60, 90, and 120 min. For ITT analysis, blood glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 min after being injected intraperitoneally with insulin of 0.75 U/kg BW to mice fasted (6 h). The trapezoidal rule was used to compute the integrated glucose areas under the curve (AUC). The following equation was used to calculate the homeostasis model of insulin resistance index (HOMA-IR): HOMA-IR = basal glucose × basal insulin/22.5 (21).

Biochemical analysis

The blood samples were centrifuged (3,000 rpm, 4°C, 10 min) to separate the serum. Serum glucose, triglyceride (TG), total cholesterol (T-CHO), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were detected using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum concentrations of insulin, interleukin-β (IL-1β), and tumor necrosis factor-α (TNF-α) were measured using commercial ELISA kits from (Meimian Biotechnology Company, Jiangsu, China).

Histological analysis

The fresh iWAT, eWAT, and BAT samples were fixed with 4% paraformaldehyde, embedded in paraffin, sliced into 5–7 μm, and stained with Hematoxylin and eosin (H and E) following standard procedures (29). The ImageJ software (NIH, USA) was used to calculate the adipocyte size. The oil red O staining of liver tissues was carried out by previously described methods (28).

Quantitative PCR analysis

Using a TRizol reagent (Takara Biotechnology, Dalian, China) to extract total RNA from selected tissues and a Synthesis Kit (Takara Biotechnology) to produce cDNA according to the manufacturer's protocols. Following the manufacturer's instructions, real-time RT-PCR was performed in duplicate using a CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalized to the housekeeping gene β-actin (30–32). The target genes, including sterol regulatory element binding protein 1 (SREBP1), peroxisome proliferators-activated receptor γ (PPARγ), fatty acid synthase (FAS), cluster of differentiation 36 (CD36), hormone-sensitive lipase (HSL), carnitine palmitoyltransferase-1 (CPT-1), uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), CCAAT/enhancer binding protein α (C/EBPα), PR domain-containing 16 (PRDM16), IL-1β, TNF-α, interleukin 6 (IL-6), and interferon-γ (IFN-γ) and their primer sequences are shown in **Supplementary Table 2**.

Gut microbiota analysis

The microbial genomic DNA of colonic digesta samples was extracted using the QIAamp-DNA Stool Mini Kit (Qiagen, Hilden, Germany). The amplification of DNA samples used common primers of variable region V3-V4 of the bacterial 16S rRNA gene with primers 338F and 806R (forward primer, 5′-ACTCCTACGGGAGGCAGCAG-3′; reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′). All the samples were sequenced using the Illumina MiSeq PE300 platform at Majorbio Biotechnology Co., Ltd. (Shanghai, China). After sequencing, raw sequence reads were quality filtered with fastp (v0.19.6) (33) and merged with FLASH (v1.2.11) (34). Then the DADA2 plugin in the Qiime2 pipeline was used to de-noise the high-quality sequences into amplicon sequence variants (ASVs) according to the recommended parameters. The taxonomy of ASVs was analyzed by the Naive Bayes Classifier implemented in QIIME2 based on the silva138/16s_bacteria database. Subsequently, the diversity and richness of microbial communities were evaluated by alpha diversity indices (Sobs, Shannon, and Simpson) using Mothur (v1.30.2). The similarity among the microbial community of different samples was analyzed by Principal coordinate analysis (PCoA) based on Bray–Curtis distance using the Vegan (v2.5-3) package. Spearman correlation analysis was carried out to explore the correlations between key intestinal microbiota and metabolites/obesity-related parameters.

Short-chain fatty acids analysis

The concentrations of SCFAs were determined using the GCMS-QP2020 system (Shimadzu, Tokyo, Japan) as previously described (35). Briefly, the colonic digesta samples were homogenized in ultra-pure water, sonicated, then centrifuged

to obtain supernatant. Subsequently, metaphosphoric acid solution, anhydrous sodium sulfate, and methyl tert-butyl ether were added to acidification, salting out, and extraction, respectively. After centrifuging and filtering, the organic phase was collected for GC-MS analysis. The gas chromatography equipped with a DB-FFAP capillary column and a flame ionization detector was utilized for chromatographic separation (injection port temperature: 250°C, carrier gas: helium, total run time: 18 min). The temperature programing is as follows: The initial temperature was 80°C for 2 min and raised to 150°C at 10°C/min for 2 min, and to 180°C at 15°C/min for 5 min. The quantification of SCFAs was performed by an external standard method as previously described (36).

Statistical analysis

The GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was utilized for statistical analyses. One-way analysis of variance (ANOVA) with Tukey's test was used to analyze all experimental data. All data were expressed as the means ± SEM. Differences were considered statistically significant at p-values<0.05 and tendencies were designated as having p-values<0.10.

Results

Brevibacillus laterosporus BL1 alleviates body weight and lipid accumulation in high-fat diet-fed mice

As shown in **Figure 1**, relative to the CON group, HFD-feeding significantly increased body weight (control diet: 25 ± 1.62; HFD: 29.49 ± 1.08; $p < 0.001$). As expected, *B. laterosporus* BL1 administration decreased body weight gain by 41.26% in obese mice ($p < 0.05$), leading to significantly lower body weight in the 8th week ($p < 0.01$). However, the supernatant of *B. laterosporus* BL1 coadministration failed to reduce body weight gain in mice fed with HFD (**Figures 1A,B**).

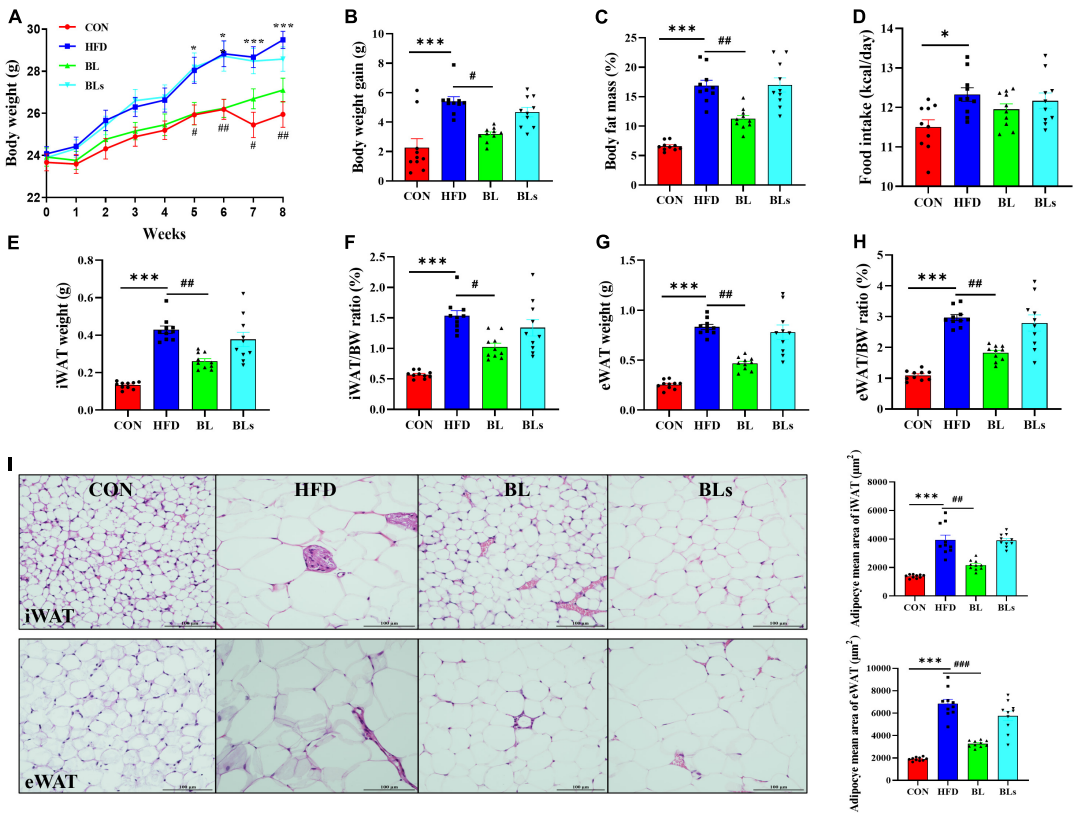


FIGURE 1
Brevibacillus laterosporus BL1 treatment reduced body weight gain and fat accumulation in HFD-fed mice. **(A)** Body weight, **(B)** body weight gain, **(C)** body fat mass, **(D)** food intake, **(E)** iWAT weight, **(F)** iWAT/BW ratio, **(G)** eWAT weight, **(H)** eWAT/BW ratio, **(I)** H and E staining of iWAT and eWAT sections. Scale bar = 100 μm. Data are presented as mean ± SEM (n = 10) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{*}p < 0.05$ and $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

Similarly, HFD-fed mice significantly increased body fat mass compared with CON-fed mice ($p < 0.001$), while the body fat mass was reduced by 33.39% in the BL group ($p < 0.01$) (**Figure 1C**). Interestingly, there was no obvious difference in food intake between the HFD and BL groups, indicating that the anti-obesity effect of *B. laterosporus* BL1 treatment was not attributable to reduced food consumption (**Figure 1D**). As presented in **Figures 1E–H**, the iWAT and eWAT weights as well as their ratios to body weight were markedly elevated by the HFD but were significantly reduced by *B. laterosporus* BL1 treatment (−39.07%, $p = 0.0017$; −43.75%, $p = 0.0013$; −33.33%, $p = 0.0113$; −38.50%, $p = 0.0015$, respectively). Moreover, HFD significantly increased the mean adipocyte sizes of iWAT and eWAT in comparison with control mice, whereas *B. laterosporus* BL1 treatment markedly reduced these values ($p < 0.01$) (**Figure 1I**).

In addition, although there was no difference in liver weight among any group, oil red O staining results showed marked lipid accumulation in HFD-fed mice, whereas this accumulation was attenuated by *B. laterosporus* BL1 supplementation (**Supplementary Figure 2**). Taken together, these results suggested that *B. laterosporus* BL1 treatment may alleviate the obesity phenotypes induced by HFD in mice, however, these effects were not observed with the intervention of supernatant of *B. laterosporus* BL1.

Brevibacillus laterosporus BL1 improves dyslipidemia and pathoglycemia in high-fat diet-fed mice

To evaluate the potential of *B. laterosporus* BL1 to regulate hyperlipidemia, lipid concentrations in serum were measured (**Figure 2A**). Relative to the control group, serum levels of TG, T-CHO, HDL-C, and LDL-C were significantly higher in HFD-fed mice ($p < 0.05$), demonstrating that the HFD group had lipid dysmetabolism. *B. laterosporus* BL1 treatment strikingly reduced serum concentrations of TG, T-CHO, and LDL-C ($p < 0.05$) but had no effect on serum HDL-C level. However, there were no alleviative effects on hyperlipidemia induced by HFD in the BLs group.

As obesity is closely correlated with hyperglycemia and hyperinsulinemia, the concentrations of fasting blood glucose and fasting serum insulin were detected. As presented in **Figure 2B**, HFD treatment showed dramatic increases in glucose and insulin levels and HOMA-IR values relative to control mice, while these decreased by *B. laterosporus* BL1 intervention. In addition, the mice in the BL group demonstrated lower area under curve values in the IGTT and ITT relative to those in the HFD group, which further suggests that *B. laterosporus* BL1 ameliorated systemic glucose tolerance and insulin resistance induced by HFD. Collectively, these data suggested a protective effect of *B. laterosporus* BL1 against HFD-induced dyslipidemia and pathoglycemia.

Brevibacillus laterosporus BL1 attenuates secretion of proinflammatory cytokines in high-fat diet-fed mice

Low-grade chronic inflammation is strongly correlated with obesity, which has been shown to induce obesity-related glucose tolerance and insulin resistance (37). To examine whether *B. laterosporus* BL1 administration ameliorated chronic inflammation in HFD-fed mice, we further analyzed proinflammatory cytokines concentrations in serum as well as hepatic mRNA expression of proinflammatory cytokines. Serum IL-1β and TNF-α levels ($p < 0.05$) markedly elevated in HFD-fed mice relative to the control group (**Figure 3A**), while both bacteria and supernatant of *B. laterosporus* BL1 treatment markedly decreased IL-1β and TNF-α levels ($p < 0.01$). Moreover, as presented in **Figure 3B**, HFD significantly upregulated hepatic mRNA expression of IL-1β, TNF-α, IL-6, and IFN-γ, whereas the expressions of these proinflammatory cytokines were significantly downregulated in the BL and BLs groups ($p < 0.05$). These data together indicated that supplementation with either the bacteria or supernatant of *B. laterosporus* BL1 may have anti-inflammatory effects in HFD-fed mice.

Brevibacillus laterosporus BL1 regulates the expression of lipid metabolism related genes in high-fat diet-fed mice

In the liver, the HFD significantly upregulated mRNA expression of lipogenic genes (SREBP1, PPARγ, and FAS) and a lipid uptake gene (CD36) and downregulated mRNA expression of lipolytic genes (HSL and CPT-1) in comparison with the control diet ($p < 0.05$). However, the upregulation of SREBP1, PPARγ, FAS, and CD36 and the downregulation of HSL and CPT-1 induced by HFD were significantly reversed by *B. laterosporus* BL1 treatment ($p < 0.05$). Also, the supernatant of *B. laterosporus* BL1 administration downregulated FAS and CD36 mRNA expression in HFD-fed mice ($p < 0.05$) (**Figure 4A**).

In the iWAT, mice fed with HFD significantly upregulated FAS and CD36 mRNA expression and significantly downregulated HSL mRNA expression relative to mice fed the control diet ($p < 0.05$). Mice treated with *B. laterosporus* BL1, but not supernatant of *B. laterosporus* BL1, exhibited downregulation of SREBP1, PPARγ, FAS, and CD36 mRNA

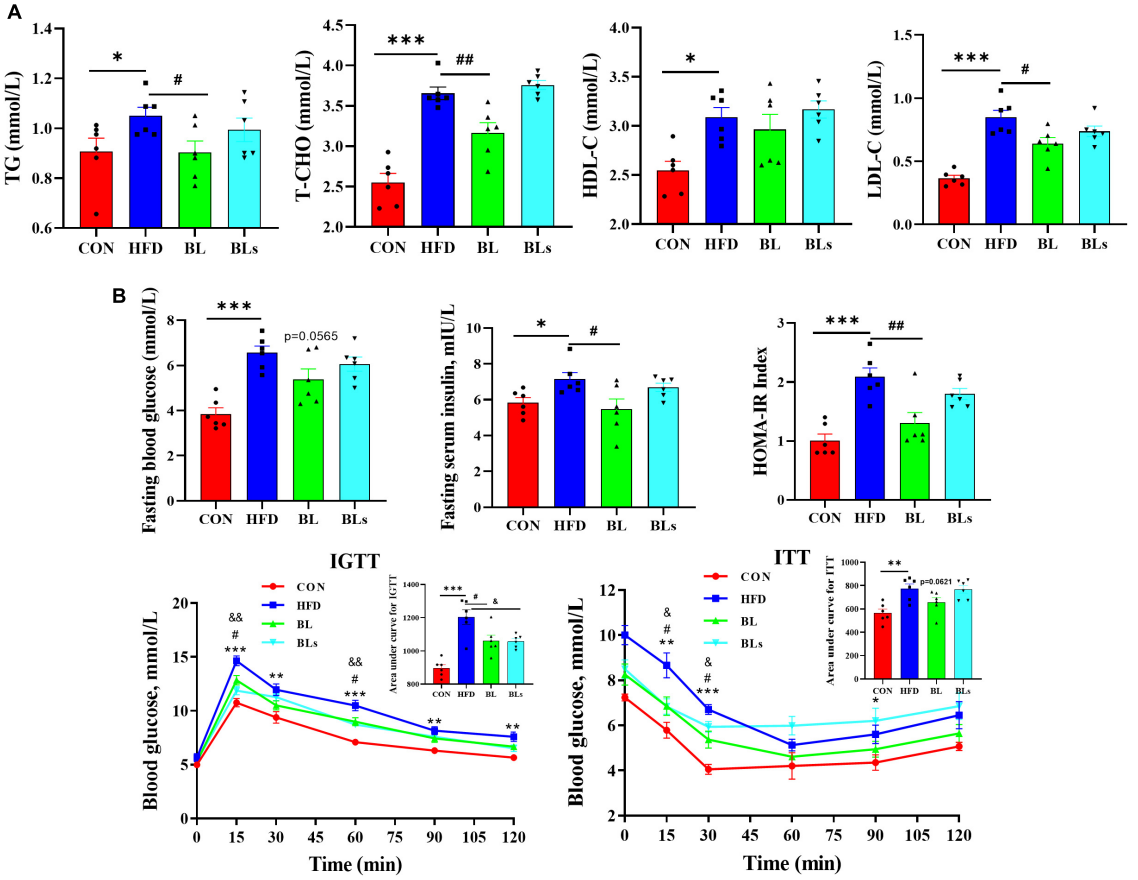


FIGURE 2
Brevibacillus laterosporus BL1 treatment improved serum lipid profile and insulin resistance in HFD-fed mice. **(A)** Serum concentrations of TG, T-CHO, HDL-C, and LDL-C, **(B)** serum concentrations of fasting blood glucose and insulin, IGTT and ITT, curves of blood glucose levels, and the calculated area under curve values. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{*}p < 0.05$ and $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between HFD and BLs are indicated by $^{\&}p < 0.05$.

expression, and upregulation of HSL mRNA expression. CPT-1 mRNA expression did not differ significantly among the four groups (Figure 4B). Overall, these data demonstrated that *B. laterosporus* BL1 exerted a protective effect against lipid accumulation through decreasing lipogenesis and increasing lipolysis in HFD-induced obese mice, and this effect was not exerted by the supernatant of *B. laterosporus* BL1.

Brevibacillus laterosporus BL1 triggers brown adipose tissue browning in high-fat diet-fed mice

The adipocyte size of BAT was the largest in the HFD group (Figure 5A), leading to lipid accumulation (that is, whitening), whereas *B. laterosporus* BL1 treatment prevented HFD-induced whitening ($p < 0.05$). Moreover, *B. laterosporus* BL1 treatment significantly increased the thermogenesis and temperature of BAT (Supplementary Figure 3). Consistently, relative to the HFD group, *B. laterosporus* BL1 administration significantly increased the mRNA expression levels of UCP1 (thermogenesis), CPT-1, PGC-1α (mitochondrial biogenesis), and PRDM16 (adipocyte browning), and significantly reduced the mRNA expression levels of C/EBPα (lipogenesis) and CD36 in BAT ($p < 0.05$) (Figure 5B). These results suggested that *B. laterosporus* BL1 triggered BAT browning to suppress lipid accumulation in mice with HFD-induced obesity.

Brevibacillus laterosporus BL1 modulates gut microbiota composition in high-fat diet-fed mice

To determine the effects of *B. laterosporus* BL1 on the intestinal microbiota compositions of HFD-fed mice, the bacterial 16S rRNA V3–V4 region was sequenced. In

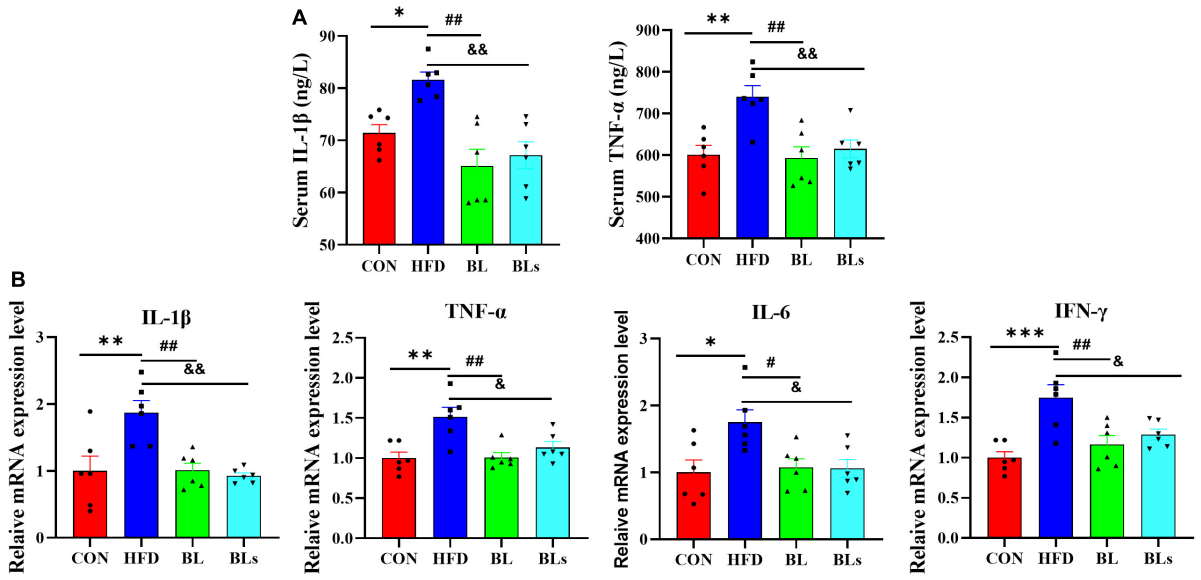


FIGURE 3
Brevibacillus laterosporus BL1 treatment alleviated the secretion of proinflammatory cytokines in HFD-fed mice. **(A)** Serum levels of IL-1β and TNF-α, **(B)** relative mRNA expression levels of IL-1β, TNF-α, IL-6, and IFN-γ were determined using real-time PCR in the liver. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between HFD and BLs are indicated by $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$.

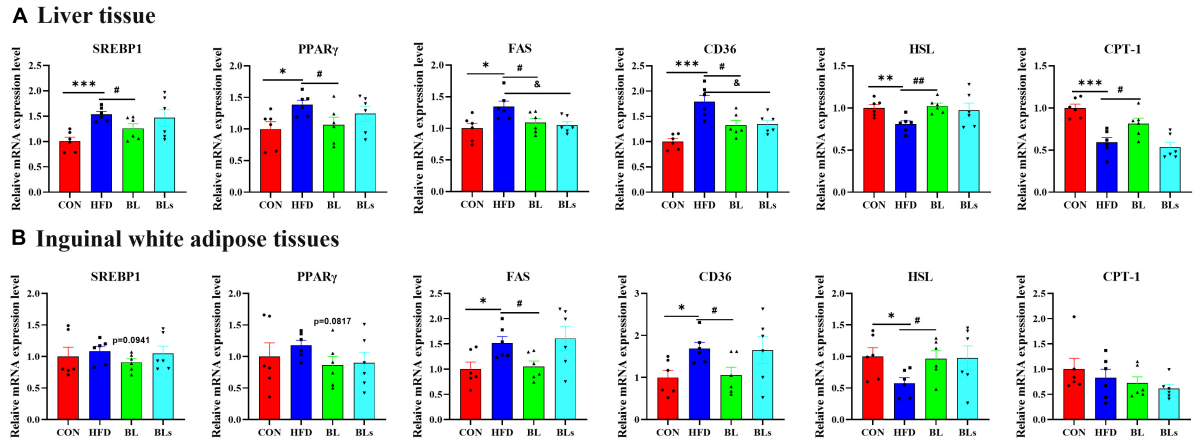


FIGURE 4
Brevibacillus laterosporus BL1 treatment regulated the mRNA expression levels of lipid metabolism-related genes in HFD-fed mice. **(A,B)** Relative mRNA expression levels of SREBP1, PPARγ, FAS, CD36, HSL, and CPT-1 in the liver and inguinal white adipose tissue. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between HFD and BLs are indicated by $^{\&}p < 0.05$.

the current study, an average of 60,433 clean reads were obtained from each sample (n = 6), and a total of 1,099 ASVs were obtained with the sequence denoising approach (Supplementary Table 3). The rarefaction curves for all samples exhibited clear asymptotes, indicating a near-complete sampling of the community (Supplementary Figure 4). As expected, mice fed with HFD showed lower richness and diversity of microbial community, as evidenced by lower Sobs, Shannon, and Simpson indices relative to the CON group. However, there was no obvious difference in α-diversity between the HFD and BL groups, indicating that *B. laterosporus* BL1 treatment did not significantly alter the taxa richness (Figures 6A–C). Venn diagrams (Figure 6D) show the shared and unique ASVs in all the treatment groups. A total of 157 of 1,099

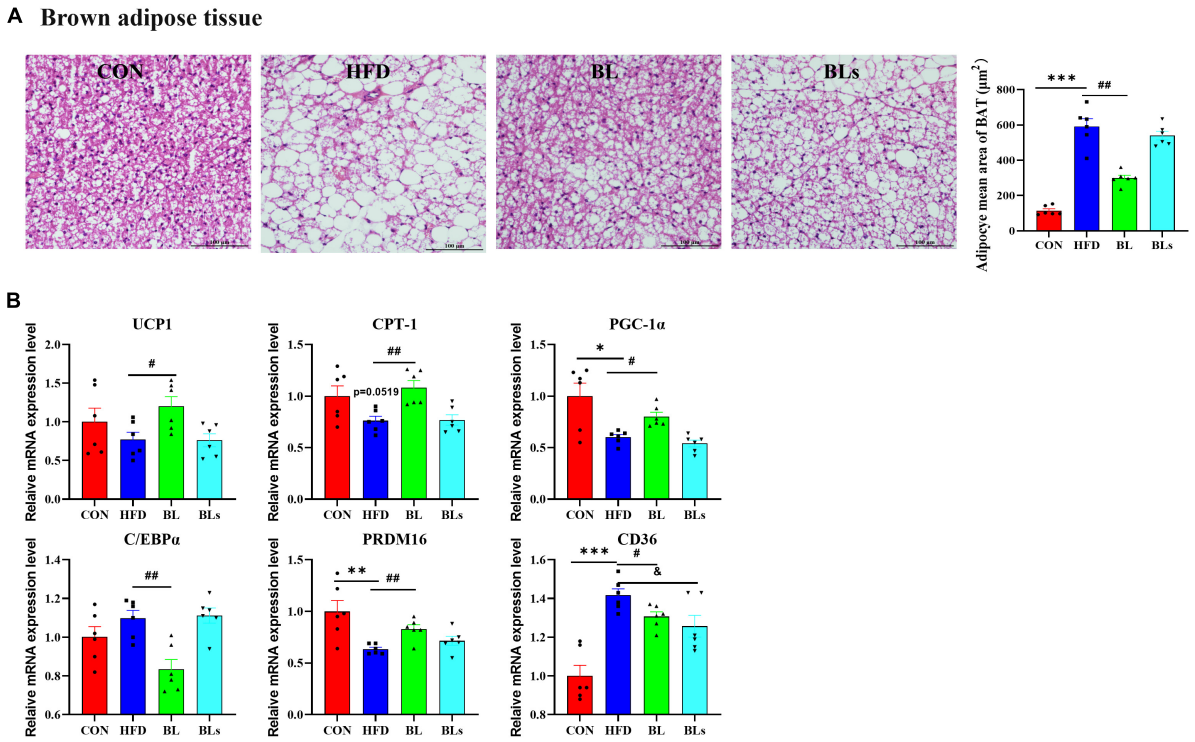


FIGURE 5
Brevibacillus laterosporus BL1 treatment triggered BAT browning in HFD-fed mice. **(A)** H and E staining of brown adipose tissue sections, scale bar = 100 μm, **(B)** relative mRNA expression levels of UCP-1, CPT-1, PGC-1α, C/EBPα, PRDM16, and CD36 in brown adipose tissue. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Significant differences between HFD and BLs are indicated by $^{\&}p < 0.05$.

ASVs overlapped among the four groups, with the CON, HFD, BL, and BLs groups having 409, 91, 131, and 102 specific ASVs respectively, which was consistent with the microbial community diversity. To better explore overall differences in intestinal microbiota structure and composition in all groups, the Bray–Curtis distance-based PCoA analysis was performed (Figure 6E, and PC1 and PC2 were 32.36–15.28%, respectively). The results revealed that mice in the CON, HFD, and BL groups showed distinct clustering of bacteria composition, while the BLs groups clustered closely with the HFD groups.

To further examine the specific alterations in overall microbial communities, the dominant flora among each sample was analyzed at different taxonomic levels. The histogram illustrating gut microbiota at the phylum level revealed that the intestinal microbiota structure in all groups was occupied by Bacteroidota and Bacillota over 90% (Figure 7A). HFD administration significantly reduced Bacteroidota relative abundance and increased Bacillota relative abundance ($p < 0.001$). Nevertheless, *B. laterosporus* BL1 intervention reversed the relative abundance of these bacterial taxa in HFD-induced mice ($p < 0.05$) (Figures 7B,C). The order level analysis revealed that the HFD group possessed higher relative Erysipelotrichales levels but lower Bacteroidales levels, and *B. laterosporus* BL1 treatment significantly reversed the relative abundance of Bacteroidales ($p < 0.05$) (Figures 7D–F). Similar results were observed for *Faecalibaculum* and norank_f_*Muribaculaceae* at the genus level (Figures 7G–I). It is noteworthy that the supernatant of *B. laterosporus* BL1 intervention had no obvious effect on intestinal microbial composition in HFD-fed mice. Therefore, these data demonstrated that *B. laterosporus* BL1, but not its supernatant, can normalize the disturbance of gut microbiota induced by HFD.

Brevibacillus laterosporus BL1 regulates short-chain fatty acid content in high-fat diet-fed mice

Increasing evidence has suggested that SCFAs, the main metabolites of dietary fiber by intestinal microbiota, regulate host lipid metabolism and intestinal microbiota. Thus, colonic SCFA contents were determined in this study. As shown in Figure 8, total SCFAs concentration was maximal in the CON group and minimal in the HFD group, and middle in the BL group. Specifically, *B. laterosporus* BL1 treatment significantly

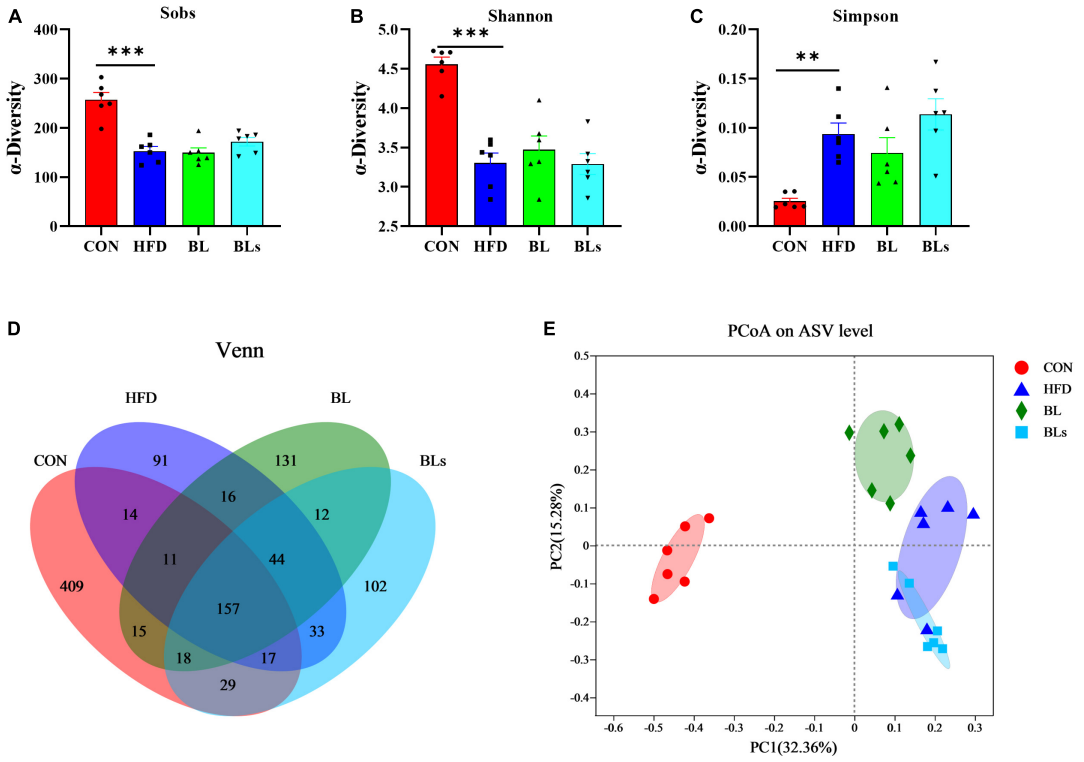


FIGURE 6
Brevibacillus laterosporus BL1 treatment altered gut microbiota diversity and composition in HFD-fed mice. **(A–C)** Sobs, Shannon, and Simpson indices in α-diversity analysis, **(D)** Venn diagrams showing the observed overlap of ASVs, **(E)** PCoA plot analysis from each sample. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$.

increased colonic concentrations of acetic acid, propionic acid, and valeric acid in HFD-fed mice ($p < 0.05$). However, *B. laterosporus* BL1 intervention did not significantly affect colonic butyric, isobutyric, or isovaleric acid levels in HFD-fed mice.

Gut microbiota correlated with short-chain fatty acids and obesity-related parameters

Given the amelioration of obesity-related complications and intestinal microbiota dysbiosis in HFD-induced obese mice by *B. laterosporus* BL1, Spearman's correlation analysis was performed to identify associations between dominant gut bacterial genera and metabolites (SCFAs)/obesity-related parameters (body weight, iWAT weight, eWAT weight, TG, T-CHO, LDL-C, HDL-C, TNF-α, IL-1β, insulin, blood glucose, and HOMA-IR) (**Figure 9**). Most gut bacteria genera were positively correlated with SCFAs and negatively correlated with most obesity-related indices. Specifically, *Lachnospiraceae*_NK4A136_group, unclassified_f__*Lachnospiraceae*, norank_f__*Lachnospiraceae*, *Odoribacter*, *Lachnoclostridium*, *Colidextribacter*, and *Lachnospiraceae*_UCG-006 were strongly positively correlated with at least four kinds of SCFAs and strongly negatively correlated with at least four kinds of obesity-related parameters. Conversely, *Faecalibaculum*, *Romboutsia*, *Blautia*, unclassified_f__*Peptostreptococcaceae*, and *Anaerotruncus* were significantly negatively correlated with at least three SCFA indices, and positively correlated with body weight, iWAT and eWAT weight, T-CHO, LDL-C, HDL-C, blood glucose, and HOMA-IR value. Moreover, *Allobaculum* was positively correlated with TNF-α but *Bacteroides* and unclassified_o__*Bacteroidales* were negatively correlated with IL-1β.

Discussion

Obesity has become an epidemic and a worldwide threat to public health and is responsible for the increased prevalence of chronic diseases such as atherosclerosis, diabetes, and cardiovascular diseases (38, 39). Overwhelming evidence has been reported that intestinal flora is closely associated with the development of obesity and its related metabolic diseases

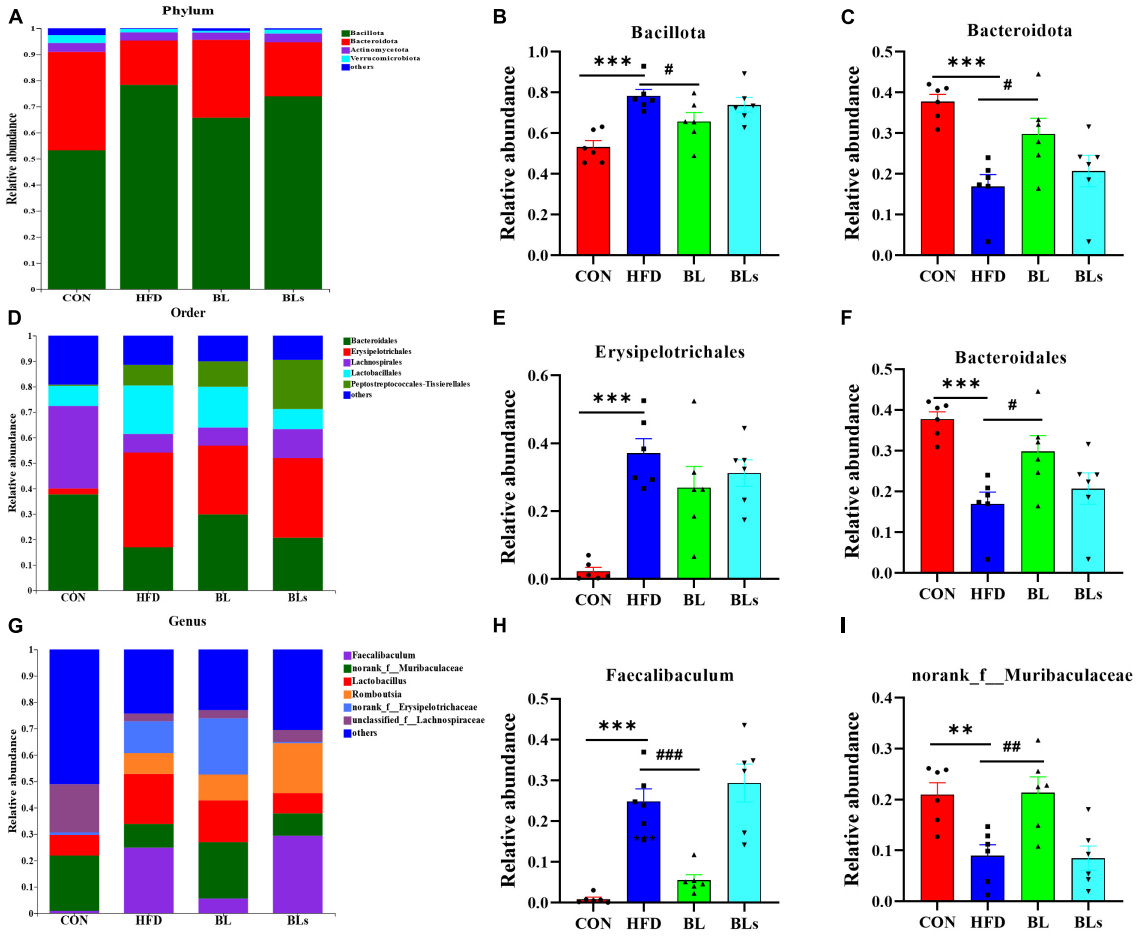


FIGURE 7
Brevibacillus laterosporus BL1 treatment modulated gut microbiota composition in HFD-fed mice. **(A)** Gut bacterial taxonomic profiling at the phylum level. The relative abundances of Bacillota **(B)** and Bacteroidota **(C)**. **(D)** Gut bacterial taxonomic profiles at the order level. Relative abundance of Erysipelotrichales **(E)** and Bacteroidales **(F)**. **(G)** Gut bacterial taxonomic profiling at the genus level. Relative abundances of *Faecalibaculum* **(H)** and norank_f_*Muribaculaceae* **(I)**. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

by influencing energy metabolism, inflammatory response, and glucose metabolism (40, 41). In recent years, increasing evidence has demonstrated that probiotics, including *Lactobacillus*, *Bifidobacterium*, and *Bacillus* can prevent obesity and its related diseases by modulation of intestinal flora in murine models and clinical trials (42–45). Thus, the regulation of gut microbiota with probiotics has attracted much attention as a potential preventive strategy against overweight and obesity. However, there are limited probiotics available as preventive agents for obesity, thus exploring novel strains with anti-obesity properties is quite necessary. Although the *B. laterosporus* BL1 strain is known for broad-spectrum antimicrobial activity, its biological activities in preventing obesity and modulating the related intestinal microbiota dysbiosis have not yet been explored. Here, the ability of *B. laterosporus* BL1 and its culture supernatant on adiposis and the underlying mechanisms associated with gut microbiota in HFD-induced obese mice were investigated. Our results found that prophylactic *B. laterosporus* BL1 bacteria treatment had protective effects against metabolic impairments and gut microbiota dysbiosis in HFD-fed mice, but these were not exerted by supernatant treatment, indicating that the bacteria rather than supernatant were responsible for the beneficial effects of *B. laterosporus* BL1. This is the first study to investigate the bioactivity of *B. laterosporus* BL1 for preventing obesity and regulating the gut microbial community. These findings indicated that *B. laterosporus* BL1 might be served as a promising probiotic to prevent obesity and its related metabolic diseases.

In HFD-fed mice, *B. laterosporus* BL1 intervention decreased body weight gain by 41.26%, which was more impactful than *Companilactobacillus* (*Lactobacillus*) *crustorum* MN047 (−29.56%) (21). Fat mass, mean size of inguinal

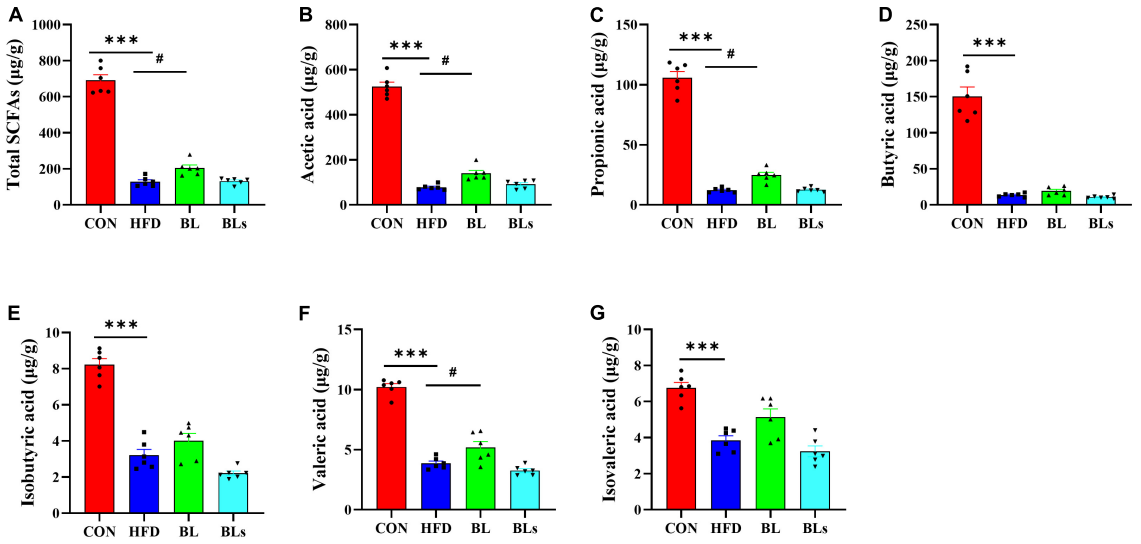


FIGURE 8
Brevibacillus laterosporus BL1 treatment improved SCFA concentrations in HFD-fed mice. **(A–G)** Total SCFAs, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid. Data are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA. Significant differences between HFD and CON are indicated by $^{***}p < 0.001$. Significant differences between HFD and BL are indicated by $^{\#}p < 0.05$.

and epididymal adipocytes, and liver lipid droplet size also significantly decreased as a result of *B. laterosporus* BL1 treatment, consistent with a previous study of the anti-obesity effects of *Bacillus licheniformis* (46). Notably, the food intake between the HFD and BL groups was not statistically significant, indicating that the beneficial effects of *B. laterosporus* BL1 on weight gain and fat accumulation were not due to a reduction in food or energy consumption. Furthermore, HFD mice presented remarkably elevated serum levels of TG, T-CHO, LDL-C, and HDL-C, while *B. laterosporus* BL1 intervention exerted an anti-hyperlipidemic effect in obese mice. Insulin resistance is one of the most common complications in obese individuals (47). As expected, our study found that *B. laterosporus* BL1 treatment significantly improved glucose tolerance and insulin resistance in HFD-induced mice, as evidenced by markedly reduced fasting blood glucose and fasting serum insulin as well as improved IGTT and ITT. Similar results were obtained in previous studies reporting that intake of a *B. licheniformis* and *B. subtilis* mixture effectively improved glucose homeostasis and conferred protection against insulin resistance (22). Therefore, these results indicated positive effects of *B. laterosporus* BL1 intervention on disorders of lipid and glucose metabolism induced by HFD.

Obesity is closely associated with chronic inflammation, which results in whole-body impaired glucose homeostasis and insulin sensitivity (48). In the setting of obesity, immune cell infiltration and proinflammatory activation increase in peripheral tissues, leading to the elevated secretion of proinflammatory cytokines. *Via* autocrine and paracrine effects of proinflammatory molecules, inflammation can interfere with whole-body insulin signaling or induce β-cell dysfunction and subsequent insulin deficiency, resulting in obesity-related insulin resistance (49, 50). In our study, *B. laterosporus* BL1 bacteria intervention remarkably decreased serum IL-1β and TNF-α levels and suppressed hepatic mRNA expression of IL-1β, TNF-α, IL-6, and IFN-γ, indicating that the amelioration of insulin resistance by *B. laterosporus* BL1 intervention may be attributable to decreases in systemic chronic inflammation. Interestingly, these anti-inflammatory effects were also observed in supernatant-treated mice, which is consistent with previous studies showing that *Lacticaseibacillus* (*Lactobacillus*) *rhamnosus* GG culture supernatant significantly inhibited hepatic inflammation and liver injury by attenuation of TNFα production in mice with fatty livers (51).

Accumulating evidence has demonstrated that HFD-induced obesity may alter the expression of lipid-related genes in adipose tissue and the liver such as SREBP1, PPARγ, FAS, CD36, HSL, and CPT-1 (52, 53). Some probiotics regulate the expression of genes related to lipogenesis and lipid metabolism to prevent obesity and ameliorate serum lipid levels (21, 46, 54). In general, SREBP1 is a key transcription factor regulating the expression of multiple lipases which participate in adipocyte differentiation and adipogenesis and then catalyze fatty acid and TG synthesis (55, 56). PPARγ, a well-known nuclear receptor, plays a crucial role in fatty acid uptake and lipogenesis, and down-regulated PPARγ may inhibit downstream enzymes catalyzing fatty acid and TG synthesis, such as FAS (23, 57). CD36 is a multifunctional membrane protein that is critical to facilitating the absorption and intracellular transport of long-chain fatty acids. Furthermore, CD36 is involved

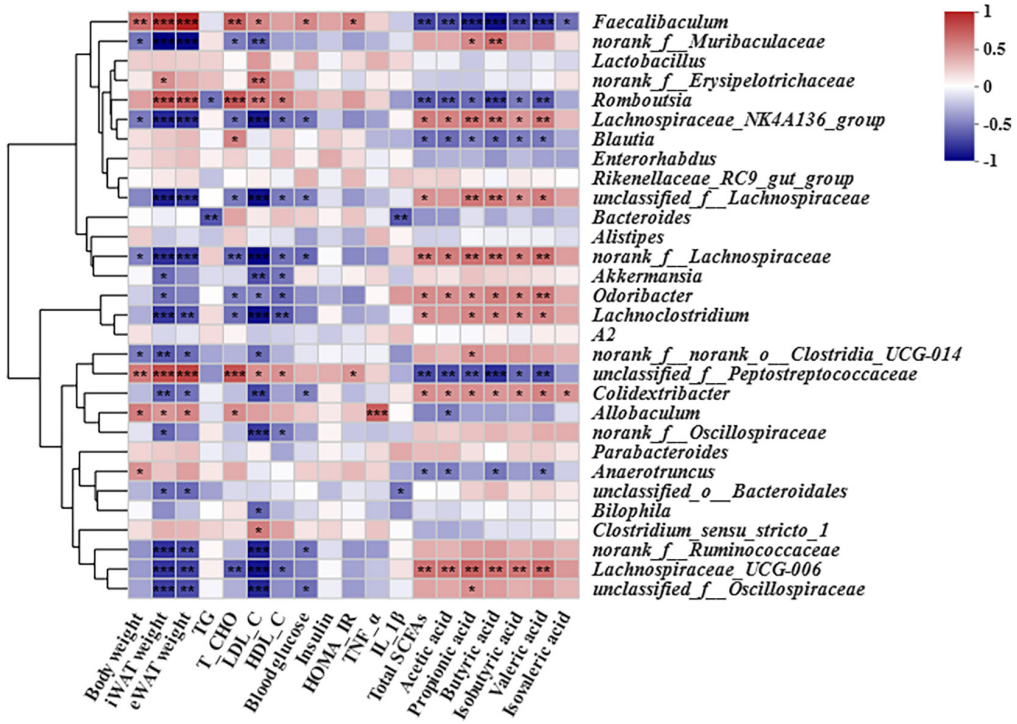


FIGURE 9
Heatmap of Spearman's correlations between gut bacteria relative abundances and obesity-related indices. The top 30 most abundant genera in each sample were used for the hierarchical clustering and heatmap analyses based on Spearman's correlation coefficients. The red and blue blocks represent positive and negative correlations, respectively. Significant correlations are indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ ($n = 6$).

in the regulation of immune responses, chronic metabolic inflammation, angiogenesis, and atherogenesis (58, 59). HSL is one of the key rate-limiting enzymes for lipolysis initiation and catalyzes TG hydrolysis to generate free fatty acid and glycerol (60, 61). CPT-1, the first rate-limiting enzyme in fatty acid β-oxidation, catalyzes the transfer of long-chain fatty acyl-CoA from coenzyme A to L-carnitine and ultimately promotes mitochondrial fatty acid oxidation (23, 62). In the present study, *B. laterosporus* BL1 treatment significantly decreased the expression of SREBP1, PPARγ, FAS, and CD36, as well as increased the expression of HSL and CPT-1 in the liver. Moreover, the expression of FAS and CD36 significantly decreased in the iWAT following *B. laterosporus* BL1 treatment, and HSL expression increased. In addition, BAT is a potential preventive target for obesity, and its activation can stimulate thermogenesis and energy expenditure, thus increasing body weight loss and potentially lowering adipose tissue inflammation (63, 64). Our present study revealed that *B. laterosporus* BL1 supplementation prevented HFD-induced whitening of BAT and increased its activity, as manifested by decreased mean adipocyte size, increased thermogenesis of BAT, and upregulated expression of key genes related to BAT activation (e.g., UCP1, CPT-1, PGC-1α, and PRDM16). Similarly, *Dysosmobacter welbionis,* a human commensal bacterium, has been reported to promote BAT activation by elevated mitochondria number and non-shivering thermogenesis (65). Therefore, these results suggested that *B. laterosporus* BL1 treatment may prevent HFD-induced obesity by reducing fat lipogenesis and accumulation as well as enhancing β-oxidation and BAT activation.

Accumulating evidence suggests that obesity-induced metabolic disorder is closely correlated with intestinal microbiota dysbiosis (29, 66). As a crucial environmental factor and a novel therapeutic target for obesity-related metabolic syndrome, the gut microbiota has been explored deeply in recent years. Moreover, several studies have reported that the beneficial effects of probiotics on HFD-induced obesity and metabolic disorder are mediated by intestinal microbiota (11, 12, 45). It is well-documented that the microbial diversity of healthy-weight individuals is higher than that of obese individuals (67). Consistent with a previous study (68), our study showed that HFD treatment significantly altered gut microbiota richness and abundance based on the results of α-diversity and Venn diagrams analyses. Although

B. laterosporus BL1 treatment did not significantly change gut microbiota α-diversity, a distinct clustering pattern in gut microbiota structure between the HFD and BL groups was observed. In addition, HFD treatment may result in increased Bacillota relative abundance and decreased Bacteroidota relative abundance (28). Bacillota and Bacteroidota are the dominant phyla in human intestinal microbiota, occupying over 90% of all sequences, and are mainly responsible for energy absorption related to gut flora (36, 69). In the current study, *B. laterosporus* BL1 intervention significantly reversed the relative abundance of Bacillota and Bacteroidota. This is similar to the findings that the prevention of *B. amyloliquefaciens* SC06 in HFD-induced obesity is accompanied by a reduction in Bacillota relative abundance and an elevation in Bacteroidota relative abundance (10). Collectively, our results suggested that *B. laterosporus* BL1 intervention helped maintain a relatively healthy microbiome, thus preventing obesity in HFD-fed mice.

Our results showed that *B. laterosporus* BL1 administration also significantly reversed some of the alterations in gut microbiota induced by an HFD, including Bacteroidales at the order level, and *Faecalibaculum* and norank_f_*Muribaculaceae* at the genus level. Bacteroidales plays a key role in the host metabolism of carbohydrates and proteins associated with gut flora, which is negatively correlated with body weight gain and fat accumulation (70). A rodent model showed that the elevation of Bacteroidales abundance by oligosaccharides from *Gracilaria lemaneiformis* may help attenuate HFD-induced obesity and metabolic syndrome (71). *Faecalibaculum* is a proinflammatory bacterium that may impair the gut barrier and is relative to various metabolic diseases, such as obesity, cardiovascular disease, and diabetes (72). Conversely, norank_f_*Muribaculaceae* is a potentially beneficial bacterium, and elevated norank_f_*Muribaculaceae* concentration helped to alleviate obesity and insulin resistance in HFD-induced mice (73). These dominant bacterial taxa, which were reversed by *B. laterosporus* BL1 intervention, may have helped prevent obesity and metabolic syndrome in the current study. Furthermore, Spearman's correlation analysis revealed that the bacteria that increased in abundance as a result of HFD administration were positively correlated with obesity-related parameters but negatively correlated with SCFAs concentration. On the contrary, *B. laterosporus* BL1 treatment promoted expansion in the relative abundance of bacterial genera that were positively correlated with SCFAs but negatively correlated with obesity-related parameters (e.g., norank_f_*Muribaculaceae*, *Odoribacter*, and *Lachnoclostridium*). Notably, our study found that SCFAs, the important signaling molecules in the communication between host and gut microbiota (74), were significantly increased by *B. laterosporus* BL1 treatment, especially acetic acid, propionic acid, and valeric acid. These results suggested that another possible mechanism of action of *B. laterosporus* BL1 preventing obesity was the promotion of SCFAs-producing bacteria and the subsequent elevation of colonic SCFA concentrations, resulting in increased body weight loss in HFD-fed mice.

In conclusion, this study suggested that prophylactic *B. laterosporus* BL1 bacteria supplementation effectively prevented body weight gain, fat accumulation, chronic inflammation, and BAT whitening, as well as attenuated lipid profiles and insulin resistance in HFD-induced mice. *B. laterosporus* BL1 bacteria intervention may also modulate the HFD-induced structural and componential alteration of gut flora, especially by increasing the relative abundance of SCFA-producing bacteria and subsequently elevating SCFA levels, which may be associated with the role of *B. laterosporus* BL1 in preventing obesity. Our study demonstrated that *B. laterosporus* BL1 is a promising probiotic candidate to prevent obesity and its related syndrome. However, HFD is characterized by a high-fat content and monotonicity compared to the human diet, this may affect the credibility and translational value of experiment results (75). Therefore, further investigations, especially long-term clinical trials, are required to confirm these findings.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA876212.

Ethics statement

This animal study was reviewed and approved by the Animal Care and Use Committee of South China Agricultural University.

Author contributions

GW and JH participated in the writing and editing of the manuscript. XM, MS, and YY contributed to the study design. DD and JD revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1050025/full#supplementary-material

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Mirco Vacca,
University of Bari Aldo Moro, Italy
Ramona Suharoschi,
University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania

*CORRESPONDENCE
Ali Chaari
alc2033@qatar-med.cornell.edu;
ali.chaari@yahoo.fr

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Can probiotic, prebiotic, and synbiotic supplementation modulate the gut-liver axis in type 2 diabetes? A narrative and systematic review of clinical trials

Yousef Al-Najjar[1], Maryam Arabi[1], Pradipta Paul[1] and Ali Chaari[2]*

[1]Medical Education Division, Weill Cornell Medicine-Qatar, Cornell University, Qatar Foundation – Education City, Doha, Qatar, [2]Premedical Division, Weill Cornell Medicine-Qatar, Qatar Foundation – Education City, Doha, Qatar

Background: Type 2 diabetes, one of the most common noncommunicable diseases, is a metabolic disorder that results in failed homeostatic control in several body systems, including hepatic function. Due to the gut microbiome's potential role in diabetes' pathogenesis, prebiotics, probiotics, and synbiotics have been proposed as complimentary therapeutic approaches aimed at microbiota readjustment.

Methods: A systematic review was conducted on PubMed, Scopus, Web of Science, Embase, and the Cochrane Library examining the effect of probiotics, prebiotics, and synbiotics on hepatic biomarkers in patients with diabetes.

Results: From 9,502 search hits, 10 studies met the inclusion criteria and were included in this review. A total of 816 participants (460 intervention and 356 control) were investigated for the effects of nine different hepatic biomarker measurements including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, bilirubin, liver steatosis, liver stiffness, fatty liver index, and gamma-glutamyl transferase levels. Of the 13 intervention groups analyzed from the 10 studies, 3 were prebiotic interventions, 3 were single species probiotic interventions, 3 were multi-species probiotic interventions, and 4 were synbiotic interventions. Nutraceuticals used in these trials included six genera of bacteria (*Lactobacillus, Bifidobacterium, Streptococcus, Acetobacter, Lactococcus*, and *Propionibacterium*), five different prebiotic formulations (inulin, inulin and beta carotene, chicory inulin enriched with oligofructose, galacto-oligosaccharides syrup, and powdered cinnamon), or a combination of these to form multi-species probiotics or synbiotics.

Conclusion: Although some studies showed insignificant changes in hepatic biomarkers, generally the results yielded a decrease in liver damage due to reduced oxidative stress, pro-inflammatory cytokines, gut dysbiosis, and insulin resistance which led to improvements in hepatic biomarker levels.

KEYWORDS

gut microbiome, dysbiosis, nutraceutical, clinical trial, liver function

Introduction

Type 2 diabetes mellitus (T2DM) is an ever-growing global health concern. In 2021, its prevalence globally in 20–79-year-old individuals was approximated to be 536.6 million individuals, representing 10.5% of global population (1). These figures are expected to rise to 783.2 million cases (12.2%) by 2045 (1). Genetic predispositions, environmental influences, metabolic disorders, and aging are strongly associated with the onset of T2DM and are therefore intertwined with these rising trends (2). In 2019, T2DM was found to be the root cause of 1.5 million deaths worldwide, with 48% of these fatal outcomes occurring in patients under the age of 70 (3). In addition to considerable mortality and morbidity, T2DM also takes a heavy financial toll on healthcare systems: the total expenditure of diagnosed T2DM was estimated to be around 327 billion USD in 2017, signifying a 26% increase over a period of 5 years (4).

The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) emphasize the need for both lifestyle changes and pharmacological drugs in the long-term control of T2DM (5). Nonetheless, drugs that are currently used over a long period of time have many drawbacks, especially those that are used for insulin replacement (6). These drawbacks include the inability to inhibit the autoimmune response that causes the impairment of pancreatic β-cells, and ineffectiveness in preventing various diabetic complications, including cardiovascular problems (7). Most antidiabetic drugs aim to stabilize blood glucose levels in patients with T2DM, but probiotics, prebiotics, and synbiotics could be used as complimentary or adjunct therapies *via* dietary intervention to treat one of the root causes – gut dysbiosis. Several studies were conducted on the effects of these supplements on the gut microbiota in diabetic patients and revealed significant improvements in patients' inflammatory and oxidative, glycemic, and lipid profiles (8–10). These improvements are due to the supplements' ability to counter bacterial translocation, reduce chronic inflammation, and enhance the body's metabolic status (11).

Disruption of the gut-liver axis in diabetes

The indigenous bacterial population of the human intestine is composed of around 100 trillion bacteria, almost 10 times more cells than there are in the human body (12). Of these bacteria, 35,000 species were identified and categorized into six phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (13). One of the primary functions of the gut microbiota is to increase the energy available from organic polymers otherwise resistant to digestion by human enzymes. For instance, the phylum Bacteroidetes is believed to aid in the digestion of plant cell wall compounds, N-glycans and O-glycans, thereby unlocking energy *via* the release of short chain fatty acids (SCFAs), which account for 7–10% of daily caloric requirements (14, 15). SCFAs, such as butyrate, acetate, and propionate, are not only largely used as a source of energy, but are also crucial modulators of several physiological pathways, including the adaptive immunity's anti-inflammatory response *via* the downregulation of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-1β (16). The metabolites made by SCFA-producing bacteria in the gut have also been implicated in the maintenance of intestinal integrity and stimulating mucus production (17).

This collectively makes the gastrointestinal tract an important component in the structural, humoral, and physiological development of the body, as well as in the progression of diseases such as T2DM, as shown in **Figure 1**. Several studies have demonstrated the association between T2DM and gut microbial dysbiosis, such as an observed decrease in the amount of SCFA-producing bacteria in conjunction with a rise in opportunistic bacteria in patients living with T2DM (18). The effects of a lack of SCFA in T2DM patients are complex and varied, ranging from impairment of receptor-mediated signaling in pancreatic beta cells, to dysregulation of the gut-brain axis and hepatic encephalopathy (19). Furthermore, it has been suggested that the translocation and ratio alterations of the intestinal microbiome in diabetic and obese patients may lead to the metabolism of otherwise insoluble and indigestible carbohydrates, significantly raising

energy harvesting and adiposity in the liver (20). Dysbiosis is also involved in chronic inflammation and oxidative stress (21). This is partly due to the production and release of endotoxins, including lipopolysaccharides (LPS), as well as the translocation of intestinal bacteria from the gut microbiota into the bloodstream (22). These bacteria and bacteria-derived LPS travel through the bloodstream, where LPS binds to Toll-like receptor 4 (TLR4) on all hepatocytes, especially Kupffer cells (23). This interaction causes the release of TNF-α, which induces the hepatic inflammatory responses and liver fibrosis associated with T2DM, and the progression from non-alcoholic fatty liver disease to nonalcoholic steatohepatitis (NASH) (24). As the liver becomes more severely damaged, enzymes such as transaminases, which are usually located inside the hepatocytes, are released into the serum from the impaired cells, increasing their levels in serum (25). On the other hand, proteins such as albumin that are normally manufactured and secreted by the liver are no longer produced due to destruction of cell machinery, leading to their decreased serum levels (26).

The various hepatic biomarkers

Medical tests used to evaluate liver function include serum biomarkers of injury [e.g., transaminases, γ-glutamyl transferase, and alkaline phosphatase (ALP)], hepatic dysfunction [e.g., liver stiffness (LS)], and antioxidants (e.g., bilirubin). Such biomarkers are utilized as proxies for liver health, one of the affected physiologies in T2DM (27).

Transaminases

Aminotransferases, or transaminases, are enzymes that catalyze the transfer of amino groups in the reversible conversion of amino acids and oxoacids (28). Among many transaminases, alanine transaminase (ALT) and aspartate transaminase (AST) are two enzymes that are clinically significant, especially in liver function tests (29). Damage to hepatocytes causes high activity levels of serum aminotransferases in T2DM and is therefore used as a diagnostic tool for insulin resistance and metabolic syndrome (30). This is due to disruption of the cell plasma membranes of liver cells, causing leakage of these enzymes (31).

Alkaline phosphatase

Aside from aminotransferases, ALP is also considered to be an independent risk factor for the development of T2DM and can be a potential biomarker for the prediction of T2DM (32). ALP occurs as a variety of isoenzymes with different roles in the body depending on tissue type. In the liver's canalicular membrane, it acts as a catalyst for the hydrolysis of organic phosphate esters. ALP is mainly produced by the liver and bone.

Total protein

Several proteins, including albumin, are produced by the liver. These proteins are important in maintaining osmotic pressure, the transfer of macromolecules, and fighting off infections. Decreased levels of total protein may be an indicator of liver damage. Specifically, serum albumin has been found to have an association with insulin resistance, which primarily induces T2DM (33).

Bilirubin

Bilirubin is an orange-yellow substance usually found in a conjugated state (34). It is naturally released during the breakdown of heme in erythrocytes by heme oxygenases (HO) (35). Bilirubin is an important molecule in the body due to its antioxidant properties, such as reducing the effect of LDL oxidation and preventing lipid peroxidation (36). T2DM has been shown to cause oxidative stress, which consequently leads to elevated levels of bilirubin (37).

Liver steatosis

Liver steatosis is an increased level of hepatocellular lipids (HCL). The causes of liver steatosis include elevated levels of free fatty acids and adipocytokines, which consequently leads to high amounts of HCL, as is commonly seen in insulin resistance and T2DM (38). The progression of liver steatosis to nonalcoholic fatty liver disease (NAFLD) can be indicated by mitochondrial and inflammatory malfunction (39).

Gamma-glutamyl transferase

Gamma-glutamyl transferase (GGT) is an enzyme that plays a role in the metabolism and homeostasis of extracellular reduced glutathione (GSH), an antioxidant (40). GGT is expressed on the luminal surface of ducts and tracts throughout the body, especially in the kidneys. A high level of GGT is considered to be a predictor of T2DM. Patients with T2DM have higher oxidative stress, leading to beta-cell dysfunction in the pancreas followed by insulin resistance and T2DM. To decrease oxidative stress, higher levels of GGT are expressed to increase the antioxidant activity of GSH (41).

In light of the key role of the gut microbiota in the development and progression of liver injury in T2DM, the use of probiotics, prebiotics, and synbiotics has been presented

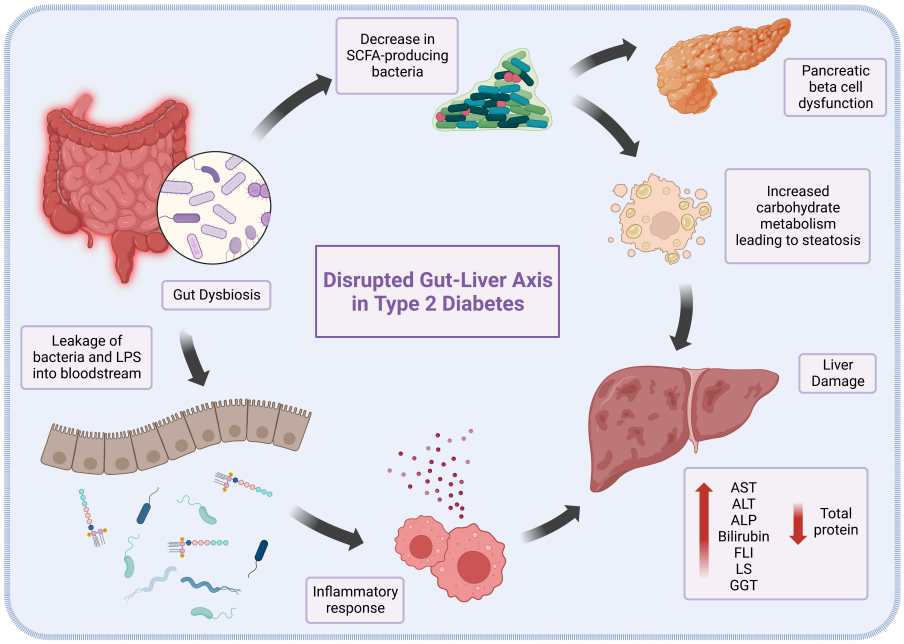

FIGURE 1
The role of gut dysbiosis in the pathogenesis and progression of type 2 diabetes-induced liver damage.

as a potential therapy for the reestablishment of homeostasis (42). Probiotics are defined as live microorganisms commonly found in food that provide health benefits to the host when administered in appropriate quantities, while prebiotics are substrates utilized by these microorganisms to grow and evoke health benefits to the host (43). Mixtures of prebiotics intended to be selectively used by the co-delivered probiotics are referred to as synbiotics (44). All three biotics work towards restoring balance in the gastrointestinal microbiome, especially following dysbiosis, and enhancing the intestinal mucosa and immunity (45). Although several studies have investigated the use of biotics in patients with T2DM, there are no reviews that compare the efficacies of different pro/pre/synbiotic combinations and dosages on hepatic biomarkers among diabetics. This review aims to explore the mechanisms and effectiveness of the use of probiotics, prebiotics, and synbiotics as interventions for the regulation of hepatic biomarkers in patients with T2DM.

Methods

Study protocol and search strategy

The protocol for this systematic review was developed using the Preferred Reporting Items for Systematic Reviews (PRISMA). A comprehensive search for published works was undertaken in PubMed, Scopus, Web of Science, Embase, and the Cochrane Library. A gray literature search was performed in ClinicalTrials.org and ProQuest Dissertations and Theses. The primary search was done in June 2020 to examine the effect of probiotics, prebiotics, and synbiotics on hepatic biomarkers in patients with T2DM. A final search was performed in April 2022 to collect any newly published data. A comprehensive breakdown of the search strategy is provided as **Supplementary Table 1**. Briefly, the search on PubMed consisted of the elements below:

> *("Probiotics"[MeSH Terms] OR "probiotics"[Title/Abstract] OR "probiotic"[Title/Abstract] OR "Prebiotics"[MeSH Terms] OR "prebiotic"[Title/Abstract] OR "prebiotics"[Title/Abstract] OR "Synbiotics"[MeSH Terms] OR "synbiotics"[Title/Abstract] OR "synbiotic"[Title/Abstract] OR "symbiotic"[Title/Abstract] OR "symbiotics"[Title/Abstract] OR "gastrointestinal microbiome"[MeSH Terms] OR "gut microbiome"[Title/Abstract] OR "gut flora"[Title/Abstract]) AND ("diabetes mellitus, type 2"[MeSH Terms] OR "T2D"[Title/Abstract] OR "type 2 diabetes"[Title/Abstract]).*

Eligibility criteria, screening, and data extraction

Inclusion Criteria: only clinical studies investigating the effect of probiotic, prebiotic, or synbiotic supplementation on hepatic biomarkers in patients diagnosed with T2DM were

included. Studies of any duration, involving adults of any age, sex, ethnicity, from any region worldwide, and published at any time, were included.

Exclusion Criteria: studies that included participants diagnosed with other types of diabetes were excluded. Further, we excluded reviews, conference proceedings, abstracts, editorials, animal studies, and other non-clinical forms of literature. Further, records with full texts in non-English languages or those that did not provide details on hepatic biomarkers were omitted. Lastly, we also excluded studies that administered non-bacterial organisms as probiotics or synbiotics.

Studies identified through multi-database searching were imported into Covidence (Veritas Health Innovation, Melbourne, VIC, Australia), which automatically detected and removed duplicates prior to manual screening. At least two independent reviewers systematically screened all remaining studies according to the eligibility criteria. Conflicts were resolved *via* consensus following discussion or by an independent reviewer. All included studies were processed for qualitative analysis and the relevant data from each study was extracted, grouped by themes, and analyzed in the discussion. Extracted data elements from each study included study characteristics, such as first author's last name, country in which the trial was conducted, study design, trial duration, and investigated biomarker; participant characteristics for both intervention and placebo/control groups, such as mean and standard deviation (SD) of age and baseline body mass index (BMI), total number of participants and ratio of sexes, presence of inclusionary comorbidities; and intervention characteristics such as type, composition, and daily dosage of nutraceutical and control/placebo substance. Changes in liver biomarkers were extracted in the most suitable form provided by the authors and color-coded. This was in the form of intragroup change from baseline to end-of-trial, intergroup mean difference (MD) between intragroup changes, or comparison of both intra-group changes. Classification of nutraceutical type was made after careful examination of nutraceutical formulation.

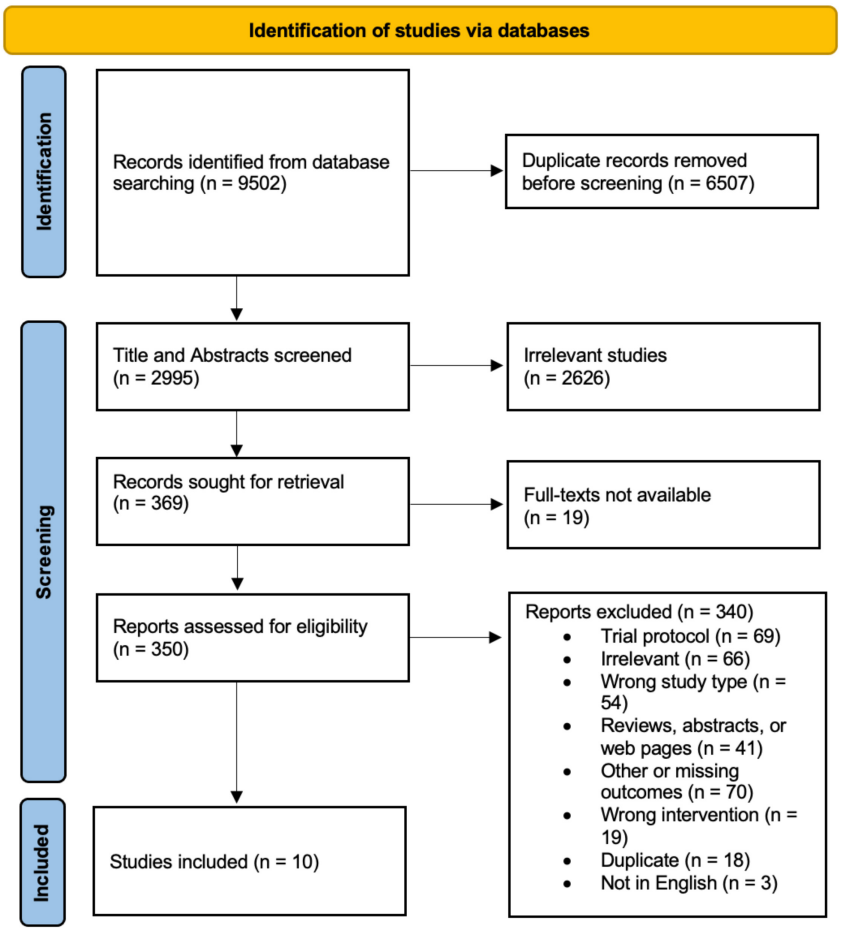

FIGURE 2
PRISMA study selection flow chart.

Risk of bias assessment

A pre-piloted Excel form, the Cochrane revised risk-of-bias tool version 2 (RoB2), was used for scoring and reporting the risk of bias associated with individual studies (46). Factors that could lead to various risk of bias, including the randomization process, allocation concealment, participant recruitment, deviations from intended intervention, missing outcome data, outcome measurement, and selection of reported results, in addition to overall bias, were rated by independent reviewers. The domains above were scored with low risk, some concerns, or high risk of bias.

Results

Search results

The PRISMA study selection protocol flowchart is shown in Figure 2. Briefly, 9,502 records were identified from database searching, of which only 10 were included in the qualitative synthesis and analysis in this review. Of the initial 9,502 records, 6,507 duplicates were removed automatically prior to manual screening. Title and abstract of the remaining 2,995 records were screened, of which 2,626 were found to be irrelevant under the eligibility criteria. Further, 19 full-texts could not be retrieved, leaving 350 reports for full-text screening. Of these, 340 were excluded for various reasons as elucidated in Figure 2, leaving 10 studies for extraction.

Trial characteristics

In total, 10 studies explored the effects of biotics on hepatic biomarkers, of which 10 studies tested for changes in AST, 10 studies tested ALT, 1 study tested liver steatosis, 6 studies tested ALP, 1 study tested LS, 1 study tested FLI, 1 study tested total protein, 3 studies on bilirubin, and 1 study tested GGT (47–56). The studies involved 816 patients, including 460 in the intervention groups and 356 in the control groups. All subjects were diagnosed with T2DM based on the studies' individual criteria. Of the studies included, six were from Iran, one from Malaysia, one from Japan, one from Ukraine, and one from Sweden. Median year of publication was 2017 (IQR 2016–2017). The median of the mean age of the intervention groups was 54.1 years (IQR 51.8–59.0), and the median of the mean BMI was 30.6 (IQR 29.8–31.7). The median of intervention periods was 8 weeks. The trials extracted included three studies with prebiotics, three studies with single species probiotics, three studies with multi-species probiotics, and four studies with single species synbiotics. Six genera of bacteria were used in the probiotics and synbiotics, namely *Lactobacillus, Bifidobacterium, Streptococcus, Acetobacter, Lactococcus*, and *Propionibacterium*. Names of the bacterial species, *Lactobacillus sporogenes* (*Bacillus coagulans*), *Lactobacillus acidophilus*, *Limosilactobacillus reuteri*, *Lacticaseibacillus casei*, *Lactococcus lactis*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Lacticaseibacillus rhamnosus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium breve*, and *Streptococcus thermophilus*, were extracted and adjusted based on the most updated nomenclature. The median dosage for probiotics in single and multi-species probiotics and single species synbiotics was 1×10^8 colony forming units per day (CFU/day; IQR: 1 10^8 to 3.5 10^{10}; range: 1 10^7 to 1 10^{11}). The prebiotics used included inulin, inulin and beta carotene, chicory inulin enriched with oligofructose, galacto-oligosaccharides syrup, and powdered cinnamon. The median mass of prebiotics administered both alone and within a synbiotic is 0.5 g/day (IQR: 0.5–9.2; range: 0.04–10).

Risk of bias and publication bias assessment

Using the Cochrane collaboration RoB2 tool, a risk of bias assessment was performed. All 10 studies had a low risk of bias in the randomization process, participant recruitment, intended intervention, missing outcome data, and outcome measurement and selection (Supplementary Figure 1).

Effects on alanine transaminase levels

Table 1 shows a summary of the fourteen interventions in 10 studies that examined the effect of biotics on ALT levels in patients with T2DM (47–56). The interventions consisted of a total of 816 patients, including 460 patients that were given a biotic and 356 given a placebo. As seen in Figure 3A, single species probiotics were administered in four trials, multi-species probiotics in three trials, prebiotics in three trials, and single species synbiotics in four trials. An increase in ALT levels was seen in five trials, while nine trials demonstrated a decrease. In the trials with an increase in ALT levels, two used single species probiotics, two used prebiotics, and one used single species synbiotics. Among the trials that showed a decrease in ALT levels, two trials administered single species probiotics, three administered multi-species probiotics, one administered prebiotic, and three administered single species synbiotics.

Effects on aspartate transaminase levels

There were 14 interventions in 10 studies on the effects of biotics on AST levels, as summarized in Table 1 (47–56). The trials included 816 patients, of which 460 patients were given a biotic and 356 patients were given a placebo. Depicted in Figure 3B, single species probiotics were used

TABLE 1 Studies investigating changes in hepatic biomarkers following intervention with probiotics, prebiotics, and synbiotics.

Type of nutraceutical	Study design and country	Participant* demographics size/sex (*n*, F/M) age (mean ± SD; years) BMI (mean ± SD; kg/m^2)		Control/ placebo substance administered	Interventional nutraceutical administered	Control/ placebo and intervention dose × frequency	Intervention duration	Effect on markers	Mean change in markers$^{\Phi}$	References
		Control/placebo	Intervention							
Probiotic (single species)	R, DB, C, CT (Iran)	*n* = 27 (12M/15F) 58.2 ± 11.8 BMI NR	*n* = 30 (10M/20F) 59.7 ± 12.2 BMI NR	Capsule containing 0.5 g of rice flour powder	Capsule containing *L. acidophilus* (10^8 CFU)	1 capsule/day	3 months	↓ AST	Markers NR	Mirmiranpour et al. (56)
								↓ ALT	Markers NR	
Probiotic (single species)	DB, R, PG, PC (Sweden)	T2D and obese patients* *n* = 15 (11M/4F) 65 ± 5 30.7 ± 4.0	T2D and obese patients*; low dose group *n* = 15 (12M/3F) 66 ± 6 30.6 ± 4.5	Capsule with mildly sweet tasting powder in an aluminum laminate stick pack	Capsule containing low-dose *L. reuteri* DSM 17938 (10^8 CFU/capsule)	1 capsule/day	12 weeks	↑ Liver steatosis (♀)	14.0 ± 8.4% fat (I, 12w) vs. 13.9 ± 8.7% fat (I, B) (♀)	Mobini et al. (47)
								↓ AST (♀)	0.38 ± 0.11 μkat/L (I, 12w) vs. 0.40 ± 0.14 μkat/L (I, B) (♀)	
								↑ ALT (♀)	0.52 ± 0.15 μkat/L (I, 12w) vs. 0.50 ± 0.17 μkat/L (I, B) (♀)	
			T2D and obese patients*; high dose group *n* = 14 (11M/3F) 64 ± 6 32.3 ± 3.4	Capsule with mildly sweet tasting powder in an aluminum laminate stick pack	Capsule containing high-dose *L. reuteri* DSM 17938 (10^{10} CFU/capsule)	1 capsule/day	12 weeks	↓ Liver steatosis (♀)	11.3 ± 8.6% fat (I, 12w) vs. 12.0 ± 9.0% fat (I, B) (♀)	
								· AST (♀)	0.40 ± 0.12 μkat/L (I, 12w) vs. 0.40 ± 0.12 μkat/L (I, B) (♀)	
								↑ ALT (♀)	0.53 ± 0.20 μkat/L (I, 12w) vs. 0.51 ± 0.15 μkat/L (I, B) (♀)	
Probiotic (single species)	R, DB, PC, CT (Iran)	Control bread (CB) *n* = 27 (5M/22F) 53.4 ± 7.5 30.5 ± 4.1	Probiotic bread *n* = 27 (5M/22F) 52.0 ± 7.2 29.8 ± 5.7	Control bread	Bread containing *L. sporogenes* (1 × 10^8 CFU/g)	40 × 3 g/day	8 weeks	↓ ALT (♀)	−1.8 ± 8.3 IU/L vs. +1.4 ± 9.7 IU/L (♀)	Bahmani et al. (48)
								↑ AST (♀)	0.01 ± 18.5 IU/L vs. +2.1 ± 14.1 IU/L (♀)	
								↓ ALP (♀)	−8.1 ± 31.73 IU/L vs. −5.4 ± 47.3 IU/L (♀)	

(Continued)

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TABLE 1 (Continued)

Type of nutraceutical	Study design and country	Participant* demographics size/sex (*n*, F/M) age (mean ± SD; years) BMI (mean ± SD; kg/m^2)		Control/ placebo substance administered	Interventional nutraceutical administered	Control/ placebo and intervention dose × frequency	Intervention duration	Effect on markers	Mean change in markers$^{\Phi}$	References
		Control/placebo	Intervention							
Probiotic (multi-species)	DB, PC, PG, RCT (Ukraine)	Patients with T2D and NAFLD *n* = 24 (NR) 57.38 ± 9.92 32.55 ± 3.62	Patients with T2D and NAFLD *n* = 26 (NR) 53.23 ± 10.09 33.19 ± 4.93	Organoleptically similar formulation as intervention	Symbiter Forte (combination of 250 mg smectite gel) and *Bifidobacterium* (1×10^{10} CFU/g), *Lactobacillus* + *Lactococcus* (6×10^{10} CFU/g), *Acetobacters* (1×10^{6} CFU/g), and SCFAs producing *Propionibacterium* (3×10^{10} CFU/g) genera	10 × 1 g/day	8 weeks	↓ ALT	−6.62 ± 13.07 IU/L or −10.32 ± 32.1%	Kobyliak et al. (49)
								↓ AST	−3.31 ± 6.88 IU/L or −6.20 ± 19.22%	
								↓ LS	−0.254 ± 0.85 kPa (−4.427 ± 12.6%) vs. +0.262 ± 0.77 (+2.38 ± 10.25%)	
								↓ FLI (ŝ)	−0.750 ± 1.23 (−1.194 ± 8.43%) vs. +3.769 ± 1.84 (+4.471 ± 12.15%) (ŝ)	
Probiotic (multi-species)	R, DB, PC, CT (Iran)	*n* = 30 (sex NS) 52.1 ± 6.9 30.7 ± 4.1	*n* = 28 (sex NS) 49.6 ± 9.9 31.9 ± 6.4	100 mg fructo-oligosaccharide with lactose/capsule	Freeze-dried *L. acidophilus* (2×10^{9} CFU), *L. casei* (7×10^{9} CFU), *L. rhamnosus* (1.5×10^{9} CFU), *L. bulgaricus* (2×10^{8} CFU), *B. breve* (2×10^{10} CFU), *B. longum* (7×10^{9} CFU), *S. thermophilus* (1.5×10^{9} CFU), and 100 mg fructo-oligosaccharide with lactose/capsule	1 capsule/day	8 weeks	↑ ALP	+18.25 ± 40.67 mg/dl	Asemi et al. (51)
								↑ ALP (ŝ)	+18.25 ± 40.67 mg/dl vs. +4.93 ± 35.91 mg/dl (ŝ)	
								↑ AST	+8.86 ± 15.11 mg/dl	
								↑ AST (ŝ)	+8.86 ± 15.11 mg/dl vs. +4.11 ± 15.11 mg/dl (ŝ)	
								↑ (x)ALT	−2.46 ± 13.10 mg/dl vs. +4.62 ± 10.81 mg/dl	

(Continued)

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TABLE 1 (Continued)

Type of nutraceutical	Study design and country	Participant* demographics size/sex (*n*, F/M) age (mean ± SD; years) BMI (mean ± SD; kg/m^2)		Control/ placebo substance administered	Interventional nutraceutical administered	Control/ placebo and intervention dose × frequency	Intervention duration	Effect on markers	Mean change in markers$^{\Phi}$	References
		Control/placebo	Intervention							
Probiotic (multi-species)	DB, R, PG, PC (Malaysia)	*n* = 68 (34M/34F) 54.2 ± 8.3 29.3 ± 5.3 *n* = 53 (PP analysis)	*n* = 68 (31M/37F) 52.9 ± 9.2 29.2 ± 5.6 *n* = 47 (PP analysis)	Organoleptically similar sachets without probiotic	Sachets containing viable microbial cell preparation of *L. acidophilus, L. casei, L. lactis, B. bifidum, B. longum*, and *B. infantis* (0.5×10^{10} CFU, each) in 250 ml water	2 sachets/day	12 weeks	· Albumin (ŝ)	45.48 ± 2.97 g/L (I, 12w) vs. 45.64 ± 3.22 g/L (I, B) (ŝ)	Firouzi et al. (50)
								↓ Total protein (ŝ)	73.03 ± 5.98 g/L (I, 12w) vs. 74.24 ± 4.93 g/L (I, B) (ŝ)	
								↑ Bilirubin (ŝ)	10.09 ± 3.70 μmol/L (I, 12w) vs. 9.77 ± 3.50 μmol/L (I, B) (ŝ)	
								↓ AST (ŝ)	25.71 ± 6.81 U/L (I, 12w) vs. 26.84 ± 77.12 U/L (I, B) (ŝ)	
								↓ ALT (ŝ)	22.33 ± 10.02 U/L (I, 12w) vs. 23.20 ± 9.65 U/L (I, B) (ŝ)	
								↓ ALP (ŝ)	67.00 ± 21.77 U/L (I, 12w) vs. 68.49 ± 23.19 U/L (I, B) (ŝ)	
Prebiotic	R, DB, C, CT (Iran)	*n* = 27 (12M/15F) 58.2 ± 11.8 BMI NR	*n* = 28 (14M/16F) 58.8 ± 12.8 BMI NR	Capsule containing 0.5 g of rice flour powder	Capsule containing 0.5 g of powdered cinnamon	1 capsule/day	3 months	↓ AST	Markers NR	Mirmiranpour et al. (56)
								↓ ALT	Markers NR	
Prebiotic	DB PC (Iran)	T2D and overweight patients* *n* = 22 (22F) 48.61 ± 9.16 29.98 ± 4.01	T2D and overweight patients* *n* = 27 (27F) 48.07 ± 8.70 31.43 ± 3.50	Maltodextrin	Oligofructose-enriched chicory inulin enriched	5 × 2 g/day	2 months	↓ AST	18.02 ± 6.41 U/L (I, 2m) vs. 24.25 ± 12.15 U/L (I, B)	Farhangi et al. (52)
								↑ ALT (ŝ)	23.25 ± 12.15 U/L (I, 2m) vs. 22.81 ± 11.04 U/L (I, B)	
								↓ ALP	183.07 ± 48.21 U/L (I, 2m) vs. 195.51 ± 65.20 U/L (I, B); also significant MD vs. control, markers NS	

(Continued)

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TABLE 1 (Continued)

Type of nutraceutical	Study design and country	Participant* demographics size/sex (*n*, F/M) age (mean ± SD; years) BMI (mean ± SD; kg/m^2)		Control/ placebo substance administered	Interventional nutraceutical administered	Control/ placebo and intervention dose × frequency	Intervention duration	Effect on markers	Mean change in markers$^{\Phi}$	References
		Control/placebo	Intervention							
Prebiotic	R, DB, PC (Japan)	*n* = 25 (17M/8F) 54 ± 12 27.2 ± 4.6	*n* = 27 (21M/6F) 55 ± 11 27.9 ± 3.6	Maltodextrin syrup	Galacto-oligosaccharide syrup	10 g/day	4 weeks	↑ ALT (ŝ)	43.0 ± 36.0 IU/L (I, 4w) vs. 40.0 ± 35.0 IU/L (I, B) (ŝ)	Gonai et al. (53)
								↑ AST (ŝ)	34.0 ± 28.0 IU/L (I, 4w) vs. 31.0 ± 23.0 IU/L (I, B) (ŝ)	
Synbiotic (single species)	DB, R, CC, CT (Iran)	*n* = 51 (16M/35F) 52.9 ± 8.1 30.15 ± 5.07	*n* = 51 (16M/35F) 52.9 ± 8.1 29.88 ± 4.77	0.38 g isomalt, 0.36 g sorbitol, and 0.05 g stevia per 1 g	*L. sporogenes* (1 × 10^7 CFU), 0.1 g inulin, 0.05 g beta-carotene with 0.38 g isomalt, 0.36 g sorbitol, and 0.05 g stevia per 1 g	9 × 3 g/day	6 × 2 weeks	↓ ALP	−12.91 ± 32.65 U/L	Asemi et al. (54)
								↓ ALP (ŝ)	−12.91 ± 32.65 U/L vs. −9.40 ± 21.17 U/L (ŝ)	
								↓ ALT (ŝ)	−0.67 ± 7.42 IU/L vs. +0.67 ± 6.21 IU/L (ŝ)	
								↑ AST (ŝ)	(+1.52 ± 11.93 IU/L vs. +2.00 ± 8.55 IU/L) (ŝ)	
Synbiotic (single species)	R, DB, C, CT (Iran)	*n* = 27 (12M/15F) 58.2 ± 11.8 BMI NR	*n* = 30 (sex NS) 58.4 ± 11.4 30.8 ± 5.9 BMI NR	Capsule containing 0.5 g of rice flour powder	Capsule containing *L. acidophilus* (10^8 CFU) and 0.5 g of powdered cinnamon	1 capsule/day	3 months	↓ AST	Markers NR	
								↓ ALT	Markers NR	
Synbiotic (single species)	R, DB, CC, CT (Iran)	*n* = 62 (sex NS) 35–70 (age NS) 30.1 ± 5.1	*n* = 62 (sex NS) 35–70 (age NS) 29.7 ± 4.6	0.38 g isomalt, 0.36 g sorbitol, and 0.05 g stevia per 1 g	*L. sporogenes* (1 × 10^7 CFU), 0.04 g inulin, 0.38 g isomalt, 0.36 g sorbitol, and 0.05 g stevia per 1 g	9 × 3 g/day	6 × 2 weeks	↑ ALP	+18.94 ± 55.50 mg/dl	Asemi et al. (55)
								↑ ALP (ŝ)	+18.94 ± 55.50 mg/dl vs. +1.09 ± 59.28 mg/dl (ŝ)	
								↑ AST	+4.29 ± 12.17 mg/dl	
								↑ AST (ŝ)	+4.29 ± 12.17 mg/dl vs. +4.36 ± 9.53 mg/dl (ŝ)	
								↑ ALT	+8.82 ± 22.54 mg/dl	
								↑ ALT (ŝ)	+8.82 ± 22.54 mg/dl vs. +3.34 ± 9.39 mg/dl (ŝ)	
Synbiotic (single species)	R, DB, C, CT (Iran)	*n* = 27 (5M/22F) 53.4 ± 7.5 30.5 ± 4.1	*n* = 27 (5M/22F) 51.3 ± 10.4 30.8 ± 5.9	Control bread	Bread containing viable and heat-resistant *L. sporogenes* (1 × 10^8 CFU) and 0.07 g inulin per gram	40 × 3 g/day	8 weeks	↓ ALT (ŝ)	−0.3 ± 10.9 IU/L vs. +1.4 ± 9.7 IU/L (ŝ)	Kobyliak et al. (49)
								↓ GGT (§)	−1.36 ± 44.22 IU/L vs. −0.76 ± 25.92 IU/L (§)	
								↑ AST (ŝ)	+1.4 ± 14.3 IU/L vs. +2.1 ± 14.1 IU/L (ŝ)	
								↓ ALP (ŝ)	−5.3 ± 60.0 IU/L vs. −5.4 ± 47.3 IU/L (ŝ)	

*All participants are T2D-diagnosed patients, unless otherwise stated. $^{\Phi}$ Order of markers compared = those of intervention (I) group first, control (B) or baseline (B) second. § Non-significant result. T2D, type 2 diabetes; NS, not specified; NR, not reported; Sp., species; SB, single-blinded; DB, double-blinded; TB, triple-blinded; R, randomized; RCT, randomized controlled trial; CC, crossover controlled; PC, placebo-controlled; PG, parallel group; CT, clinical trial; UCS, uncontrolled study; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LS, liver stiffness; FLI, fatty liver [illegible] LDH, lactate dehydrogenase; γGGT, gamma-glutamyl transferase.

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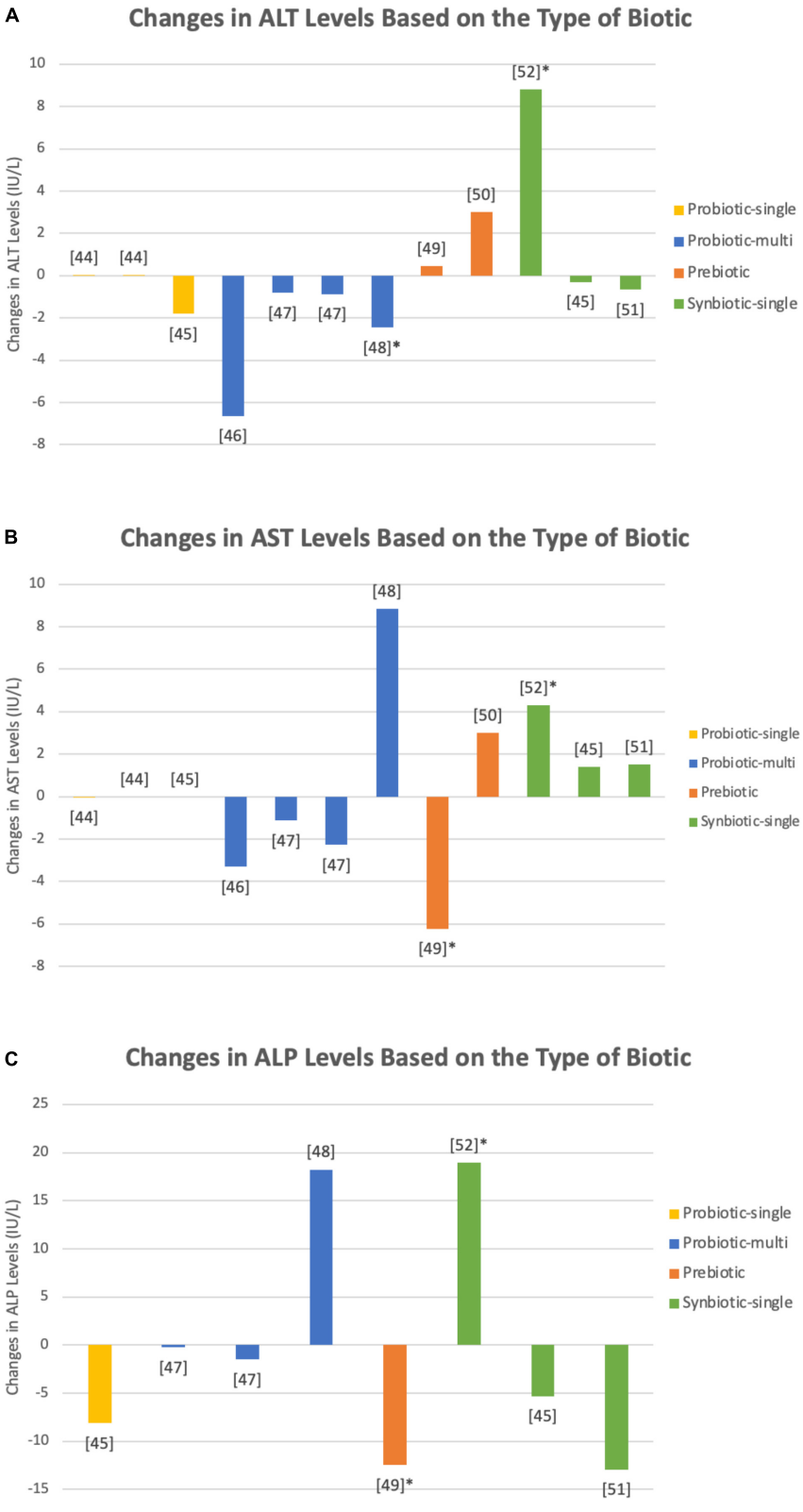

FIGURE 3
Changes in **(A)** ALP, **(B)** ALT, and **(C)** AST levels upon administration of single species probiotics (yellow), multi-species probiotics (blue), prebiotics (orange), and single species synbiotics (green) in nine studies (47–55). Bars marked with an asterick represent significant changes in the respective biomarker levels. Both single species *L. reuteri* (47) and *B. coagulans* (48) showed insignificant alterations in ALP, ALT, and AST

(Continued)

FIGURE 3 (Continued)
levels. Kobyliak et al. (49) used a multi-species probiotic formulation, comprised of Bifidobacteria and Lactobacilli among others (species not specified), that demostrated a decrease in liver enzymes, however the decrease was insignifcant. Firouzi et al. (50) used a similar multi-species probiotic and tested its effect over two intervention periods, 6 and 12 weeks, both of which yielded no notable alterations in biomarkers. Asemi et al., on the other hand, found a statistically relevant decrease in ALT levels upon administration of a multi-species probiotic containing species of Lactobacilli, Bifidobacteria, and Streptococci (species not specified) (51). Farhangi et al. utilized chicory inulin enriched oligofructose and measured a significant reduction in AST levels (52). Single species synbiotics employed by Asemi et al. (55) combined *B. coagulans* (1×10^7) and inulin (0.04 g), yielding a significant increase in each of ALT, AST, and ALP levels. Synbiotic formulations administered by Asemi et al. (54), containing *B. coagulans* (3×10^7 CFU), inulin, and beta carotene (0.45 g), and Bahmani et al. (48), containing *B. coagulans* (1×10^8 CFU) and inulin (8.4 g), caused no statistically relevant adjustments in hepatic enzyme levels. Mirmiranpour et al. reported significant decreases in ALT and AST levels when subjects were given both prebiotic powdered cinnamon and a synbiotic combination of 1×10^8 CFU of the probiotic *L. acidophilus* with 0.5 g of powdered cinnamon, but no data points were documented in the paper (56).

in four trials, multi-species probiotics were used in three trials, prebiotics were used in three trials, and single species synbiotics were used in four trials. Out of the 14 interventions, 6 reported an increase in AST levels upon administration of pro/pre/synbiotics, 7 reported a decrease, and 1 reported no change. Of the interventions that demonstrated an increase in AST levels, one used single species probiotics, one used multi-species probiotics, one used prebiotic, and three used single species synbiotics. Of the interventions with a decrease in AST levels, two offered single species probiotics, two multi-species probiotics, two offered prebiotics, and one offered single species synbiotics. No change in AST levels was observed when patients were given a single species probiotic.

Effects on alkaline phosphatase levels

As summarized in **Table 1**, seven interventions in six studies examined the effect of different biotics on ALP levels (48, 50–52, 54, 55). A total of 555 patients with T2DM were employed, 290 participants in the intervention groups and 265 participants in the control group. **Figure 3C** portrays one intervention that examined the effects of prebiotics, one that examined single species probiotics, two that examined multi-species probiotics, and three that examined single species synbiotics. Overall, two of the seven interventions reported an increase in ALP levels upon administration of biotics, and five reported a decrease. Of the studies that observed an increase in the marker's levels, one used multi-species probiotics, and one used a single species synbiotic. Of the interventions that found a decrease, one utilized prebiotic, one utilized single species probiotics, one utilized multi-species probiotics, and two utilized single species synbiotics.

Effects on liver steatosis

Only two interventions in one study reported the effects of biotics on liver steatosis (**Table 1**) (47). The study employed 44 participants with T2DM, 29 of which were in the intervention group and 15 were in the control group. Both trials used the same type of single species probiotic, with an increase in HCL in one trial that used a dose of 10^{10} CFU ($n = 14$) and a decrease in HCL in the second trial that used a lower dose of 10^8 CFU ($n = 15$).

Effects on liver stiffness and fatty liver index

One intervention from one study tested the effects of multi-species probiotics on both LS and fatty liver index (FLI) (**Table 1**) (49). The intervention consisted of 50 patients with T2DM, 26 of which were given the probiotics and 24 were given a placebo. Both LS and FLI decreased after 8 weeks of taking the probiotics.

Effects on total protein levels

Table 1 shows one intervention from one study that looked into the changes in total protein levels in T2DM patients after taking multi-species probiotics (50). A total of 136 patients were employed in these trials, 68 of which were administered multi-species probiotics and 68 took a placebo. The trial yielded a decrease in total protein levels after a 6- and 12-week intervention period.

Effects on bilirubin levels

There were three interventions in three studies that demonstrated the effects of biotics on bilirubin levels (**Table 1**) (50, 51, 55). The interventions collectively consisted of 318 participants, 158 of whom were in the intervention group and 160 in the control group. Two trials used multi-species probiotics, while the third trial tested single species synbiotics. One of the interventions reported an increase in bilirubin levels using multi-species probiotics. The other two reported a decrease; one trial also used multi-species probiotics, while the other used single species synbiotics.

Effects on gamma-glutamyl transferase levels

Only one intervention from one study reported the effects of biotics, specifically multi-species probiotics on the levels of GGT (**Table 1**) (49). A total of 50 participants were employed, 26 of which were given the multi-species probiotic and 24 of which were given a placebo. GGT levels decreased after administration of multi-species probiotics across an 8-week interventional period.

Discussion

There is a growing interest in the relationship between the gut microbiome, its dysbiosis and the pathophysiology of various diseases, including T2DM. In turn, there has been a recent increase in the number of studies investigating the effect of pro/pre/synbiotics as microbiome-modulating agents to utilize their potential therapeutic potential against the metabolic imbalances observed in T2DM patients (57). Thus, there is a significant need to qualitatively summarize, analyze, and provide future directives for investigation in this field. To our knowledge, this is the most comprehensive and detailed systematic review investigating the effect of pro/pre/synbiotics on hepatic biomarkers in clinical trial participants with T2DM.

Current and future directives for probiotics

Single-species probiotic formulations, especially including *Lactobacillus* species, have been consistently investigated for their effect on various metabolic diseases. Bahmani et al. performed a double-blind, placebo-controlled trial on 81 diabetic patients to examine the effects of both probiotic and synbiotic bread on liver enzymes, among other biomarkers (48). The consumption of probiotic bread containing *L. sporogenes* (1 10^8 CFU), currently referred to as *B. coagulans,* caused an insignificant decrease in ALP ($p = 0.97$) and ALT ($p = 0.48$) levels, and an insignificant increase in AST levels ($p = 0.88$). Although these results may be limited by the short intervention period (8 weeks), some scientists have questioned whether these spores are excreted intact from patients and therefore have no probiotic effect (58). While the vegetative state of this bacterium facilitates transportation and quality of the probiotic, *B. coagulans* may not survive long in the harsh conditions of the gut. Hibernating in its dormant form, the probiotic was shown to have weak tolerance, if not sensitivity, to bile, delaying its proliferation by up to 60 min (59). Additionally, one study suggested that *B. coagulans* has weak adhesion to the intestinal epithelium of piglets, being lost a week after administration (59). Such characteristics may explain the insignificant effects on the above-mentioned liver enzymes. Nonetheless, Bahmani et al. did find a significant increase in nitrous oxide (NO) ($p < 0.001$) and decrease in malondialdehyde upon administration of *B. coagulans* ($p = 0.001$), which may indicate a potential application in liver regeneration and reduced lipid oxidation (60). Mirmiranpour et al. reported a significant decrease in ALT and AST levels upon daily administration of 1 10^8 CFU of the probiotic *L. acidophilus* for 3 months (56). Although the exact mechanism for this decrease is not well understood, several studies highlighted the effect of *L. acidophilus* on the inhibition of pro-inflammatory cytokines (56, 61). Lv et al. fed rats *L. acidophilus* (3 10^9 CFU) for 7 days and observed an initial alleviation of histological hepatic injury, in addition to a suppression of macrophage inflammatory cytokines, leading to a reduction in serum ALT, AST, ALP, and bile acids (62). *L. acidophilus* also plays a role in the minimization of gut dysbiosis. On the other hand, Mobini et al. reported various changes over the course of 12 weeks in AST, ALT, and liver steatosis levels upon administration of different doses of the probiotic *L. reuteri* (1 10^8 CFU and 1 10^{10} CFU), although the changes were not significant (47). The insignificant results could be due to some of the study's limitations, including the subjects' consumption of metformin, an antidiabetic drug, which is known to affect gut microbiota composition, as well as the relatively small cohort size (47).

Based on the studies analyzed above, the use of single species probiotic *L. acidophilus* (1 10^8 CFU) demonstrated a significant improvement in hepatic function compared to other species within the same genus. In fact, it has been demonstrated that *Lactobacillus* has an effect on various diseases, especially liver diseases (61, 63). The use of different species of *L. acidophilus* on mice showed that it could improve intestinal barrier function, restore the composition of the gut microbiota, increase SCFA levels to that of the control group, suppress inflammatory responses in the liver and regulate glucose and lipid metabolism in the liver, hence improving T2D (64). One of the potential mechanisms that might explain the beneficial effect of *L. acidophilus* is that this organism can reshape the composition of the gut microbiota, leading to an increase in butyric acid that targets the liver (64, 65).

Multi-species probiotics have also been investigated for their effects in the physiological modulation of the intestinal microbiota as a result of the diversity in the administered species. One study revealed insignificant changes in ALT, AST, ALP, bilirubin, and total protein levels ($p = 0.199$, $p = 0.441$, $p = 0.209$, $p = 0.739$, and $p = 0.190$, respectively) when T2DM participants were administered 6 10^{10} CFU of a multi-species probiotic containing *L. acidophilus*, *L. casei*, *L. lactis*, *B. bifidum*, *B. longum*, and *Bifidobacterium infantis* over 12 weeks (50). As opposed to the anti-inflammatory and antioxidant properties observed in the administration of single species *L. acidophilus,* when given in addition to several other probiotic species, *L. acidophilus* may have an antagonistic effect (66). A study

conducted by Kwoji et al. highlighted the complexity of the interactions between various multi-species probiotics and their effects on human health, inhibition of pathogens, and treatment of disease (66). While *L. acidophilus* alone yields a significant reduction in several hepatic biomarkers due to decreased cellular injury and apoptosis, consequently leading to mitigation of the release of these intracellular enzymes (67), its administration with other species may not produce such effects and may require further investigation.

Another study conducted by Asemi et al. examined the effects of daily administration of multi-species probiotics containing a combined dose of 3.72 10^{10} CFU of *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *B. longum*, *B. breve*, and *S. thermophilus* over an 8-week intervention period (51). Asemi et al.'s trials yielded insignificant changes in ALP, AST, and bilirubin levels (p = 0.19, p = 0.23, and p = 0.91, respectively) but, as seen in **Figure 3A**, a significant decrease in ALT levels (p = 0.02). Multi-species probiotics have complex interactions that, while unfavorable at times, may have combined benefits for overall human health (66), detectable as improvements in liver biomarker levels, namely ALT and AST. Although Asemi et al. portrayed an increase in the levels of AST as well as uncertainty regarding the changes of other liver biomarkers, these changes were insignificant. Finally, Kobyliak et al. provided 1 10^{11} CFU of multi-species probiotic daily containing Bifidobacteria, Lactobacilli, Lactococci, Acetobacters, and SCFA-producing Propionibacteria (species not specified) (49). Although the study reported decreases in ALT, AST, FLI, LS, and GGT after an 8-week intervention period, none of these changes were significant (p = 0.991, p = 0.420, p = 0.521, p = 0.401, and p = 0.088, respectively). While it is true that many probiotics may have synergistic effects when administered with one another, some probiotics alone and in conjunction with others may prove to have antagonistic effects on the host's microbiota (68). Since the authors never specified the specific species of bacteria used in the intervention, certain harmful *Lactobacillus* species may have been used in conjunction with possibly beneficial species to yield inconsistent findings, hence explaining the insignificant reduction of the previously listed markers (68). Furthermore, the dosage could have also contributed to the insignificant results as the combined dose is marginally higher than the recommended ranges provided by Islam et al. which may explain a potential reduction in these liver biomarkers (69).

While it is unclear that specific combinations of probiotics yielded beneficial hepatic effects, the species *L. acidophilus*, *L. casei*, and B. *longum* were consistently used throughout all three studies and may be associated with an improvement of hepatic function (49–51). However, specific doses and intervention durations may need adjustment to clarify the effects of these variables and optimize the effects of the multi-species intervention. More in-depth investigations may be needed to assess the intricate mechanisms governing the complex interactions between multi-species probiotics to understand the end results on various liver biomarkers.

Current and future directives for prebiotics

Prebiotics have been investigated in several studies as low-risk, low-cost supplements to conventional T2DM treatments (**Figures 3A–C**). Farhangi et al. studied the effects of chicory inulin enriched with oligofructose on liver function tests, as well as glucose and calcium homeostasis, in female T2DM patients (52). Upon the administration of 10 g of the prebiotic for 2 months, a significant decrease was recorded in both ALP and AST levels compared to baseline (p = 0.05 and $p < 0.001$, respectively), but there was an insignificant increase in ALT levels (p = 0.39). Inulin and oligofructose are functional foods commonly found in plants, and they have been theorized to aid in important physiological processes, including modulating the gut microbiota's composition (70). The underlying mechanism of action of inulin oligofructose is the selective "fertilization" of SCFA-producing bacteria, such as Bifidobacteria, Lactobacilli, and Bacteroides (52). Chicory inulin is broken down by bacterial groups *via* β-fructofuranosidase, generating fermented byproducts such as acetate, lactate, and propionate (71–73). These substances play key roles in maintaining homeostasis, reducing inflammation, and alleviating insulitis (74). Gut microbiota changes, reduction in endotoxemia and insulin resistance, and improvement in glycemic control were also observed by Ho et al. (75), who provided oligofructose enriched inulin to children with T1D. These findings present chicory inulin enriched with oligofructose as a potential complement to current biomedical treatments. Galacto-oligosaccharide, another type of prebiotic, was given to T2D patients by Gonai et al., but no notable changes were recorded in hepatic biomarkers (53). Gonai et al. also investigated and compared the gut microbiota's composition and metabolites in T2DM patients versus a control group, as well as the effect of galacto-oligosaccharide ingestion on lipid blood profile and glucose indices in T2DM patients (53). Analyses revealed a significantly lower abundance of Bifidobacteriaceae in T2DM patients before treatment, as well as Actinobacteria, Lachnospiraceae, and Firmicutes ($p < 0.05$), and these results have been supported by other studies (76). On the other hand, Lachnospiraceae were found to be positively correlated with AST and ALT levels, which may be indicative of high lipid metabolism corresponding to a metabolic disturbance (77, 78). After taking the galacto-oligosaccharides, the levels of the above-mentioned bacteria in T2DM patients changed significantly ($p < 0.05$), promoting eubiosis and helping with the regulation of liver function. The assessment showed no significant change in AST and ALT levels upon consumption of the prebiotic; however, this may be due to comparatively

shorter intervention durations and sample sizes. Significant decreases in ALT and AST, after a 3 month follow-up, were also reported by Mirmiranpour et al. when a daily dose of 0.5 g of powdered cinnamon was given to subjects (56). Cinnamon has been shown to have therapeutic effects when consumed in adequate amounts. A study by Shekarchizadeh-Esfahani et al. concluded that administering cinnamon at daily dosages of <1,500 mg for at least 12 weeks significantly reduced ALT levels (p = 0.002) (79). Although cinnamon's effect on liver function remains unclear, several studies reported cinnamon's interaction with peroxisome proliferator-activated receptor (PPAR) which ultimately improves insulin resistance, down-regulates pro-inflammatory cytokine levels, and decreases serum AST levels (79, 80). Longe et al. also looked at the hepatoprotective properties of cinnamon on alloxan induced diabetic rats (81). The results were promising, yielding decreased ALT, AST, and ALP levels, as well as other improvements in glycemic and lipid profiles.

Overall, observations of the effects of prebiotics on hepatic functions has yielded remarkable results. The use of prebiotics, such as cinnamon and chicory inulin enriched with oligofructose, neutralized symptoms of liver damage, consequently decreasing liver enzyme levels, and also helped restore gut microbiota eubiosis.

Current and future directives for synbiotics

Synbiotics have been investigated for their synergistic potential stemming from combination of pro/prebiotics. A study by Asemi et al. reported insignificant changes in the liver enzymes AST, ALP, and ALT upon intake of beta-carotene fortified single species synbiotic comprising *B. coagulans* (3 10^7 CFU), inulin, and beta carotene (0.45 g) (54). Bahmani et al. also did not find any significant changes in liver enzymes when a synbiotic formulation of *B. coagulans* (1 10^8 CFU) and inulin (8.4 g) was used (48). Although Farhangi et al. demonstrated a significant decrease in liver enzyme levels upon administration of chicory inulin enriched with oligofructose alone in diabetic patients, combining the prebiotic with the probiotic *B. coagulans* and beta carotene in the synbiotic produced no notable changes in hepatic biomarkers (52). This lack of significant reduction of serum liver enzymes may be explained by the short duration periods as well as the dose of the probiotic and prebiotics. There are currently limited data on the potential benefits of *B. coagulans* on the gut microbiota and liver function. Interestingly, though, when Asemi et al. used a lower daily dose of both the *B. coagulans* (1 10^7) and inulin (0.04 g) on T2DM patients, there was a significant increase in serum concentrations of ALP (p = 0.009), ALT (p = 0.003), and AST (p = 0.007), with a significant drop in bilirubin levels (p = 0.007) (55). Further analyses may be needed to determine the potential use and mechanism of this synbiotic combination in T2DM patients. Be that as it may, significant decreases in ALT and AST levels were reported by Mirmiranpour et al. following a trial that combined daily administration of 1 10^8 CFU of the probiotic *L. acidophilus* with 0.5 g of powdered cinnamon over 3 months (56). The results were remarkable as they did not outperform *L. acidophilus* and cinnamon alone. Despite several other studies indicating the benefit of synbiotics in improving antioxidant and anti-inflammatory indices, these studies did not obtain their results from diabetic patients (82–84). While cinnamon and *L. acidophilus* may work additively or synergistically as potential therapeutic compliments in patients with T2DM, their interactions may be altered based on the patients' comorbidities and atypical hepatic functions. A meta-analysis discussing the effects of probiotics and synbiotics on liver and renal biomarkers in T2DM patients obtained results running mostly in parallel to this paper, with some exceptions (85): even though Abdollahi et al. concluded with insignificant changes in liver biomarkers overall, their results were based on a smaller sample size with limited focus on the isolated effects of the nutraceuticals studied (85).

While the use of synbiotics was not effective in improving hepatic function, exceptions applied to the use of cinnamon and *L. acidophilus*, although their use together yielded similar results to their use as isolated components. Further studies are needed to investigate the mechanism of action of symbiotic components to understand their potential conflicting effects when administered together.

Limitations and strengths

There are a few limitations to this study. First, due to the great heterogeneity between the types of nutraceuticals, their composition and dosage, the duration of intervention, and the diversity of the trial participant characteristics, it was difficult to ascertain the optimum combination of the above factors that provided the greatest effect on hepatic biomarkers. Second, our search strategy for prebiotics captured only those trials that explicitly self-identified the use of prebiotics; this has a potential to omit other sources (86, 87). Moreover, we did not identify adverse events following administration for nutraceuticals, although prior studies have shown minimal complications. Finally, the sample sizes of most trials are small. Future high-quality studies should have larger sample sizes, longer durations, and more regionally diverse participants. However, this study has multiple strengths. It is one of the most comprehensive reviews of the effect of pro/pre/synbiotics on liver profiles in T2D patients, whereas other reviews largely focus on probiotics only or in addition to synbiotics. Bias in study selection was minimized *via* independent screening and

extraction of studies, and RoB assessment revealed low risk of bias for most parameters within the studies used.

Conclusion and future prospects

This systematic review examined the potential medicinal uses of probiotics, prebiotics, and synbiotics in improving liver function in patients with T2DM. Present evidence reveals that insulin resistance, oxidative stress, and gut dysbiosis contribute to fluctuations in hepatic biomarkers. Supplementation of some biotic formulations, such as prebiotic chicory inulin enriched with oligofructose and multi-species probiotics, demonstrated statistically significant improvements in liver function, specifically in the levels of liver enzymes. However, several studies showed no significant changes or significant increases in these biomarkers upon administration of specific species and types of probiotics and prebiotics. Such contradictory data may be due to differences in doses, intervention periods, or species of probiotics used. Nevertheless, more research needs to be done to better assess the best dose-response relationships for the biotics mentioned in this paper.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

AC designed the study, critically supervised the project, revised and reviewed the manuscript, and initially screened studies. YA-N and MA analyzed the data, updated the search, wrote the majority of the manuscript, generated the figures, and edited the manuscript. PP initially screened and extracted studies, generated the tables, wrote parts of the manuscript, and reviewed all of it. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1052619/full#supplementary-material

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Hong Kong SAR, China

REVIEWED BY
Yao-Tsung Yeh,
Fooyin University, Taiwan
Zhoujin Tan,
Hunan University of Chinese Medicine,
China

*CORRESPONDENCE
Tristan Chalvon-Demersay
tristan.chalvon-demersay@
metex-noovistago.com

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Selective nourishing of gut microbiota with amino acids: A novel prebiotic approach?

Martin Beaumont[1], Eugeni Roura[2], William Lambert[3], Conny Turni[4], Joris Michiels[5] and Tristan Chalvon-Demersay[3]*

[1]GenPhySE, Université de Toulouse, INRAE, ENVT, Castanet-Tolosan, France, [2]Centre of Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, QLD, Australia, [3]METEX ANIMAL NUTRITION, Paris, France, [4]Centre for Animal Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, QLD, Australia, [5]Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Prebiotics are dietary substrates which promote host health when utilized by desirable intestinal bacteria. The most commonly used prebiotics are non-digestible oligosaccharides but the prebiotic properties of other types of nutrients such as polyphenols are emerging. Here, we review recent evidence showing that amino acids (AA) could function as a novel class of prebiotics based on: (i) the modulation of gut microbiota composition, (ii) the use by selective intestinal bacteria and the transformation into bioactive metabolites and (iii) the positive impact on host health. The capacity of intestinal bacteria to metabolize individual AA is species or strain specific and this property is an opportunity to favor the growth of beneficial bacteria while constraining the development of pathogens. In addition, the chemical diversity of AA leads to the production of multiple bacterial metabolites with broad biological activities that could mediate their prebiotic properties. In this context, we introduce the concept of "Aminobiotics," which refers to the functional role of some AA as prebiotics. We also present studies that revealed synergistic effects of the co-administration of AA with probiotic bacteria, indicating that AA can be used to design novel symbiotics. Finally, we discuss the difficulty to bring free AA to the distal gut microbiota and we propose potential solutions such as the use of delivery systems including encapsulation to bypass absorption in the small intestine. Future studies will need to further identify individual AA, dose and mode of administration to optimize prebiotic effects for the benefit of human and animal health.

KEYWORDS

prebiotics, amino acids, aminobiotics, gut microbiota, gut health

Introduction

The gastrointestinal tract is colonized by a complex microbial community composed of hundreds species of bacteria, fungi, protozoa, and yeasts, collectively referred to as the gut microbiota (1–3). In humans like in other monogastric animals, the gut microbiota has major physiological functions for the host, including resistance against colonization by pathogens, degradation of undigested proteins and complex carbohydrates, regulation of nutrient absorption, metabolism, and immunity among others. Disruption of the gut microbiota balance (dysbiosis) has been linked to numerous human diseases such as inflammatory bowel disease, obesity, cancer, diabetes, and autism (4). In farm animals, dysbiosis has also been associated with impairment of the gut development and nutrient absorption, infection by enteric pathogens, inflammation and, ultimately, reduced performance, health, and welfare (2, 4, 5). Diet constitutes the main environmental factor able to modulate the gut microbiota composition and function (6, 7). Dietary constituents may provide competitive advantages to selected microorganisms according to their metabolic requirements and capacities. Complex carbohydrates derived from plants are the main nutrients affecting the gut microbiota and dietary intervention targeting intestinal bacteria have mainly used fermentable fibers, leading to the concept of prebiotics (7).

The term prebiotic was initially defined in 1995 by Gibson and Roberfroid as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon, and thus attempt to improve host health" (8). This definition of prebiotics applied mostly to non-digestible oligosaccharides such as inulin-type fructans and fructo- or galacto-oligosaccarides that promote the growth of *Bifidobacterium* and *Lactobacillus* spp. associated with protection against pathogens and beneficial immunomodulatory and metabolic effects (8, 9). Based on recent advances in the field of gut microbiota, the definition of prebiotics was updated in 2017 by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (10). This expanded definition of prebiotics possibly includes non-carbohydrate substances. For instance, polyphenols are now recognized as a novel class of prebiotics since they are able to provide a health benefit to the host through a modulation of the gut microbiota composition and/or activity (11).

Although less studied than non-digestible carbohydrates and polyphenols, dietary proteins and amino acids (AA) can also influence host health through the regulation of immunity, gut barrier function, oxidative stress but also microbiota composition and its production of bioactive metabolites (12, 13). Thus, this review highlights the potential utilization of AA as a novel class of prebiotics. The structural and chemical diversity of AA represent an opportunity for targeting a broader range of gut bacteria than standard prebiotics by targeting their metabolic requirements/capacities. Moreover, AA are precursors of a broad range of bioactive bacterial metabolites, much more diverse than those derived from saccharides (14). To explore the concept of AA as prebiotics, we first review briefly the metabolism of AA by intestinal bacteria followed by a detailed description of the effects of dietary AA supplementation on intestinal bacteria, metabolites, and the consequences for the host. We also present the potential health benefit of AA supplementation co-administered with beneficial live bacteria (i.e., probiotics). Finally, we discuss technological strategies that may be required to deliver free AA to gut bacteria bypassing the proximal small intestine.

Amino acid requirements and avoidance in bacterial populations

Host and microbiota-derived proteases hydrolyze dietary and endogenous (host or microorganism-derived) proteins in the intestinal lumen into peptides and AA that can be used by gut bacteria after uptake (14). Available AA can be used by intestinal bacteria for protein synthesis or as carbon and energy sources (15). Luminal AA of dietary and endogenous origin are the main constituents of bacterial protein in the pig ileum, indicating it is likely that *de novo* synthesis of AA by the intestinal bacteria of the foregut of non-ruminants is limited (16). The capacity to synthetize AA differs greatly across bacterial taxa (14). For instance, *Escherichia coli* encodes genes for the biosynthesis of all 20 α-AA while *Lactobacillus* has limited capacity for AA biosynthesis and thus relies on the uptake of extracellular AA (14, 17). It has been proposed that some commensal and pathogenic gut bacteria might have lost biosynthetic pathways for AA due to the high availability of AA in the gut environment (18). It is also important to consider that the autotrophy for AA in gut bacteria can be strain dependent (14). In addition to exogeneous AA, some species of intestinal bacteria such as *E. coli,* use ammonia as the preferred nitrogen source (19). Intestinal bacteria utilize AA in a species-dependent manner, as demonstrated in bacteria derived from the pig microbiota (20, 21). For example, *in vitro* incubation with ^{14}C-labeled AA demonstrated that intestinal *E. coli* and *Klebsiella* spp. and *Streptococcus* spp. have differential utilization of AA for protein synthesis (20). The bacterial intracellular AA composition also seems to be species specific compatible with the concept of different AA requirements (22). Interestingly, the saccharolytic pathogen *Campylobacter jejuni* uses a limited range of AA (serine, aspartate, asparagine, glutamate, glutamine, and proline) (23). Further to the roles of protein synthesis and energy source, luminal AA also play an important signaling role

in the gut ecosystem, which may have significant impact on the development of enteric pathogens (24). The sensing of arginine by Enterohemorrhagic *E. coli* (EHEC) induces the expression of virulence genes (25). In contrast, the presence of some AA in the medium can impair growth of specific bacteria strains, such as high concentrations of valine and leucine inhibiting *E. coli* growth (26). Overall, the bacterial use of exogenous AA can be classified as nutritionally essential (as a protein building block), non-essential (as energy source), preferred, or avoided/toxic for the growth of specific bacterial species as shown in **Table 1**.

Metabolite release from amino acid catabolism by intestinal bacteria

The microbial catabolism of AA may impact the host intestinal lumen caused by the release of multiple metabolites resulting from a combination of deamination, decarboxylation, or desulfurization reactions (12). The deamination of AA releases ammonia which may negatively impair mitochondrial respiration, resulting in decreased cell proliferation and barrier function in the intestinal epithelium (27, 28). In addition, the degradation of cysteine by the gut microbiota releases hydrogen sulfide which can reduce mitochondrial respiration and increase inflammation when present at high concentration (29). Microbial catabolism of tyrosine releases *p*-cresol which can induce DNA damages and reduce mitochondrial respiration in intestinal epithelial cells and has been implicated in renal, cardiovascular and neurological disorders (30). Imidazole propionate, a bacterial metabolite derived from histidine, was shown to disrupt insulin signaling and was implicated in diabetes (31). Based on these observations, AA fermentation (also called putrefaction) has often been considered detrimental for health (32).

In contrast, other bacterial metabolites derived from AA were shown to have beneficial effects for host health. Deamination and decarboxylation of glutamate, threonine, alanine, lysine, glycine, and aspartate produces short-chain fatty acids (SCFA; acetate, propionate, and butyrate) which have protective effects against infection and enhance the gut barrier function and immunity (12). The microbial catabolism of branched-chain AA (BCAA) releases branched chain fatty acids (isovalerate, isobutyrate, isocaproate, and 2-methylbutyrate) that can serve as an energy substrate for epithelial cells and promote the barrier function *in vitro* (12, 13, 33). Valerate, a bacterial metabolite derived from proline but also from propionate and ethanol, directly inhibits the growth of *Clostridioides difficile* but not of commensal bacteria (34). Decarboxylation of arginine and lysine produces the polyamines agmatine, spermine, and cadaverine, respectively (35). These metabolites influence mitochondrial function, epithelial proliferation, gut barrier function, and have trophic effects on the developing gut (35). However, the specific effects of gut microbiota-derived polyamines has not been clearly distinguished from those derived from the diet or from host cells and detrimental effects of polyamines have also been described (36). The histidine-derived metabolite histamine reduced the secretion of pro-inflammatory cytokines *ex vivo* (37). The gut microbiota also produces numerous catabolites from tryptophan, including indole, indole-3-propionate, indole-3-aldehyde (38). These bacterial metabolites have protective effects for the gut barrier since they reduce epithelial permeability and inflammation (38, 39) and can also contribute to the effects of the gut microbiota on the brain (40). Interestingly, neurotransmitters (GABA, histamine, serotonin, dopamine) which may have an important role in the gut-brain axis, can also be produced by the gut microbiota through AA catabolism (14). Thus, many AA-derived bacterial metabolites have protective effect on host health.

In summary, AA are used by intestinal bacteria in a species (or strain) specific manner, which highlights the potential of specific AA used as prebiotics for promoting the selective growth of beneficial over pathogenic gut microbes. Moreover, stimulating the production of protective AA-derived metabolites by the gut microbiota has the potential to mediate beneficial effects of AA used as prebiotics on host health.

Dietary amino acids as modulators of the gut microbiota and consequences for host health

Modification of dietary protein intake would be the most straightforward approach to modify AA supply to the gut microbiota. Increasing protein intake results in a larger amount of dietary protein reaching the distal part of the gut where the microbiota is mostly located (14, 41, 42). The digestibility and AA profiles of dietary protein also influences the availability of AA for the gut microbiota. In general, plant are less digestible than animal proteins and the biological value of the AA composition is commonly higher in the latter compared to the first (43). Modifying dietary protein intake in terms of quality and quantity can also change AA availability for the gut microbiota, which might have both detrimental and beneficial consequences for health (44, 45). It has been suggested in the literature that casein or whey protein, particularly rich in BCAA, could protect against obesity and modulate microbiota in humans and rodents. Similarly, lean seafood or meat with high amounts of aromatic acids, glycine, and taurine could be associated with increased energy expenditure, anti-obesogenic effect, and modulation of microbiota (44). It is also well described in the literature that high-protein intake has been associated with

TABLE 1 AA required, utilized, preferred, or avoided according to bacterial species.

Bacteria species	AA required	AA that can be utilized	AA preferred	AA that cannot be utilized	AA avoided or no growth observed	References
Commensal bacteria						
Acidaminococcus fermentans	Val, Phe, Tyr, Ser, Cys, Arg, His, Trp, Glu	Ala, Arg, Leu, Pro, Thr, Met, Lys, Asp			Pro, Lys, Asp, Asn	(66)
Bacteroides fragilis			Asn, Asp, Gln, Glu, Gly, Ser, Thr, His			(67)
Clostridium sticklandii		Orn, Lys, His, Asp, Val	Arg, Ser, Thr, Cys, Pro, Gly			(68)
Lactobacillus arabinosus	Cys, Met, Trp, Leu. Val, Glu, Thr	Tyr, Phe				(69)
Lactobacillus mesenteroides	Glu, Val					(70)
Lactobacillus citrovorum	Glu, Val					(70)
Lactobacillus mesenteroides	Glu, Val, Ile					(70)
Lactobacillus dextranicum	Glu, Val, Ile					(70)
Lactobacillus brassicae	Glu, Val, Ile, Leu, Cys					(70)
Lactobacillus buchneri	Glu, Val, Ile, Leu, Cys, Met					(70)
Lactobacillus pentosus	Glu, Val, Ile, Leu, Cys					(70)
Lactobacillus arabinosus	Glu, Val, Ile, Leu, Trp					(70)
Lactobacillus brasscitrovorumicae	Glu, Val, Ile, Leu, Arg, Trp, His					(70)
Lactobacillus dextranicum	Glu, Val, Ile, Leu, Cys, His, Thr, Trp, Met					(70)
Lactobacillus fermenti	Glu, Val, Ile, Leu, Met, Trp, Phe, Tyr					(70)
Lactobacillus mannitopoeus	Glu, Val, Ile, Leu, Met, Arg, Trp, Phe, Tyr				Arg	(70)
Lactobacillus delbrueckii	Glu, Val, Ile, Leu, Arg, Trp, Cys, Tyr, Ser					(70)
Lactobacillus casei	Glu, Val, Ile, Leu, Cys, Arg, Trp, Cys, Tyr, Ser					(70)
Lactobacillus gayonii	Glu, Val, Ile, Leu, Arg, Trp, His, Phe, Tyr					(70)
Streptococcus faecalis	Glu, Val, Ile, Leu, Arg, Trp, His, Thr, Tyr, Lys, Ala					(70)
Lactobacillus citrovorum	Glu, Val, Ile, Leu, Arg, Trp, Cys, His, Thr, Phe, Gly, Ala					(70)
Lactobacillus pentoaceticus	Glu, Val, Ile, Leu, Met, Arg, Trp, Cys, His, Thr, Phe, Tyr, Gly, Lys					(70)
Lactobacillus mesenteroides + *Lactobacillus brevis*	Glu, Val, Ile, Leu, Met, Arg, Trp, Cys, His, Thr, Phe, Tyr, Gly, Asp, Lys					(70)
Megasphaera elsdenii		Ile, Val, Leu	Ser, Thr			(71)
Streptococcus bovis		Gln				(72)
Veillonella spp.			Arg, Gln, Glu, Lys, Orn			(73)

(Continued)

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TABLE 1 (Continued)

Bacteria species	AA required	AA that can be utilized	AA preferred	AA that cannot be utilized	AA avoided or no growth observed	References
Potential pathogens						
Campylobacter jejuni			Asn, Asp, Gln, Glu, Pro, Ser			(74)
Clostridium difficile	Gly, Ile, Phe, Thr	Phe, Trp, Arg, Tyr, Ala, Asp, Val	Ser, Met, Leu, Pro		Glu, Lys	(75)
Clostridium perfringens			Arg, Ile, Leu, Lys, Thr			(75)
E. coli	Ser, Asp, Cys, Asn	Glu, Lys	Asp, Ser		Val, Leu, Ile, Trp, His	(26, 76, 77)
Fusobacterium nucleatum			Arg, Asp, Asn, Gln, Glu, Gly, His, Lys, Orn, Thr			(78)
Fusobacterium varium				Glu, His, Lys, Ser		(79)
Klebsiella pneumoniae			Arg, Asn, Gln, Glu, His, Lys, Met			(20)
Pasteurella	Met, Cys, Glu	Leu				(80)
Peptostreptococcus spp.			Gln, Leu, Phe, Ser, Thr			(81)
Pseudomonas		Ala, Glu, Asp		Met, Cys, Thr		(76)
Pseudomonas aerunginosa		Lys				(76)
Salmonella enterica		Asp, Glu, Gly, Pro, Ser, Ala, Arg				(82)
Staphylococcus aureus	Pro, Arg, Val, Cys, Phe (for enterotoxin production)					(83–85)
Veillonella spp.	Lys, Orn					(86)

AA, amino acid; Ala, alanine; Arg, arginine; Asp, Aspartate; Asn, Asparagine; Cys, Cysteine; Glu, glutamate; Gln, glutamine; Gly, Glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, Serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

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TABLE 2 Effect of free amino acid supplementation on microbiota and health outcomes in humans and mice.

AA	Supplemental level as compared to control	Subject	Duration (d)	Segment	Phylum	Genus	Species	Diversity	Health	References
Gln	30 g/d	Overweight or obese patients	14	Feces	↓ *Actinobacteria Firmicutes*	↓ *Pseudobutyrivibrio Veillonella, Dorea, Dialister*	NA	NA	NA	(87)
BCAA	14 g twice daily	Hemodialysis patients	120	Feces	—	—	↓ *Bifidobacterium dentium Lacticaseibacillus paracasei*	=	=	(88)
Arg	0.5%	Mice	14	Jejunum	↑ *Bacteroidetes* ↓ *Firmicutes*	↑ *Lactobacillus*	NA	NA	↓jej TLR-6, Crs4c, Spla2 ↑jej TLR-8, IFN-γ, MUC2, MUC4, J-Chain, Cryptdin 1, 4, 5, Crsc1c, Ang4	(89)
Arg	0.5%	**Mice**	14	Ileum	↑ *Bacteroidetes* ↓ Firmicutes	↑ *Streptococcus* ↓ *Lactobacillus*	NA	NA	↑ il TLR-4, TLR-6, TLR-8, IL1-β, TNF-α, IFN-γ, J-Chain, Cryptdin 1, 4, 5, Crs1c, Ang4, Reg3γ, Lyz2	(89)
Asp	0.5, 1, 2%	Mice	14	Ileum	=	NA	NA	NA	↑ il Cryptdin-1 (2%), PigR (2%) ↓ il IL-17 (1%), IFN-γ (1%), Muc2 (0.5, 1%)	(90)
Asp	0.5, 1, 2%	Mice	14	Feces	↓ *Firmicutes: Bacteroidetes (0.5%, 1%)* ↑ *Firmicutes: Bacteroidetes (2%)*	NA	NA	NA		(90)

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10.3389/fnut.2022.1066898

TABLE 2 (Continued)

AA	Supplemental level as compared to control	Subject	Duration (d)	Segment	Phylum	Genus	Species	Diversity	Health	References
Gln	1%	Mice	14	Jejunum	↓ *Firmicutes*	↑ *Streptococcus* *Bifidobacterium*	NA	NA	↑ jej MUC4, Cryptdin 1, 4, 5, Reg3γ ↓jej Crs4c ↑ il TLR-4, Reg3γ, Cryptdin-4, IL1-β, TNF-α, IL-17 ↓ il TLR-5	(91)
				Ileum	↓ *Firmicutes*	↓ *Streptococcus* *Lactobacillus*	NA	NA		
Trp	0.1%	Acetic acid-induced colitis mouse model	7	Colon	=	↓ *Turicibacter* *Candidatus* *Clostridium* *Coprococcus*	NA	=	↑ col TNFα, IL23 ↓ col IL-22	(92)

AA, amino acid; Arg, arginine; Asp, Aspartate; BCAA, branched-chain amino acids; Gln, glutamine; Trp, tryptophan; NA, non-available; jej, jejunum; il, ileum; col, colon; Ang4, angiogenin 4; Crs, cryptdin-related sequence; IL, interleukin; IFN, interferon; Lyz2, Lysozyme 2; PigR, polymeric Ig receptor; Reg3γ, regenerating islet-derived 3g; Spla2, secretory group II A phospholipase A2; TLR, toll-like receptor; TNF, tumor necrosis factor; ↑, significantly increased compared to control group; ↓, significantly decreased compared to control group; =, similar to control group.

negative effects on gut health, such as inflammatory bowel disease in humans and postweaning diarrhea in piglets (46). In contrast, reducing crude protein level in piglet's diets has been associated with reduced diarrhea score as reported in a recent meta-analysis (47). As discussed above, pathogenic, and commensal bacteria utilize AA in a specific manner and detrimental or protective metabolites are produced from AA by the gut microbiota. Thus, changing protein intake does not facilitate a targeted supply of AA to the gut microbiota to promote health. Alternatively, dietary supplementation with specific AA is a targeted approach with potential beneficial consequences for host health through a selective growth promotion of beneficial bacteria and through the production of protective metabolites.

Accumulating evidence is showing that dietary supplementation with free AA can modulate the microbiota composition and activity both *in vitro* (33) and *in vivo* (**Tables 2–4**). This is associated with consequences for the host as reported in mice and humans (**Table 2**), pigs (**Table 3**), and chickens (**Table 4**). Most studies reveal an effect of AA supplementation on the gut microbiota diversity or composition. Thus, the comparison between studies presented in **Tables 2–4** does not reveal general trends regarding the effects of AA supplementation on the gut microbiota. The divergence of the results obtained after AA supplementation can be explained by multiple differences between studies including the AA tested, host species, gut segment, dose, and duration of AA supply and exposition or not to an inflammatory or infectious challenge. It is interesting to notice that some effects of the AA supplementations were observed in the large intestine, despite that free-AA are anticipated to be absorbed mostly in the upper part of the intestine. It can be hypothesized that a high level of free-AA might temporally overwhelm the absorptive capacity of the small intestine. A surprising observation is that no linear dose-dependent effects were observed on the microbiota in studies testing the same AA at different level of supplementation (**Table 3**). Thus, further studies are clearly needed to define more clearly what are the effects of AA supplementation on the gut microbiota. This future work should help determine which AA have the most favorable effects on the gut microbiota and what is the effective level of supplementation. In addition, only few studies presented in **Tables 2–4** have investigated the effects of AA supplementation on the production of AA-derived metabolites, the latter being potentially more predictable than the effects on the composition of the microbiota due to functional redundancy (i.e., different bacterial communities can express similar metabolic capabilities).

The evaluation of host parameters focused on intestinal barrier function, immunity and metabolism revealed modulations associated with the modification of the gut microbiota induced by AA. However, it is not clear yet if these modifications at the gut level are beneficial for the host,

which is a requirement to fulfill the definition of prebiotics. In farm animals, the effects of AA supplementation on the microbiota were associated with either no or beneficial effects on growth performance. An important perspective will be to demonstrate that the modulation of the gut microbiota by AA is directly involved in the effect observed at the host level.

Combination between amino acids and probiotics (symbiotic approach)

The potential synergistic effect of AA supplementation with probiotics has been investigated. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (46). Combining AA and probiotics corresponds with the concept of "symbiotic" which is "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (48). One potential mechanism is that AA could promote the survival of the exogenous microbial species (i.e., the probiotic) in addition of being used as substrates for their growth. For instance, the catabolism of glutamine has been shown to improve the acid tolerance of *Lactobacillus* (49, 50). Therefore, the association of glutamine and the probiotic bacteria *L. plantarum* was expected to increase its survivability and thereby improve its positive effect on gut health. Accordingly, the authors showed that the combination of glutamine (1–4 mM) and *L. plantarum* decreased the translocation of *E. coli* in weanling rabbit ileal loops (51). They observed a synergistic effect indicating that the provision of the two compounds (AA and probiotics) together was more efficient than the anticipated sum of effects resulting from each compound alone. Other studies investigated the effects of the combination of arginine and *Lactobacillus* in rats with acute liver injury. The authors showed that the co-administration of *L. plantarum* and arginine reduced the hepatocellular necrosis and inflammatory cell infiltration, whereas the effect of individual administration of probiotics or arginine alone had lower effects than the two together (52). The potential mechanism of this synergistic effect may involve the metabolization of arginine by *L. plantarum* into polyamines, nitric oxide or its utilization as an energy source. Another study showed that a supplementation with *L. plantarum* and arginine 10 days before an LPS-challenge induced a synergistic reduction of liver damage and inflammation (53). The authors hypothesized that probiotics could direct arginine toward polyamines rather than NO synthesis by decreasing inflammation level and promoting cell proliferation and healing in the liver (53). Altogether, these results suggest that symbiotics composed of AA and probiotics have the potential to promote health to a larger extent than the simple sum of both dietary supplements alone. Multiple combination of probiotics and AA should be tested in future studies, notably by selecting the most promising association based on the capacity of the probiotic strain to metabolize specific AA (**Table 1**).

Discussion

Based on the data presented above, it appears AA supplementation can modulate the gut microbiota composition, its metabolic activity and these effects can be associated with benefits for the host. Thus, we consider that dietary AA supplements could fulfill the requirements to be considered as a novel class of prebiotics, the "Aminobiotics." Additional studies are still required to support this promising concept since important questions remain open.

First, most of the doses of AA supplementation used in the literature are relatively high, raising the question of whether the observed effects are linked to direct effects of AA on the microbiota or to indirect effects mediated by the host (i.e., after absorption), or a combination of both. For example, it has been shown that AA supplementation can increase the expression of intestinal β-defensin (an endogenous small cationic polypeptide that functions as a broad-spectrum antimicrobial) by blocking nuclear factor kappa-B (NF-κB) and MAPK inflammatory signaling pathways and activating the mammalian target of rapamycin signaling pathway (mTOR) (54, 55). Similarly, several AA have been reported to modulate the secretion of immunoglobulin A (IgA) in the intestine (56, 57) which could in turn affect the gut microbiota. Therefore, it is very likely that dietary AA affect gut microbial composition both directly (i.e., as a substrate) and indirectly (i.e., through modulation of host factors). Second, only nine AA out of the 20 proteinogenic ones have been tested and they were tested only a limited number of times which urges to be cautious when drawing conclusions. Third, microbiota analysis was not always the main endpoint of the studies which can also generate some biases.

Another challenge will be to develop strategies to supply AA reaching the lower gut were most of the microbiota develops. Indeed, when provided in a free form, AA are rapidly absorbed in the proximal small intestine (58). In contrast, the microbiota density is higher in the distal part of the digestive tract, mostly in the ileum, caecum, and colon (59). One potential strategy to circumvent this lack of space/time synchrony between dietary free AA release and microbiota, would be to delay the release of specific free AA by using protective delivery systems such as fat matrix encapsulation. The encapsulation of AA with probiotics may hypothetically optimize their use by the target communities of bacteria. Another possibility to deliver AA to the gut microbiota would be to combine AA with polyphenols that

TABLE 3 Effect of free amino acid supplementation on microbiota, performance and health outcomes in pigs.

AA		Supplemental level (%) as compared to control	Age (d) or BW at start	Duration (d)	Segment	Phylum	Genus	Diversity	Metabolites	Performance and health	References
Glu or MSG	MSG	3	25 ± 1.3 kg	30	Jejunum	=	↓ *Prevotella* *Peptostreptococcus* *Clostridium coccoides*	NA	NA	=Perf	(93)
	MSG	3	25 ± 1.3 kg	30	Ileum	↑ *Firmicutes* *Bacteroidetes*	↑ Prevotella	NA	NA	=Perf	(93)
	MSG	3	25 ± 1.3 kg	30	Cecum	=	↑ *Roseburia*	NA	NA	=Perf	(93)
	MSG	3	25 ± 1.3 kg	30	Colon	↑ *Firmicutes*	↑ *Faecaliabacterium prausnitzii* *Fusobacterium prausnitzii* ↓ *Peptostreptococcus productus* *Methanobrevibacter smithii*	NA	NA	=Perf	(93)
	Glu	1	77.1 ± 1.3 kg	60	Colon	NA	=	=	↑ *Propionate*, *Valerate*	↓ Body fat	(94)
	Glu	0.5	28 days	28	Ileum	=	↑ *Prevotella* *Anaerovibrio* ↓ *Clostridium* *Terrisporobacter*	=	NA	= Perf ↑ Duo Goblet cells, Villus height/crypt depth ↑ Jej Goblet cells ↑ Il Villus area, Claudin 1, 2, 3, occludin, muc1, IL1β, IL-6, IFNγ, MCP1 ↓ Il TNFα ↓ Ser IL1β	(95)

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TABLE 3 (Continued)

AA		Supplemental level (%) as compared to control	Age (d) or BW at start	Duration (d)	Segment	Phylum	Genus	Diversity	Metabolites	Performance and health	References
BCAA	BCAA	0.6	28 days	14	Colon	NA	NA	=	=	↑: BWG ADFI ↓: FCR ↑ Duo Villus height ↑ Jej Villus height ↑ Il Villus height	(96)
	Leu	1	77.1 kg	60	Colon	↑ Firmicutes, Actinobacteria	↑ *Lactobacillus* *Coriobacteriaceae* *Collinsella* ↓ *Ruminiclostridium, Clostridiales_vadinBB60*	=	↑ Butyrate Propionate	↓ Body fat, cholesterol, triglycerides ↑ HSL, CPT-1 (adipose tissue)	(97)
	BCAA	1.94	28 days	28	Feces	=	=	=	↓5-aminovaleric acid (blood)	↑: Feed intake Energy expenditure	(98)
Arg		1	77.1 ± 1.3 kg	60	Colon	NA	↓ *Actinobacteria*	=	↑Valerate	NA	(94)

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TABLE 3 (Continued)

AA	Supplemental level (%) as compared to control	Age (d) or BW at start	Duration (d)	Segment	Phylum	Genus	Diversity	Metabolites	Performance and health	References
Trp	0.2, 0.4	24	28	Cecum	↑ *Bacteroidetes* ↓ *Firmicutes*	↑ *Prevotella (0.2%)* *Roseburia (0.2%)* *Succinivibrio (0.2%)* ↓ *Clostridium sensu stricto (0.4%)* *Clostridium XI (0.4%)* *Lactobacillus (0.4%)*	↑ (0.2%)	↑ Isobutyrate (0.2, 0.4%) Isovalerate (0.2, 0.4%) ↑ IAA (0.4%)	↑: BWG ADFI = : FCR ↑ Cec Ahr (0.2, 0.4%), CYP1A1 (0.2, 0.4%) ↓ Cec IL-8 (0.4%), TNFα (0.2, 0.4%) ↑ Col Ahr (0.2, 0.4%), CYP1A1 (0.2, 0.4%), CYP1B1 (0.4%), ZO-1 (0.2, 0.4%), Occludin (0.2%) ↓ Col IL-8 (0.4%)	(99)
Trp	0.2, 0.4	24	28	Colon	NA	NA	NA	↑ Propionate (0.2, 0.4%) Isobutyrate (0.2%) Isovalerate (0.2%) Tryptamine (0.2, 0.4%) ↑ IAA (0.2, 0.4%) ↑ Indole (0.2%)		(99)
Trp	0.2, 0.4	24	28	Jejunum	↓ *Firmicutes* *Bacteroidetes* ↓ *(0.2%),* ↑ *(0.4%)*	↑ *Lactobacillus* *Clostridium XI* ↓ *Clostridium sensu stricto* *Streptococcus*	↑ (0.2, 0.4%)	NA	↑ Jej ZO-1 (0.2, 0.4%), ZO-3 (0.2, 0.4%), Claudin-1 (0.2, 0.4%), Occludin (0.4%), beta defensin-2 (0.2, 0.4%), beta defensin 3 (0.2, 0.4%), sIgA (0.2%)	(100)
Trp	0.21 vs. 0.27	24	21	Jejunum	NA	NA	=	NA	NA	(101)
Trp	0.21 vs. 0.27 Mildly ETEC susceptible	24	21	Jejunum	NA	NA	↑	NA	NA	(101)
Trp	0.21 vs. 0.27 ETEC-susceptible	24	21	Jejunum	NA	NA	↑	NA	NA	(101)

AA, amino acid; Arg, arginine; BCAA, branched-chain amino acids; Glu, glutamate; Gln, glutamine; Leu, leucine; MSG, monosodium glutamate; Thr, threonine; Trp, tryptophan; Na, non-available; Duo, duodenum; jej, jejunum; il, ileum; col, colon; Ser, serum; Perf, performance; BW, body weight; BWG, body weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio; Ahr, aryl hydrocarbon receptor; ETEC, Enterotoxigenic E. coli; CYP1, Cytochrome P450, family; CPT-1, carnitine palmitoyl transferase-I; HSL, hormone-sensitive lipase; IL, interleukin; IAA, indole-3-acetic acid; MCP-1, monocyte chemoattractant protein-1; PigR, polymeric Ig receptor; Reg3γ, regenerating islet-derived 3g; SigA, secretory immunoglobulin A; Spla2, secretory group II A phospholipase A2; TLR, toll-like receptor; TNF, tumor necrosis factor; ZO, zonula occludens; ↑, significantly increased compared to control group; ↓, significantly decreased compared to control group; = , similar to control group.

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TABLE 4 Effect of free amino acid supplementation on microbiota, performance and health outcomes in chickens.

AA	Supplemental level (%) as compared to control	Challenge	Age (d)/BW at start	Duration (d)	Segment	Phylum	Genus	Diversity	Performance and health	References
Trp	0.2, 0.4	Transportation stress	21	21	Cecum	NA	↑ *Enterococci* (0.2, 0.4%) *Bifidobacteria* (0.4%) ↓ *E. Coli* (0.2, 0.4%) *Clostridia* (0.2, 0.4%) *Enterobacteria* (0.2, 0.4%) *Campylobacteria* (0.2, 0.4%)	NA	↑ADFI (0.2, 0.4%) Ser serotonin (0.2, 0.4%) ↓ Ser corticosterone (0.2, 0.4%) HSP70 (0.2, 0.4%)	(102)
Trp	0.1, 0.2		0	42	Cecum		↑ *Anaerobacter* (d21) Sporoacetigenium (d42) ↓ *Streptococcus* (d21)	= (d21) ↑ (d42)	NA	(103)
Trp	0.04, 0.08, 0.12		1	42	Ileo-cecal	NA	↑ *Lactobacillus (0.04%)* ↓ *E. Coli (0.04%)*	NA	↑ABWG (0.04%) ↓ FCR (0.04%) ↑ Jej Villus height (0.04%), Villus width (0.04%)	(104)
Thr	0.08, 0.16, 0.24		1	42	Ileo-cecal	NA	=	NA	= Perf	(105)
Thr	0.1, 0.3		1	21	Cecum	NA	↑ *Lactobacillus (0.3%)* ↓ *Salmonella (0.3%)* *E. coli (0.3%)*	NA	= Perf ↑ Jej Villus height (0.3%), goblet cells (0.1, 0.3%), Villus height/Crypt depth (0.1, 0.3%), IgG (0.1, 0.3%), IgM (0.1%), sIgA (0.1%) ↓ Jej MDA (0.1, 0.3%) ↑ Il Villus height (0.1, 0.3%), goblet cells (0.1, 0.3%), Villus height/Crypt depth (0.1, 0.3%), MUC2 (0.3%), SIgA (0.3%) ↓ Il IL1β (0.3%), IFNγ (0.3%) ↓ Ser MDA (0.3%)	(106)

(Continued)

10.3389/fnut.2022.1066898

TABLE 4 (Continued)

AA	Supplemental level (%) as compared to control	Challenge	Age (d)/BW at start	Duration (d)	Segment	Phylum	Genus	Diversity	Performance and health	References
Trp	0.3		1	23	Ileum		↑ *Lactobacillus*	=	↓ jej Crypt depth	(107)
Arg	0.3	Salmonella typhimurium	1	23	Ileum	↓ *Proteobacteria*	↑ *Candidatus Arthromitus* ↓ *Escherichia–Shigella*	↓	↓ jej Crypt depth ↓ Ser IL1β, IL-8, LITNF ↓ Jej IL-8 ↑ Jej IL-10	(107)
Arg	0.12, 0.24, 0.36, 0.48		1	30	Ileum	↑*Firmicutes* (0.24, 0.48%) ↓ *Proteobacteria* (0.24, 0.48%)	↑ *Rombutsia*(0.24, 0.48%) ↓ *Candidatus Arthromitus* (0.24%) *Clostridium sensu stricto* (0.24, 0.48%)	NA	↑BWG (0.12, 0.24, 0.36, 0.48%) ADFI (0.48%) ↓ FCR (0.12, 0.24, 0.36, 0.48%) ↑Jej GSH-PX (0.12, 0.24, 0.36, 0.48%), T-AOC (0.36, 0.48%), HMOX1 (0.24, 0.36%), NRF2 (0.36%), IgG (0.36, 0.48%) ↑Il GSH-PX (0.24, 0.36, 0.48%), T-AOC (0.36, 0.48%), HMOX1 (0.36, 0.48%), NRF2 (0.36%), sIgA (0.24, 0.36, 0.48%) ↓ Jej MDA (0.36, 0.48%) ↓ Il IL1β (0.24, 0.36, 0.48%), MyD88 (0.36, 0.48%), TLR4 (0.48%)	(108)
Arg	0.4	Clostridium perfringens	1	21	Ileum	↑ *Firmicutes* ↓ *Proteobacteria* *Bacteroidetes* *Plantomycetes* *Verrucomicrobia* *Nitrospirae* *Acidobacteria* *Chloroflexi*	NA	↓	↑ Jej Villus height ↓ *C. Perfringens* lesion scores	(109)

(Continued)

10.3389/fnut.2022.1066898

TABLE 4 (Continued)

AA	Supplemental level (%) as compared to control	Challenge	Age (d)/BW at start	Duration (d)	Segment	Phylum	Genus	Diversity	Performance and health	References
Arg	0.61		1	28	Ileum/cecum	NA	↓ *il, cec C. perfringens*	NA	↑plasma D-Xylose ↑Jej sIgA, Claudin-1, IFN-γ ↓ FD4 flux	(110)
Arg	0.61	Eimeria + Clostridium perfringens	1	28	Ileum/cecum	NA	↓ *il C. perfringens*	NA	↑plasma D-Xylose ↑Jej sIgA, Claudin-1, IL-10, IFN-γ, NOD1 ↑ Il Occludin, IFN-γ, NOD1 ↓ FD4 flux	(101)

AA, amino acid; Arg, arginine; Thr, threonine; Trp, tryptophan; Na, non-available; Duo, duodenum; jej, jejunum; il, ileum; col, colon; cec, cecum; Ser, serum; Perf, performance; BWG, body weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio; FD4, Fluorescein Isothiocyanate-dextran; GSH-PX, glutathione peroxidase; HMOX1, Heme Oxygenase 1; HSP, heat shock protein; Ig, immunoglobulin; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; NRF2, Nuclear factor (erythroid-derived 2)-like 2; MyD88, Myeloid differentiation primary response 88; NOD, Nucleotide Binding Oligomerization Domain; LITNF, lipopolysaccharide-induced tumor necrosis factor-alpha factor; SigA, secretory immunoglobulin A; Spla2, secretory group II A phospholipase A2; T-AOC, total antioxidant capacity; TLR, toll-like receptor; TNF, tumor necrosis factor; ZO, zonula occludens; ↑, significantly increased compared to control group; ↓, significantly decreased compared to control group; =, similar to control group.

escape absorption in the small intestine. AA bound to polyphenols might potentially reach the colon and be degraded by the gut microbiota. A recent study showed that the supplementation of piglets with a mix of 0.1% AA (L-arginine, L-leucine, L-valine, L-isoleucine, L-cystine) and 100 ppm grape polyphenols increased the concentration in the caecum of bacterial metabolites derived from AA (e.g., isovalerate and 2-methylbutyrate) (49). Moreover, it is important to consider that the intestinal AA absorption capacity by the host is region dependent. Concurrent with higher expression of brush-border exopeptidases in the distal part of the small intestine (60), the absorption capacity for free AA by enterocytes is the highest in mid- to lower small intestine (61, 62). The latter is mediated by a complex system of brush-border Na^+ dependent and independent transporters with considerable overlap and competition between AA (63). In addition, differences in rate of absorption between AA are noticeable (61). Therefore, any strategy to supply free AA to location-specific microbiota should be carefully designed.

AA supplementation can also be associated with deleterious effects on the gut microbiota. For example, in poultry, it has been reported that the consumption of diets high in glycine such as fish meal or gelatin are associated with increased populations of pathogenic *C. perfringens* (64). In line with these results, a study by Dahiya et al. reported that birds fed glycine at high levels (34.3 or 47.7 g/kg) in an encapsulated form to slowly release the AA along the entire length of the gut exhibited a higher number of *C. perfringens* and a lower number of *Lactobacillus* in the ileum compared to birds fed low levels of encapsulated glycine (7.6 or 21.0 g/kg). This higher colonization was associated with higher intestinal lesions and reduced performance (65). This reinforces the importance of carefully selecting both the AA supplement type and dose to modulate microbiota to deliver a healthy outcome.

Conclusion

Dietary supplementations with free AA modulate the microbiota composition and metabolic activity in association with consequences for host health. These properties indicate that AA have the potential to be used as a novel class of prebiotics ("Aminobiotics"). The successful utilization of AA as prebiotics to selectively nourish gut microbiota still requires to (i) select the most appropriate AA and dose of supplementation, (ii) develop strategies to deliver the desired AA profile to the microbiota, and (iii) demonstrate that the modulation of the microbiota by AA is directly involved in benefits for host health. Overall, utilization of AA as prebiotics, alone or in combination with probiotics, will expand the nutritional tools available to target the gut microbiota for human and animal health.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

TC-D, WL, ER, JM, and MB contributed to the conception and structure of the manuscript. TC-D, MB, and CT created and organized the tables. TC-D wrote the first draft of the manuscript. TC-D, WL, ER, JM, CT, and MB contributed to manuscript writing and revision. All authors approved the final version for submission.

Conflict of interest

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

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland, Finland

REVIEWED BY
Xionge Pi,
Zhejiang Academy of Agricultural Sciences, China
Daniele Maria-Ferreira,
Pelé Pequeno Príncipe Research Institute, Brazil
Maria Florencia Zacarias,
CONICET Santa Fe, Argentina

*CORRESPONDENCE
Zhihong Ren
renzhihong@icdc.cn

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Latilactobacillus sakei Furu2019 and stachyose as probiotics, prebiotics, and synbiotics alleviate constipation in mice

Yanan Guo, Liqiong Song, Yuanming Huang, Xianping Li, Yuchun Xiao, Zhihuan Wang and Zhihong Ren*

State Key Laboratory for Infectious Disease Prevention and Control, Research Units of Discovery of Unknown Bacteria and Function (2018 RU010), Chinese Center for Disease Control and Prevention, National Institute for Communicable Disease Control and Prevention, Chinese Academy of Medical Sciences, Beijing, China

Introduction: Slow transit constipation (STC) is a common disorder in the digestive system. This study aimed to evaluate the effects of stachyose (ST) and *Latilactobacillus sakei* Furu 2019 (*L. sakei*) alone or combined on diphenoxylate-induced constipation and explore the underlying mechanisms using a mouse model.

Methods: ICR mice were randomly divided into five groups. The normal and constipation model groups were intragastrically administrated with PBS. The ST, *L. sakei*, and synbiotic groups were intragastrically administrated with ST (1.5 g/kg body weight), alive *L. sakei* (3×10^9 CFU/mouse), or ST + *L. sakei* (1.5 g/kg plus 3×10^9 CFU/mouse), respectively. After 21 days of intervention, all mice except the normal mice were intragastrically administrated with diphenoxylate (10 mg/kg body weight). Defecation indexes, constipation-related intestinal factors, serum neurotransmitters, hormone levels, short-chain fatty acids (SCFAs), and intestinal microbiota were measured.

Results: Our results showed that three interventions with ST, *L. sakei,* and synbiotic combination (ST + *L.* sakei) all alleviated constipation, and synbiotic intervention was superior to ST or *L. sakei* alone in some defecation indicators. The RT-PCR and immunohistochemical experiment showed that all three interventions relieved constipation by affecting aquaporins (AQP4 and AQP8), interstitial cells of Cajal (SCF and c-Kit), glial cell-derived neurotrophic factor (GDNF), and Nitric Oxide Synthase (NOS). The three interventions exhibited a different ability to increase the serum excitatory neurotransmitters and hormones (5-hydroxytryptamine, substance P, motilin), and reduce the serum inhibitory neurotransmitters (vasoactive intestinal peptide, endothelin). The result of 16S rDNA sequencing of feces showed that synbiotic intervention significantly increased the relative abundance of beneficial bacteria such as *Akkermansia*, and regulated the gut microbes of STC mice. In conclusion, oral administration of ST or *L. sakei* alone or combined are all effective to relieve constipation and the symbiotic use may have a promising preventive effect on STC.

KEYWORDS

slow transit constipation, *Latilactobacillus sakei*, stachyose, synbiotics, intestinal flora

Introduction

Slow transit constipation (STC) is a common clinical symptom of gastrointestinal dysfunction and is characterized by persistent dry stools, difficult bowel movements, and infrequent or incomplete defecation (1, 2). Constipation commonly occurs in the elderly, women, and people with high stress (3, 4). The overall incidence of constipation in the world is about 14%, and the incidence of constipation varies in different regions (5). In some parts of Africa, the incidence of constipation is as high as 30% (5). Mild constipation may induce hemorrhoids and anal fissures, and severe constipation may induce proctitis and even colorectal cancer (6). In people with heart disease, high blood pressure, and cirrhosis, constipation can even cause cardiac arrest, ruptured blood vessels, and massive bleeding (7). In addition, long-term constipation may lead to mental and psychological problems and severely affect people's health and quality of life (8).

Gut motility is controlled by several factors. The enteric nervous system (ENS) is the primary regulator of gut motility, followed by the autonomic nervous system (ANS) and central nervous system (CNS) (9). Both the ENS and CNS can produce 5-hydroxytryptamine (5-HT), which is a key neurotransmitter and mediates enteric nervous reflexes to initiate secretion and propulsive motility and acts on vagal afferents to regulate gut mortality (10). The ENS can interact with the gut microbiota *via* serotonin 5-HT (11). Gut microbiota and the metabolic products of bacterial fermentation are critical to the maturation and stimulation of the ENS then affect gut transit (12). Although it has been reported that constipation can change the composition of gut microbiota (13), several studies have shown that constipation could be relieved by regulating the intestinal microbiota and the metabolites (14), promoting gut-brain communication (15), and enhancing the intestinal peristalsis *via* the c-Kit pathway of interstitial cells of Cajal (ICCs) (9). ICCs are regarded as the pacemaker of gastrointestinal peristalsis and constipation is often accompanied by abnormalities in ICCs (16).

The prebiotics that has been confirmed so far mainly include oligosaccharides, dietary fiber, inulin, and others (17), which can promote specific beneficial bacteria growing rapidly in the gut. Stachyose (ST) belongs to the raffinose family of oligosaccharides and is formed by combining two α-galactoses with 1,6-glycosidic bonds on the glucosyl side of sucrose (18). Studies have shown that ST can relieve the symptoms of constipation in patients and significantly enhance the amount of Bifidobacteria and Lactobacilli and reduce the fecal *Clostridium perfringens* concentration (19). ST has been clinically proven to relieve constipation and it acts mainly by increasing the abundance of short-chain fatty acids (SCFAs) (20). *Latilactobacillus sakei*, Gram-positive and facultative anaerobic bacteria, was first discovered in Japanese sake (21). *L. sakei* is often used in fermented foods and has also been isolated from homemade fermented bean curd, kimchi, pig ears, and other local specialties in Sichuan (22). *L. sakei* Furu 2019 (*L. sakei*) was isolated from a traditional Chinese food called fermented bean curd which was considered to be beneficial "Chinese cheese." Therefore, *L. sakei* is usually regarded as a transient resident of the human gastrointestinal tract because it can survive under harsh conditions. *L. sakei* has been reported to have several health benefits. *L. sakei* is currently known to have anti-inflammation and weight loss effects, for example, *L. sakei* ADM14 can alleviate high-fat diet-induced obesity and alter the gut microbiota in mice (23). *L. sakei* WIKIM31 can prevent the weight gain induced by the high-fat diet by modulating lipid metabolism and suppressing inflammation (24). *L. sakei* S1 can improve trinitrobenzene sulfonic acid-induced colitis by inhibiting NF-kappaB signaling in mice (25, 26).

Clinically, constipation is often treated with laxatives such as cisapride. However, drug treatment often has side effects (27). Thus, it is of need to develop an effective and safe treatment to relieve constipation. Some probiotic products have been used to treat constipation, such as live *Bacillus coagulans* capsules and *Bifidobacteria* capsules (28). The important advantage of these probiotics is that probiotics are gentler and safer (28). The effects of probiotics on the composition of the microbiota, SCFA production, and gut motility of constipation are still relatively poorly understood. Some prebiotics such as oligosaccharides could also alleviate constipation (29). It has been proposed that synbiotics may exert a better effect on host health (30). Since there is no information available thus far regarding the effect of *L. sakei* or ST alone or combined on constipation, we conducted this study to evaluate the efficacy of *L. sakei* and ST as probiotics, prebiotics, and synbiotics in alleviating constipation in a mouse model and also to further investigate the underlying mechanisms.

Materials and methods

Ethics statement

This study was approved by the Ethics Review Committee of the National Institute for Communicable Disease Control and Prevention at the Chinese Center for Disease Control and Prevention (Beijing, China).

Reagents and preparation of *L. sakei*

Diphenoxylate-atropine containing 2.5 mg of diphenoxylate hydrochloride and 0.025 mg of atropine sulfate per tablet (H22022037, Changhong Pharmaceutical Co., Ltd, Changchun,

Abbreviations: STC, slow transit constipation; ST, stachyose; *L. sakei*, *Latilactobacillus sakei*; SCFAs, short-chain fatty acids; ICCs, interstitial cells of Cajal.

China) was used to establish the constipation mouse model in this study. The ingredients of Stachyose (ST) used in this study include stachyose (no <70%), sucrose (12 ± 2%), raffinose (5 ± 1%), mannotriose (2 ± 0.5%), fructose (2 ± 0.5%), glucose (1 ± 0.2%), verbascose (2 ± 0.5%), water (no more than 5%), and 1% ash. ST was provided by the China National Research Institute of Food and Fermentation Industries. A stock solution (0.5 g/kg body weight) of ST in sterile PBS was microfiltered (0.22 μm sterile disc, Merck Millipore Ltd, USA) and kept at 4°C before use. Ten grams of gum Arabic (Beijing Lebesi des Biotechnology Co., Ltd., ALBJ1L) was added into 80 mL of ultrapure water. Then 10 g of activated carbon powder (Beijing Pringle Science and Trade Co., Ltd., C7261) was added into the Arabic solution and boiled three times to make the final 100 mL ink stock solution as an indicator of defecating the first black stool. *L. sakei* was isolated from a traditional Chinese food called fermented bean curd and was routinely grown in Man-Rogosa-Sharpe (MRS) medium in a 37°C carbon dioxide incubator (Forma CO2, Thermo Fisher Scientific, Waltham, MA, USA). A culture of *L. sakei* in the logarithmic growth phase was washed and resuspended with sterile PBS for oral inoculation of mice.

Animal experiment

SPF male ICR mice (24–26 g, 6-week-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed in the Animal Center of China CDC and had free access to water and food under a 12/12-h dark/light cycle at a constant temperature of 22–24°C. After 7 days of acclimation, 40 mice were randomly divided into five groups: normal, constipation, prebiotics, probiotics, and synbiotics. The normal and constipation model groups were given 0.3 mL of PBS every morning. The prebiotics, probiotics, and synbiotics groups were intragastrically administrated 0.3 mL of ST (1.5 g/kg), alive *L. sakei* (3×10^9 CFU/mouse), and the mixture of ST and *L. sakei* (1.5 g/kg plus3 $\times$ 10^9 CFU/mouse), respectively. After 21 days of intervention, all mice were fast for 16 h. On day 22, all mice except the normal mice were intragastrically administrated with diphenoxylate (10 mg/kg) to establish the constipation mouse model according to previous publications (31, 32). After 30 min, all mice were gavaged with 0.25 mL of ink. Then mice were given the water and diet and put in metabolic cages individually. Within 5 h after oral administration of the ink, defecation index was recorded. After 16 h-fast, on day 23, fresh feces were collected for 16S rRNA sequencing. Then all mice except the normal mice were given 0.3 mL of diphenoxylate (5 mg/kg). After 25 min, all mice were sacrificed and the entire bowel was removed and its length was measured. The blood was collected to obtain serum for quantification of neurotransmitters. The colons were collected and kept in 4% paraformaldehyde and RNAlater (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Fresh cecal content was collected and preserved at −80°C for quantification of $SCFA_S$.

Determination of defecation index in mice

On day 22, within 5 h after oral administration of the ink, the defecation index including the time of expelling black stool for the first time, the number of excreted feces, and the wet weight of the feces were recorded. Collected feces were continuously dried in an oven at 100°C for 2 h, and the dry weight of feces was measured to calculate the moisture content of the feces using the following formula (33).

$$R = \frac{W_{(wet)} - W_{(dry)}}{W_{(wet)}} \times 100\% \quad (1)$$

Determination of intestinal propulsion index in mice

The entire bowel was removed and its length was measured. The following formula was used to calculate the intestinal propulsion rate where S_1 represents the total length of the intestinal tract, and S_2 represents the distance from the pylorus to the front end of the ink.

$$D = \frac{S_2}{S_1} \times 100\% \quad (2)$$

Quantitative PCR detection of mRNA expression

Total RNA of the colonic tissues was extracted using TRIzol (Invitrogen, US) and the reverse transcription reactions were performed to generate cDNA by reverse transcription kit (Takara, Japan) according to the manufacturer's instructions. The cDNA products were subjected to RT q-PCR in an ABI 7500 Real-Time PCR System (ABI, United States). The amplification reaction conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s followed by 40 cycles. The relative levels of mRNA expression were calculated using the delta-delta CT method. Duplicated wells were set up for each sample and the average Ct value was determined from two runs. The gene expression level of β-actin was used as the internal control. ΔCt = Ct (target gene) – Ct (control gene), and relative gene expression was calculated as $\Delta\Delta Ct = \Delta Ct$ (treated)–ΔCt (blank). The relative gene expression levels were converted to $2^{-\Delta\Delta Ct}$. The primers are listed in Table 1.

TABLE 1 List of primers used in the qRT-PCR assay for constipation-related intestinal factors.

Gene	Primer sequence
β-Actin	Forward: 5′-CGGACACGGACAGGATTGACA
	Reverse: 5′-CCAGACAAATCGCTCCACCAACT
Aqp4	Forward: 5′-GCAGACAAGGTGCAACGTGGTT
	Reverse: 5′-GGCGGAAGGCAAAGCAGTATGG
Aqp8	Forward: 5′-AGCAGGAGCAGGTGGCAGAA
	Reverse: 5′-TCCAAAGGCACGAGCAGGGT
GDNF	Forward: 5′-GGGGTATGGAGAAGTTGGCTAG
	Reverse: 5′-CTATGAGAATGCTGCCGAAAA
SCF	Forward: 5′-TCAGGGACTACGCTGCGAAAG
	Reverse: 5′-AAGAGCTGGCAGACCGACTCA
c-Kit	Forward: 5′-CATAGCCCAGGTAAAGCACAAT
	Reverse: 5′-GAACACTCCAGAATCGTCAACTC
NOS	Forward: 5′-TCAGCGGTGA TAGGA TAAAGCA
	Reverse: 5′-CGCTGTGCTAAGTAGCCCTCG

Immunohistochemical analysis

The colons were fixed with 4% formaldehyde. Sections were first deparaffinized in xylene and passed through various concentrations of alcohol. Sections were incubated with 5% BSA (Wuhan Boster, China) for 30 min. The primary antibodies including anti-c-Kit antibody (CST, USA), anti-SCF antibody (Absin, China), anti-AQP4 antibody (Abcam, USA), and anti-AQP8 antibody (Absin, China) were added and incubated overnight at 4°C. Then the sections were rewarmed at room temperature for 1 h and washed three times with PBS buffer. The secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (Beyotime, China) was added dropwise and incubated at 37°C for 30 min. After washing 3 times with PBS for 10 min, DAB color development was performed. Hematoxylin was then used for counterstaining, differentiation, and reverse blue. After dehydration, washing, and mounting, the sections were photographed with Computer image processing system CMOS (OLYMPUS,Japan)and analyzed using Image-Pro Plus software (Media Cybernetic, US). The intensity of positive immunostaining and tissue area were determined by human-computer interaction. The average optical density was calculated as the ratio of integrated optical density versus tissue area. These results were evaluated by an experienced person who was blind to the samples in terms of treatment. The average optical density was statistically analyzed using GraphPad Prism5.

Quantification of SCFAs

$SCFA_S$ were determined by Beijing Nuohezhiyuan Biotechnology Co., Ltd. Briefly, 30 mg of mouse cecum content was suspended in 900 μL of 0.5% phosphoric acid (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China). After centrifugation at 14,000 g 4°C for 10 min, 800 μL supernatant was mixed with an equal volume of ethyl acetate. After the mixture was centrifuged at 14,000 g for 10 min, 600 μL of the upper organic phase was collected for GC-MS analysis. Six hundred microliters of a mixed standard solution composed of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and hexanoic acid with eight concentrations were added with 25 μ L 500 μM Methyl valerate as internal standard. The injection volume was 1 μL, and the split ratio was 10:1. Temperature program was as follows: the initial temperature was set at 90°C; the temperature was increased to 120°C at 10°C/min; then the temperature was increased to 150°C at 5°C/min; finally, the temperature was increased to 250°C at 25°C/min and maintained for 2 min. The carrier gas was helium and the carrier gas flow rate was 1.0 mL/min. Agilent 7890A/5975C GC-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an Agilent DB-WAX (Agilent Technologies, Co., Ltd., US) capillary column (30 m × 0.25 mm ID × 0.25 μm) was used to determine the SCFA contents. MSD ChemStation software was used to obtain the chromatographic peak area and retention time. The contents of short-chain fatty acids in the samples were calculated based on the standard curve.

Quantification of neurotransmitters

Serum was collected to measure 5-hydroxytryptamine (5-HT), vasoactive intestinal peptide (VIP), substance P (SP), motilin (MTL), and endothelin (ET) using serum test kits (Beijing Yisheng Zhaobo Biotechnology Co., Ltd.). Briefly, a total of 50 μL standard substance or 1:5 diluted samples were added on the enzyme-labeled coating plate, respectively, and the plates were sealed and incubated at 37°C for 30 min. Then the plates were washed 6 times with a washing solution. Fifty microliters of enzyme-labeled reagent was added into each well except for control wells. After incubation and washing, 50 μL of chromogenic reagents A and B were added and mixed then placed in the dark at 37°C for 10 min to develop color. Finally, 50 μL of stop solution was added into each well then the absorbance (OD value) was measured at 450 nm wavelength.

16S rRNA sequencing and intestinal microbiota analysis

Fresh fecal samples were collected as previously described and 16S rRNA sequencing was performed on the V3–V4 gene region to analyze the mouse fecal microbiota. MagPure Stool DNA KF kit B (Magen, China) was used to extract the DNA from feces. DNA was quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, USA) and the quality was checked by agarose gel electrophoresis. The variable region V3–V4 of the

bacterial 16S rRNA gene was amplified with the degenerate PCR primers 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Libraries were characterized by an Agilent 2100 Bioanalyzer (Agilent, USA). The amplicons and quality control of the raw data were conducted on the Illumina Hiseq 2500 platform (BGI, China). Clean data was performed using USEARCH (V10.0.240)5 and VSEARCH (V2.8.1)6 for reads splicing, filtering of low-quality reads, chimera removal, and operable taxonomic unit (OTU) construction. The α diversity and principal coordinates analysis (PCoA) was calculated and visualized by R software (V3.6.1; https://github.com/microbiota). Differential OTUs were identified and statistically analyzed by LEfSe (http://huttenhower.sph.harvard.edu/galaxy/) online using non-parametric factorial Kruskal–Wallis and Wilcoxon rank-sum tests. The threshold was set at 2.0 for discriminative features. The original data can be downloaded from the NCBI SRA database (PRJNA877764).

Statistical analysis

Statistical analysis of data was performed with GraphPad Prism 5 software (GraphPad Inc., San Diego, CA, USA). All continuous variables were presented as mean ± standard deviation (SD), and category variables were expressed as *n* (%). Statistical differences between groups were determined by one-way analysis of variance (ANOVA) for normal distribution data. Otherwise, the non-parametric Kruskal–Wallis rank-sum test was used followed by Duncan's test for multiple comparisons for alpha diversity analysis of the gut microbiota. $P < 0.05$ was considered statistically significant.

Result

Effects of ST or *L. Sakei* as prebiotics, probiotics, and synbiotics on defecation parameters and gastrointestinal transit

The constipation-related indices in the diphenoxylate-induced constipation mouse model include the fecal weight, fetal number, fecal water content, the first black fecal defecation time, and gastrointestinal transit. The shorter time of defecating the first black stool indicates more rapid motility throughout the intestine and a stronger intestinal transport capacity. The higher GI transit rate indicates greater small intestinal motility. In this study, diphenoxylate treatment effectively induced constipation model as indicated by lower feces weight (105 ± 62.7 vs. 468.67 ± 162.96 mg), fewer feces number (8 ± 3.58 vs. 24.5 ± 7.56), less water content of feces (40 ± 6.1 vs. 78.33 ± 7.47%), longer time of first black feces defecation (70.83 ± 20.84 vs. 195.83±10.96 min), and lower gastrointestinal transit rate (35.33 ± 3.77 vs. 58.23 ± 6.79%) compared with the normal control mice (Figures 1A–E). After intervention with ST or *L. sakei* alone or combined, the five constipation-related indices in the three intervention groups were all significantly different from the constipation model group and close to the normal control group (Figures 1A–E). For example, the feces weight in the ST group (284.83 ± 66.62 mg), *L. sakei* group (252.8 ± 45.98 mg), and synbiotic group (552.4 ± 267.16 mg) were significantly higher than that in the constipation model group (105 ± 62.7 mg; $P < 0.05$). These results indicate that while all three interventions can alleviate the symptoms of constipation, the synbiotic combination of ST and *L. sakei* is more effective in some indicators than ST or *L. sakei* intervention.

Effects of ST or *L. sakei* alone or combined on mRNA expression levels of constipation-related intestinal factors

Aquaporin (AQP) channels play a central role in regulating fluid homeostasis in the colon. Several AQP channels were detected in human colon epithelial cells. AQPs are primarily expressed in human colon epithelial cells. Our results showed that *AQP4* and *AQP8* expression in the colon of constipation mice was significantly higher than that of normal mice, while *AQP4* and *AQP8* expression was significantly down-regulated in the colon of three intervention groups (Figures 2A,B). In addition, activation of the C-kit receptor on the surface of interstitial Cajal cells (ICC) is closely related to ICC function, and the stem cell factor (SCF)/C-kit signaling pathway plays an important role in stimulating intestinal motility. We found that *c-Kit* and *SCF* expression in the colon of constipated mice was significantly lower than that of normal mice. The levels of *SCF* and *c-Kit* in ST or *L. sakei*, and synbiotics groups were all significantly higher than those in the constipation group ($P < 0.05$). The differences among the three intervention groups were not significant (Figures 2C,D, $P > 0.05$). The increasing expression of *GDNF* (glial cell-derived neurotrophic factor) in mice helps gastrointestinal innervation, which affects intestinal motility and avoids constipation. Our results showed that the expression of *GDNF* in the constipation group was significantly lower than that in the normal group ($P < 0.05$) and *L. sakei* treatment significantly increased the expression of *GDNF*. The expression of GDNF in both the ST and the synbiotic group was increased but did not reach a significant difference compared with the constipation group (Figure 2E). It has been reported that colonic motility is negatively correlated with the production of nitric oxide (NO) and the expression of NOS, the only enzyme involved in NO synthesis. We found that the expression level of *NOS* in the normal group was significantly lower than that in the constipation group. Both ST or *L. sakei* interventions significantly decreased the expression levels of *NOS* ($P < 0.05$) while the symbiotic intervention has less effect on the expression of NOS (Figure 2F, $P > 0.05$).

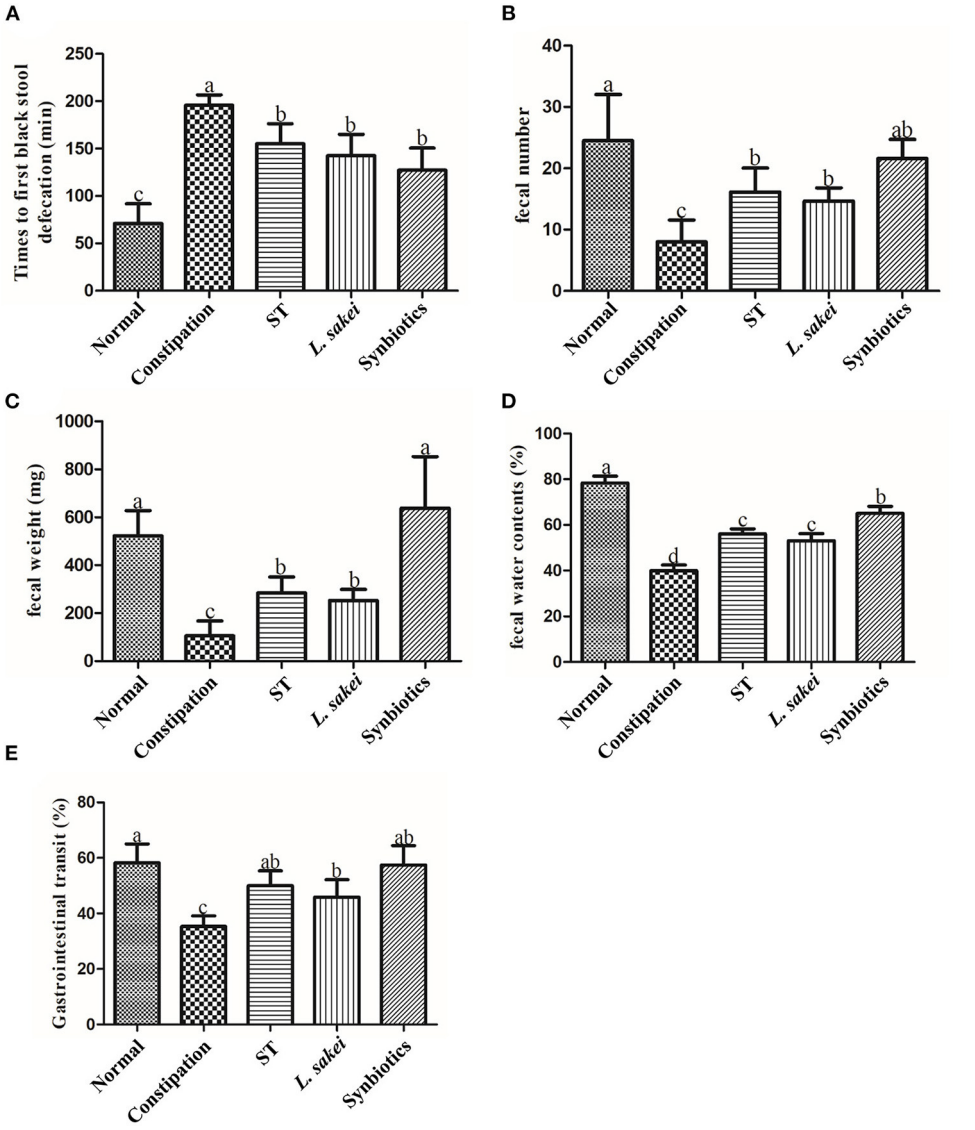

FIGURE 1
Effects of ST or *L. sakei* alone or combined on defecation parameters and gastrointestinal transit in mice. **(A)** Times to first black stool defecation; **(B)** Fecal number; **(C)** Fecal weight; **(D)** Fecal water contents; **(E)** Gastrointestinal transit. Data represent the mean ± SD ($n = 6$). Statistical analysis was conducted using One-way ANOVA followed by Tukey's multiple comparisons test for each group. The bars bearing different letters indicate a significant difference, $P < 0.05$.

Effects of ST or *L. sakei* alone or combined on protein levels of constipation-related intestinal factors

Immunohistochemical results of protein levels of AQP4, AQP8, c-Kit, and SCF in colon tissue were shown in Figure 3A, which were observed under a 200× microscope and the average optical density value was calculated. The result showed that the protein levels of AQP4 and AQP8 in the colon of constipation mice were significantly higher than those in the normal group. Compared with constipation mice, the mice in the three intervention groups all had significantly lower levels of AQP4 and AQP8 in colons except for the effect of prebiotic ST treatment on AQP4 level (Figures 3B,C, $P < 0.05$). The protein expressions of c-Kit and SCF in the colon of constipation mice were significantly lower than those in the normal group, and the down-regulation of c-Kit and SCF by constipation was increased in all three intervention groups (Figures 3D,E, $P < 0.05$). These results indicate that intervention of ST or *L. sakei* alone or combined could affect the protein level of aquaporins and the number of ICCs to relieve constipation.

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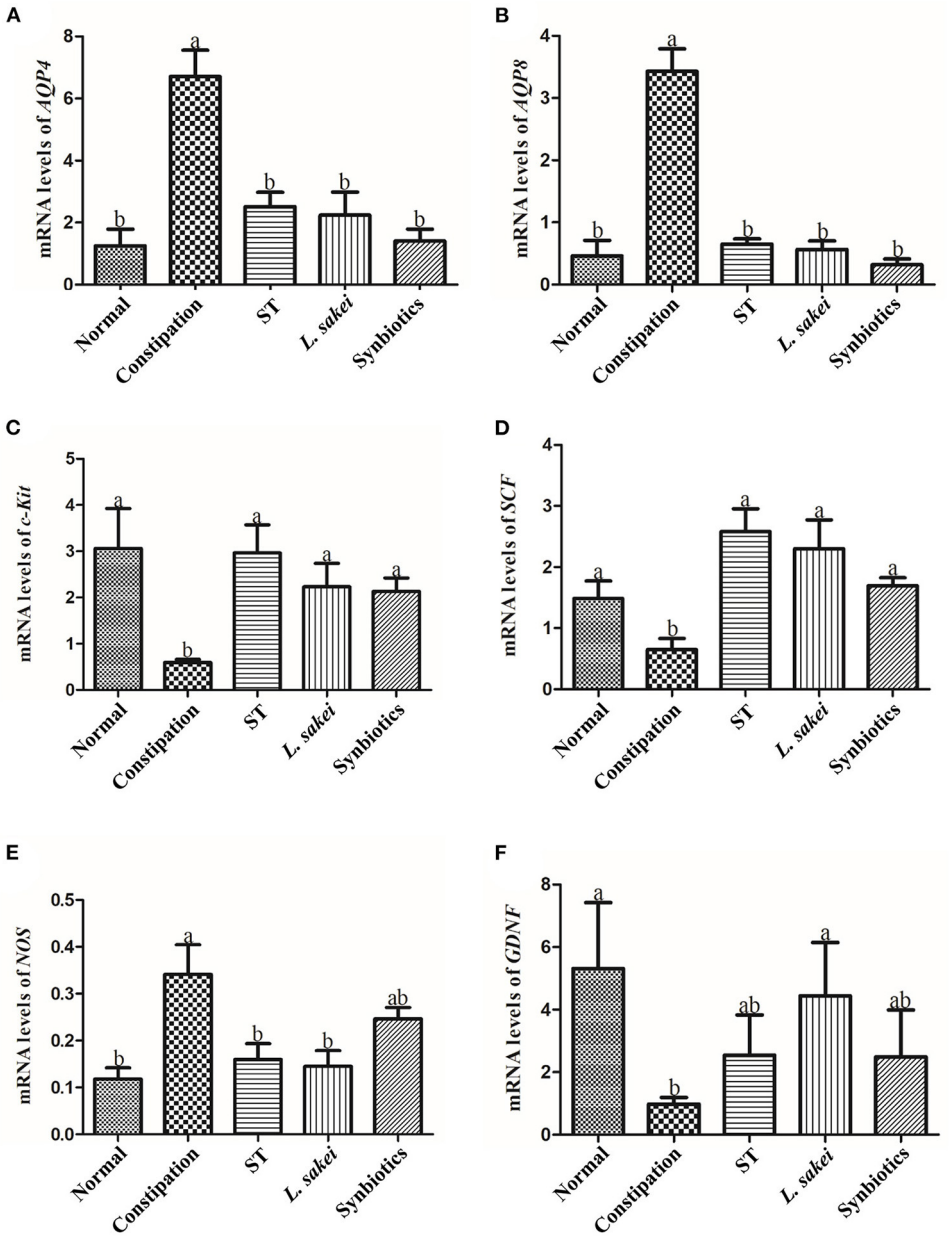

FIGURE 2
The effect of ST or *L. sakei* alone or combined on the mRNA expression levels of constipation-related intestinal factors in mice. **(A)** Changes in mRNA levels of *AQP4*; **(B)** Changes in mRNA levels of *AQP8*; **(C)** Changes in mRNA levels of *c-Kit*; **(D)** Changes in mRNA levels of *SCF*; **(E)** Changes in mRNA levels of *NOS*; **(F)** Changes in mRNA levels of *GDNF*. Data represent the mean ± SD ($n = 6$). Statistical analysis was conducted using One-way ANOVA followed by Tukey's multiple comparisons test for each group. Bars bearing different letters indicate a significant difference, $P < 0.05$.

Effects of ST or *L. sakei* alone or combined on neurotransmitter and hormone levels in the serum of constipation mice

By measuring the excitatory neurotransmitters serotonin (5-HT), substance P (SP), and the gastrointestinal hormone motilin (MTL), and the inhibitory neurotransmitters vasoactive intestinal peptide (VIP) and endothelin (ET), we explored whether treatment with ST or *L. sakei* alone or combined can restore intestinal function by affecting the content of intestinal neurotransmitters. The results showed that the levels of excitatory neurotransmitters and hormones including 5-HT (271.32 ± 10.16 ng/L); SP (61.05 ± 0.76 ng/L), and MTL

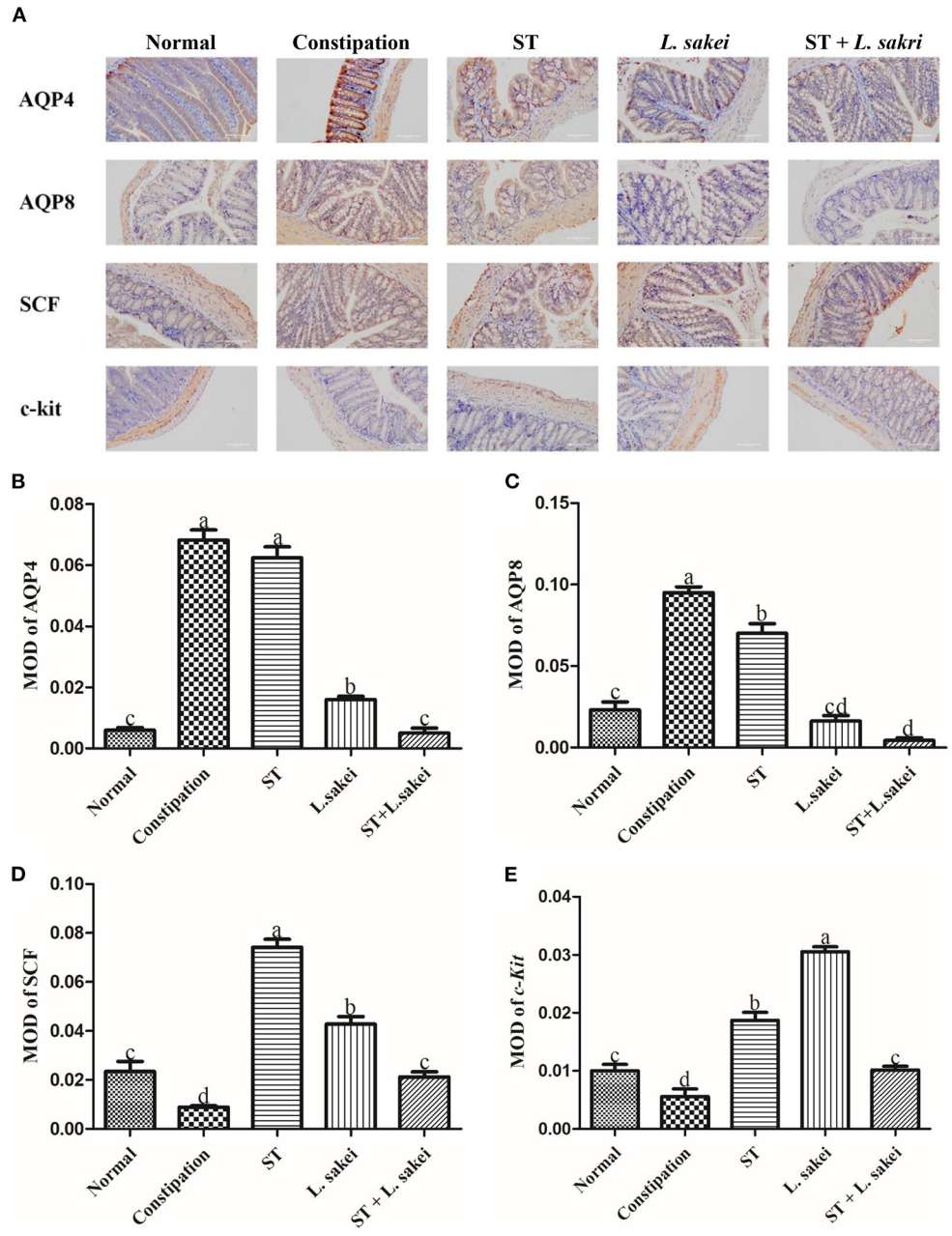

FIGURE 3
The effect of ST or *L. sakei* alone or combined on the protein levels of constipation-related intestinal factors in mice. **(A)** Protein expressions of AQP4, AQP8, c-kit, and SCF in the colons of mice were detected by immunohistochemistry and were observed under a 200×00 microscope. The mean optical density (MOD) of AQP4 **(B)**, AQP8 **(C)**, c-kit **(D)**, and SCF **(E)** was calculated and evaluated by an experienced person who was blind to the samples in terms of treatment. Data represent the mean ± SD ($n = 6$). Statistical analysis was conducted using One-way ANOVA followed by Tukey's multiple comparisons test for each group using GraphPad Prism5. Bars bearing different letters indicate a significant difference, $P < 0.05$.

(449.88 ± 11.25 ng/L) in the serum of constipation mice were significantly lower than those in the normal group (5-HT 280.14 ± 8.74 ng/L; SP 63.35 ± 1.65 ng/L; MTL 473 ± 17.1 ng/L, $p < 0.05$). Among the three intervention groups, only the symbiotic intervention significantly increased the content of 5-HT (287.34 ± 10.16 vs. 271.32 ± 10.16 ng/L). In addition, three interventions all increased the production of SP (ST 64.1 ± 2.46 ng/L, *L. sakei* 68.69 ± 2.02 ng/L, and Symbiotic 71 ± 0.94 ng/L vs. PBS 61.05 ± 0.76 ng/L) and MTL (ST 548.52 ± 28.26 ng/L, *L. sakei* 523.24 ± 20.25 ng/L,

and Symbiotic 457.69 ± 20.05 ng/L vs. constipation 449.88 ± 11.25 ng/L; Figures 4A–C). We noticed that the synbiotic intervention had the strongest up-regulation effect on 5-HT and SP. For inhibitory neurotransmitters, the levels of VIP (233.81 ± 5.26 ng/L) and ET (198.98 ± 4.36 ng/L) in the serum of constipation mice were increased compared with normal mice (VIP 207.16 ± 6.12 ng/L, ET 167.87 ± 4.57 ng/L). Among the three intervention groups, only *L. sakei* intervention down-regulated the expression of VIP (224.08 ± 2.43 vs. 233.81 ± 5.26 ng/L; Figures 4D,E). Administration of ST or *L. sakei* affected all the above-mentioned neurotransmitters except VIP. All three interventions restored and decreased the content of ET. Our data showed that the administration of ST or *L. sakei* alone or combined can differently affect the production of neurotransmitters and hormones.

Effects of ST or *L. sakei* alone or combined on short-chain fatty acids (SCFAs)

The abundance of SCFAs in the cecal content of all mice was measured in this study. The results showed that there was no significant difference in the abundance of SCFAs between the normal group and the constipation group. The mice supplemented with ST had a significantly higher amount of acetic acid (1105.87 ± 147.84 μg/g), propionic acid (306.86 ± 78.3042 μg/g), isovaleric acid (23.33 ± 6.91 μg/g), isobutyric acid (26.56 ± 4.03 μg/g), and valeric acid (39.81 ± 8.79 μg/g) while the mice supplemented with *L. sakei* had a significantly higher amount of propionic acid (313.53 ± 49 μg/g), acetic acid (1177.42 ± 198.4 μg/g), butyric acid (291.86 ± 80.05 μg/g), and valeric acid (37.69 ± 5.65 μg/g) compared with constipation mice treated with PBS (acetic acid 779.83 ± 178.15 μg/g, propionic acid 208.90 ± 41.51 μg/g, isovaleric acid 10.79 ± 1.87 μg/g, isobutyric acid 20.73 ± 2.22 μg/g, butyric acid 134.76 ± 69.49 μg/g, valeric acid 19.78 ± 6.50 μg/g). Administration of synbiotics only increased the amount of propionic acid (277.87 ± 54.47 μg/g) and butyric acid (304.33 ± 145.85 μg/g; Figures 5A–F). These results showed that both ST or *L. sakei* alone or combined could relieve constipation by increasing the abundance of SCFAs.

Effects of ST or *L. sakei* alone or combined on the diversity of gut microbes

The effects of ST and *L. sakei* on the diversity of gut microbes were analyzed. As shown in Figures 6A–C, the α-diversity of gut microbes in the constipation group was significantly greater than that in the normal and three intervention groups. It indicates that intervention with ST or *L. sakei* could reverse the changes in the diversity and species richness in mice caused by constipation. The beta diversity of gut microbes was analyzed at the phyla (Figure 6D) and genus level (Figure 6E). The gut microbes of the normal group and the constipation group were significantly different at the phylum level. Among the three intervention groups, the symbiotic group was the most different and the prebiotic group was the least different from the constipation group (Figure 6D). At the genus level, the beta diversity of gut microbes in the normal group, the constipation group, the prebiotic group, and the probiotic group was close to each other while the symbiotic group was different from the other four groups (Figure 6E), which indicates that the combination of ST and *L. sakei* could significantly change the diversity of gut microbes in constipated mice.

Effects of ST or *L. sakei* alone or combined on the structure of intestinal microbiota in mice

At the phylum level, the microbiota composition and relative abundance of each group are shown in Figure 7A. The relative abundance of Verrucomicrobiota in the constipation group was significantly lower than the normal and three intervention groups. The relative abundance of Verrucomicrobiota in both *L. sakei* and ST + *L. sakei* mice was even higher than that of the ST and normal mice, indicating that intervention with *L. sakei* can significantly increase the relative abundance of Verrucomicrobiota and reverse the reduction by constipation. At the genus level, compared with the normal group, the relative abundance of *Bacteroides* and *Alloprevotella* had a trend of increasing in the constipation group, and the relative abundance of *Clostridium_XlVa*, *Prevotella*, *Alistipes*, and *Akkermansia* tended to decrease. The intervention of ST tended to partially restore the relative abundance of *Prevotella*, *Alloprevotella*, *Alistipes*, and *Akkermansia*, *L. sakei* tended to partly restore the relative abundance of *Bacteroides*, *Clostridium_XlVa*, *Alistipes*, and *Akkermansia*, and ST + *L. sakei* tended to partially restore the relative abundances of *Bacteroides*, *Alistipes*, and *Akkermansia* (Figure 7B).

To determine the difference in the abundance of species among populations, we performed the LEfSe analysis (Figure 7C). We found that the characteristic gut microbes of the normal group were Rikenellaceae, Lachnospiracea_incertae_sedis, Lactobacillaceae, *Bacilli*, *Lactobacillus,* and Lactobacillales. The characteristic gut microbes of the constipation group were *Bacteroides* and Bacteroidaceae. The characteristic gut microbes of the prebiotic group were *Ruminococcus.* The characteristic gut microbes of the probiotic group were *Proteobacteria* and the characteristic gut microbes of the synbiotic group were Porphyromonadaceae, *Akkermansia*, Verrucomicrobiales, Verrucomicrobiota, Verrucomicrobiaceae, and, Verrucomicrobiae. Together, these

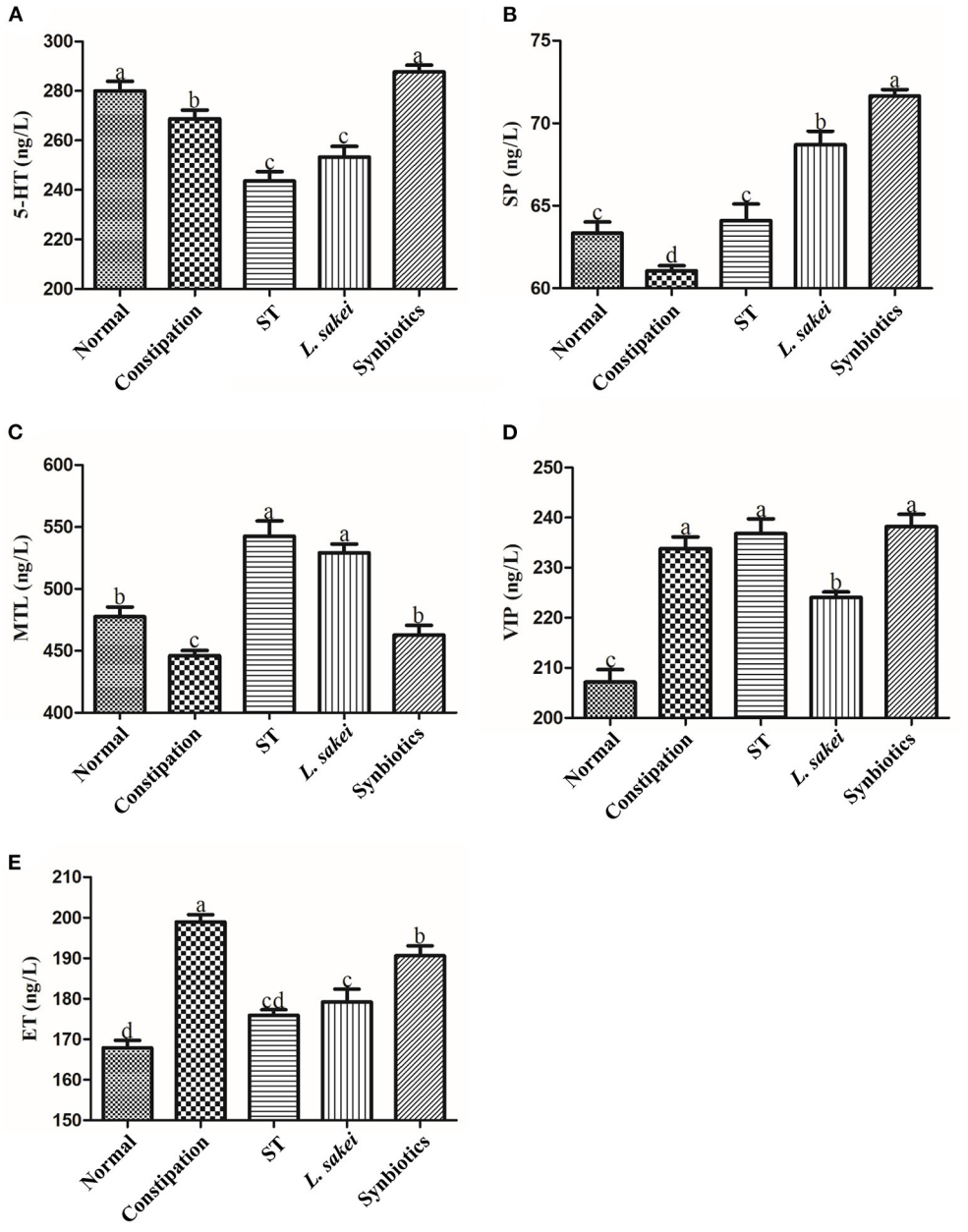

FIGURE 4
Effects of ST or *L. sakei* alone or combined on intestinal hormone levels in mice. **(A)** 5-HT level; **(B)** SP level; **(C)** MTL level; **(D)** VIP level; **(E)** ET level. 5-HT, 5-hydroxytryptamine; SP, substance P; MTL, motilin; VIP, vasoactive intestinal peptide; ET, endothelin. Statistical analysis was conducted using One-way ANOVA followed by Tukey's multiple comparisons test for each group. Bars bearing different letters indicate a significant difference, $P < 0.05$.

results suggest that the combination of ST and *L. sakei* probably relieves constipation by altering the abundance of beneficial bacteria and harmful bacteria.

Discussion

Alteration in intestinal peristaltic contractions can result in prolonged intestinal transit time, prolonged bacterial fermentation time, and prolonged water absorption time, which is associated with reduced fecal water content and decreased defecation. The defecation process is associated with multiple factors including neurotransmitters, hormones, gut microbes, interstitial cells of Cajal, and intestinal motility factors (34). Abnormalities in any of these factors can lead to constipation. According to the previous studies using constipation models, we set the initial observation indicators such as the first defecation

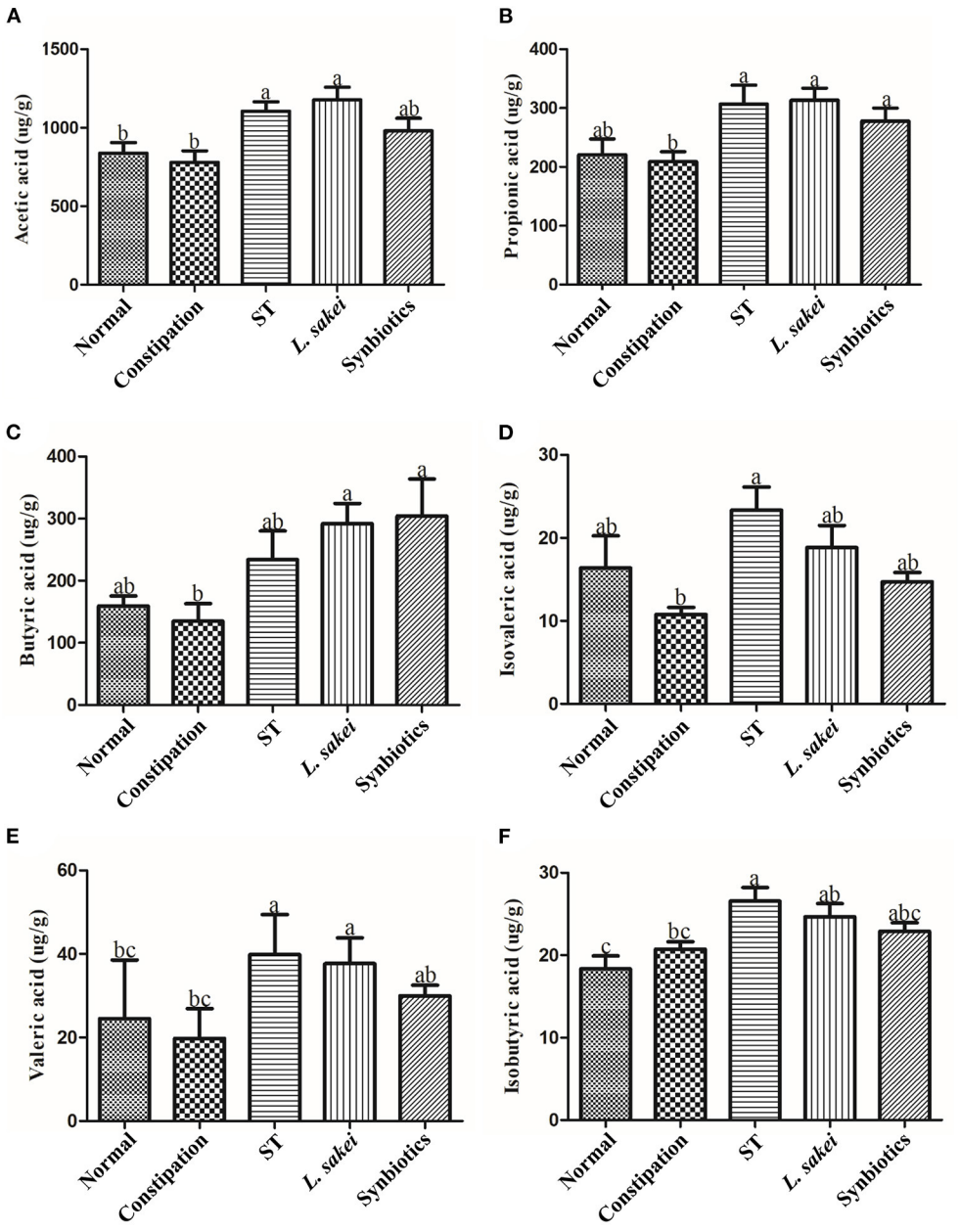

FIGURE 5
The effect of ST or *L. sakei* alone or combined on short-chain fatty acid levels of constipation-related intestinal factors in mice. **(A)** Acetic acid; **(B)** Propionic acid; **(C)** Butyric acid; **(D)** Isovaleric acid; **(E)** Isobutyric acid; **(F)** Valeric acid. Data represent the mean ± SD ($n = 6$). Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons test for each group. Bars bearing different letters indicate a significant difference, $P < 0.05$.

time, stool wet weight, stool quantity, stool water content, and small intestine propulsion rate in this study (35–38). Studies have shown that the beneficial effect of ST on intestinal diseases is mainly reflected in two aspects, i.e., broad effects, such as on bowel parameters and fecal water content; and specific effects, such as SCFAs and intestinal effects of neurotransmitters (30). Our results showed that three interventions with ST or *L. sakei* alone and combined all alleviated constipation, and synbiotic intervention was superior to ST or *L. sakei* alone in some defecation indicators presumably because ST not only has a laxative effect on the intestinal tract itself, but it also promotes the growth of *L. sakei* and other probiotics in the intestinal tract.

Aquaporin (AQP) channels are involved in regulating fluid homeostasis in the colon (39). The expression level of AQP

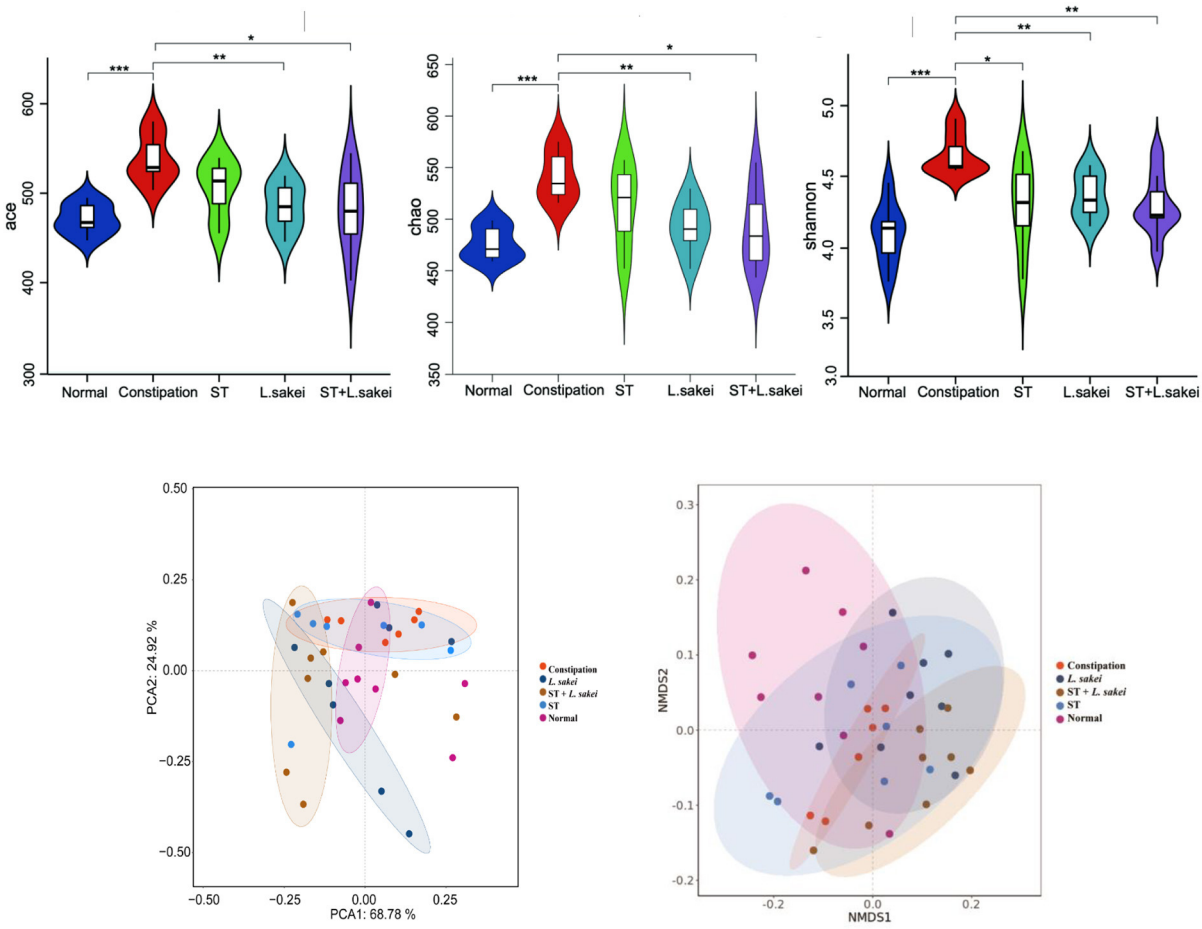

FIGURE 6
The effect of ST or *L. sakei* alone or combined on the alpha diversity analysis and beta diversity analysis in mice microbiota. **(A)** Ace index; **(B)** Chao index; **(C)** Shannon index; **(D)** PCA score plot at the phylum level; **(E)** NMDS plot at the genus level. Data represent the mean ± SD (n = 6–8). Wilcoxon signed-rank test of non-parameter statistics was used for constipation group vs. normal group, ***$p < 0.001$. Wilcoxon signed-rank test of non-parameter statistics was used for ST, *L. sakei*, and ST + *L. sakei* groups, vs. constipation group *$p < 0.05$, **$p < 0.01$.

in the gut is particularly important for fecal conditions (39). When the expression level of a specific AQP is too high, the gut will over-absorb water from the feces, and thus reduce the water content of the feces resulting in difficult fecal excretion and constipation (39). We detected the expression of AQP by RT-PCR and IHC and found that the expressions of AQP4 and AQP8 in the intestines of mice in the constipation group were significantly increased. Intervention of ST or *L. sakei* alone or combined down-regulated the expression levels of AQP4 and AQP8, and the combination of ST and *L. sakei* had the greatest effect on AQP expression. We, therefore, speculated that down-regulating the expression of AQP4 and AQP8 by ST or *L. sakei* may contribute to increasing the water content of feces. Downregulation of SCF and c-Kit in constipation mice could lead to a decrease in ICCs (40). ICCs are a type of intestinal pacemaker cell closely associated with the development of STC (40). In this study, ST and *L. sakei* could act on c-Kit and its ligand SCF to increase the number of ICCs, thereby promoting intestinal EMG activity and smooth muscle contraction to relieve constipation.

Studies have shown that when GDNF (glial cell-derived neurotrophic factor) is knocked out in mice, the mice lose gastrointestinal innervation, which affects intestinal motility (41). Therefore, increasing the expression of GDNF helps prevent and relieve constipation. In addition, NOS is the only enzyme involved in nitric oxide (NO)synthesis. With the increase of NOS, NO diffuses to smooth muscle cells, where it increases cyclic guanosine monophosphate (cGMP) resulting in the decrease of intracellular Ca^{+} concentration, relaxation of smooth muscle, weakness of conductance, and inhibition of gastrointestinal motility (42). Therefore, reducing the production of NO and the expression of NOS may help alleviate constipation (42). Our results showed that intervention with *L. sakei* increased the expression level of GDNF, and

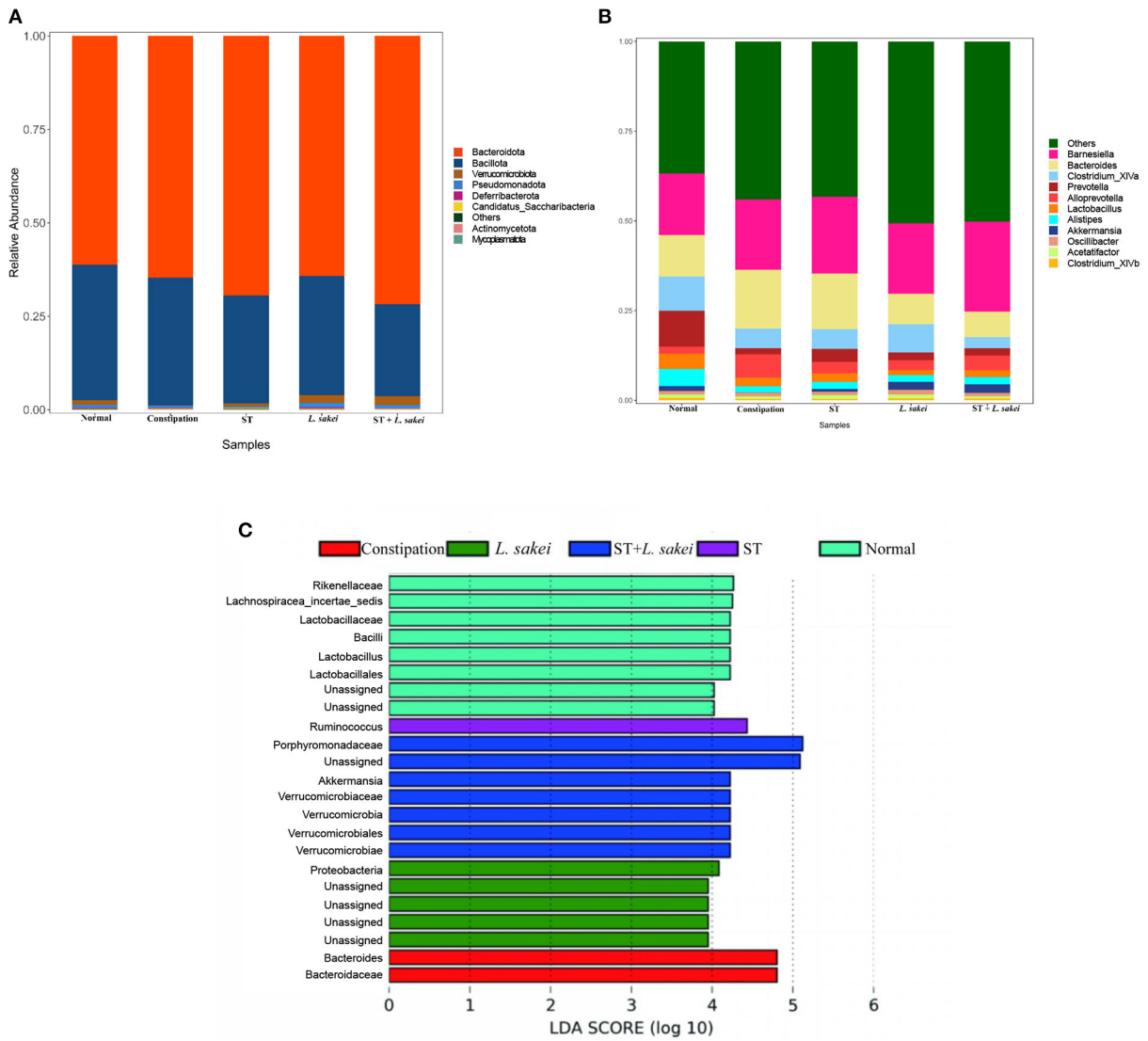

FIGURE 7
The effect of ST or *L. sakei* alone or combined on the phylum- and genus-level structures of the gut microbiota. **(A)** Relative abundance bar plot at the phylum level; **(B)** Relative abundance at the genus level; **(C)** LEfSe analysis of gut microbiota. Data represent the mean ± SD ($n = 6–8$). Wilcoxon signed-rank test of non-parameter statistics was used for constipation group vs. normal group, ***$p < 0.001$. Wilcoxon signed-rank test of non-parameter statistics was used for ST, *L. sakei*, and ST + *L. sakei* groups, vs. constipation group *$p < 0.05$.

supplement with ST or *L. sakei* reduced the expression level of NOS, indicating that ST or *L. sakei* may relieve constipation and accelerate intestinal motility by affecting the expression of GDNF and NOS.

We measured some serum factors related to intestinal motility and found that diphenoxylate-induced constipation could reduce the expressions of 5-HT, MTL, and SP in serum and increase the expressions of ET and VIP. Ninety-five percent of 5-HT in the body is produced by enterochromaffin cells. The regulation of the intestinal system by the enteric nervous system requires the mediation of 5-HT. Large amounts of 5-HT activate primary afferent neurons in the submucosa, stimulate neurotransmitter release, and cause regular intestinal contractions (43). Therefore, 5-HT plays a major role in controlling intestinal motility. MTL is a hormone in the digestive tract and plays a role in promoting the motility of the gastrointestinal tract and the transportation of water and electrolytes in the gastrointestinal tract. Studies have found that this physiological effect is mainly through the stimulation of inter-digestive myoelectric activity, which promotes the contraction of gastric force and segmental movement of the small intestine (44). SP is a ubiquitous excitatory

neurotransmitter and is usually released by intrinsic neurons or the vagus nerve in the digestive tract. SP is not only involved in the transmission of pain sensation but also binds to the receptor NK1 to regulate intestinal motility (45). On the other hand, both ET and VIP are inhibitory neurotransmitters. They are closely related to vasoconstriction. It has been reported that many elderly people with constipation also suffer from cardiovascular and cerebrovascular diseases (46). Excessive VIP levels can relax smooth muscle, and lower VIP levels can induce intestinal spasms. This study showed that the synbiotics intervention increased the levels of 5-HT, MTL, and SP, and decreased the levels of VIP and ET. This provides evidence to support the use of ST and *L. sakei* in combination as synbiotics.

SCFAs are important metabolites produced by intestinal microbiota. One study reported that treatment with loperamide led to significant reductions in the fecal levels of acetic acid, propionic acid, butyric acid, and valeric acid compared to the normal group, but there were no significant changes in fecal levels of isobutyric acid and isovaleric acid (47). Another study reported that people with constipation have significantly lower levels of fecal propionic acid and butyric acid compared with those in the normal population (48). There was no significant difference in the level of SCFAs in the cecum content between the normal and constipation mice in our study probably because 24h diphenoxylate treatment was too short to alter the metabolism of microbiota to produce SCFAs. The different profiles of SCFAs between the cecum and fecal content may be another reason for inconsistent results. Moreover, it was reported that the administration of *Lactiplantibacillus plantarum* NCU116 significantly improved the symptoms of constipation in mice and led to significant increases in acetic acid and propionic acid levels in their feces (49). But it was also reported that five strains of *L. rhamnosus* failed to recover the fecal levels of SCFAs (46). The roles of SCFAs in the alleviation of constipation remain unclear. Our results showed that both ST and *L. sakei* could slightly increase the level of SCFAs but how SCFAs play an important role in preventing constipation remain to be studied.

Gut functions are closely related to the composition of gut microbiota. At the phylum and genus levels, the relative abundance of *Bacteroidota* in the model group was increased in our study. Our result is inconsistent with one clinical study showing that *Bacteroidota* were significantly reduced in the feces of constipated children (50). The conflicting result is probably due to the different compositions of gut microbiota between mice and humans. Therefore, the relationship between *Bacteroidota* and constipation is not yet clear. By comparing the gut microbiota of African children on a predominantly vegetarian diet with those of European children on a "Western diet," the abundance of *Prevotella* decreased significantly in the European group of children, further suggesting that *Prevotella* abundance is positively correlated with dietary fiber content and decreased abundance of *Prevotella* is associated with the onset of constipation (50, 51). Our result showed that the abundance of *Prevotella* was decreased in the constipation model group and increased after ST intervention. Therefore, we speculate that ST may relieve constipation by increasing the abundance of *Prevotella* in the intestine. Studies have found that the abundance of *Alistipes* is involved in gastrointestinal function, and can produce SCFAs and reduce intestinal inflammation (52). *Alistipes* in the three intervention groups were all increased to a certain extent. The LEfSe results showed that the characteristic gut microbiota of the ST group was *Ruminococcus*, which is known to metabolize some refractory plant components and generate SCFAs to provide energy for the host, and it was related to the relief of functional constipation (53). The relative abundance of *Akkermansia* in the constipation group decreased, and its relative abundance increased after treatment, which is consistent with previous results (54). *Akkermansia* has been widely studied to significantly improve obesity, inflammation, colon cancer, and autism (55–58), but its relationship to constipation is unclear. Some studies have found that *Akkermansia* can degrade mucin and positively regulate the thickness of the intestinal mucus layer and the integrity of the intestinal barrier (59). Therefore, we speculated that *Akkermansia* may relieve constipation by improving intestinal inflammation and repairing the intestinal mechanical barrier.

Conclusions

Our results from the defecation experiments and intestinal propulsion experiments in mouse models suggest that oral administration of ST or *L. sakei* alone or combined are all effective to relieve constipation. Improvement of constipation may be mediated through different mechanisms of reducing intestinal inflammation, altering associated protein expression, modulating neurotransmitter release, and improving gut microbiota.

Data availability statement

The data presented in the study are deposited in the https://www.ncbi.nlm.nih.gov/, repository, accession number PRJNA876368.

Ethics statement

This study was approved by the Ethics Review Committee of the National Institute for Communicable Disease Control and Prevention at the Chinese Center for Disease Control and Prevention (Beijing, China).

Author contributions

YG and ZR conceptualized the experiments and wrote the paper. YG, LS, YH, YX, and XL conducted the experiments. YG and YH analyzed the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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EDITED BY
Hani El-Nezami,
The University of Hong Kong, Hong Kong SAR, China

REVIEWED BY
Tien Sy Dong,
UCLA Health System, United States

*CORRESPONDENCE
Hardinsyah Hardinsyah
hardinsyah2010@gmail.com

†These authors have contributed equally to this work and share senior authorship

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Can salivary microbiome become a biodetector for type-2 diabetes? Opinion for future implications and strategies

Hardinsyah Hardinsyah [1*†], Fahrul Nurkolis [2†], Rudy Kurniawan [3], William Ben Gunawan [4], Piko Satria Augusta [5], Astuti Setyawardani [6], Rafiv Fasya Agustianto [7], Msy Firyal Nadya Al Mahira [5], Ghevira Naila Praditya [5], Deogifta Graciani Lailossa [5], Dewangga Yudisthira [5], Salsabila Farradisya [5] and Hero Barazani [5]

[1]Division of Applied Nutrition, Faculty of Human Ecology, Department of Community Nutrition, IPB University, Bogor, Indonesia, [2]Department of Biological Sciences, Faculty of Sciences and Technology, State Islamic University of Sunan Kalijaga (UIN Sunan Kalijaga Yogyakarta), Yogyakarta, Indonesia, [3]Alumnus of Department of Internal Medicine, Faculty of Medicine, University of Indonesia–Cipto Mangunkusumo Hospital, Jakarta, Indonesia, [4]Alumnus of Department of Nutrition Science, Faculty of Medicine, Diponegoro University, Semarang, Indonesia, [5]Medical Study Programme, Faculty of Medicine, Brawijaya University, Malang, Indonesia, [6]Medical Student of Faculty of Medicine, University of Jember–Soebandi Regional Hospital, Jember, Indonesia, [7]Medical Student of Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

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1. Introduction

Microbiome has become a topic that is developing rapidly in the health sector for the past 2 decades (1). Previous research has found that the microbiome was associated with a variety of diseases, such as metabolic, gastrointestinal, and immunity disorders (2). The microbiome is a collection of genomes or genetic materials from all microorganisms, symbiotics, and pathogens that live in vertebrates such as bacteria, viruses, archaea, fungi, and small protists (1, 3). About 100 trillion microbiomes called the gut microbiome to reside in the digestive tract (4). The gut microbiome plays a role in nutrient and drug metabolism, immune modulation, and maintenance of gut integrity (3).

In addition to the gut, the second highest number of microbiomes is located in the mouth and called oral microbiome or salivary bacteriome, considering that there is a link between the two both physically and chemically (5, 6). An oral cavity is an ideal place for the survival of the oral microbiome since it has an average temperature of 37°C and saliva with a stable pH of 6.5–7, causing the bacteria to hydrate transporting the nutrients to microorganisms (7). The oral cavity is the first part of the gastrointestinal tract and the place where it meets with food, exogenous microbes, and allergens before it gets into the gastrointestinal tract. The presence of direct exposure in the absence of fibrous epithelium makes the oral cavity susceptible to infection (8). Those conditions highlight the important role of the microbiome in maintaining ecological balance and preventing oral cavities (9). One of the mechanisms is resistance to the colonization of pathogens by defeating pathogenic species and lowering the chance of integration by exogenous pathogens. Some microbiomes are also able to fight acids produced by caries-causing bacteria by increasing the pH of saliva through the production of alkaline metabolic byproducts (8, 10). Disorder in this system may cause dysbiosis triggered by various factors such as diet, inflammatory response, systemic disorders, and alcohol which will induce oral diseases (5, 8).

Recently, it has been found that the human microbiome has an important role in the occurrence and development of diabetes mellitus (11). Several studies have shown that type 2 diabetes mellitus (T2DM) is associated with changes in the diversity and number of bacteria in supragingival plaques, and oral microbiota changes have also been found in various glycemic states (12, 13). The phylum *Actinobacteria* was found to have decreased in the T2DM group compared to the control group, and the increase was associated with a reduced risk of T2DM (11, 14). The phylum *Actinobacteria* is also associated with the prevalence of obesity, suggesting that the oral microbiome may have an important role in the etiology of diabetes (14). The number of microbiotas of the genus *Prevotella* was decreased in the T2DM group, and *Prevotella spp.* was reduced in high-glucose salivary conditions (11, 12). Bacteria of the genus *Rothia* experienced a decrease in T2DM and potential pre-diabetic conditions (11, 15). Bacteria in the phylum *Firmicutes*, one of which is the *Streptococcus*, was significantly increased in the T2DM and pre-diabetic groups compared to the non-diabetic group (11, 13, 16).

To the best of our knowledge, there has been no review study or opinion article related to the use of oral or salivary microbiome as a biodetector of T2DM disease. Therefore, the main purpose of this critical opinion is to summarize the findings regarding the oral microbiome as a biodetector of T2DM and explain its opportunities, implications, and strategies for future use.

2. Oral microbiome in general

The human mouth is inhabited by various microorganisms such as bacteria, viruses, fungi, and protozoa called oral microbiota. The diversity of the human oral microbiota is one of the effects of accelerated regeneration and non-keratinized epithelium types found in the oral cavity (17, 18) since these factors increase the process of molecular absorption, implying that the microbiota is more likely to reach other organs and has a broad metabolic effect. In addition, the diversity of the oral microbiota can also be caused by the high intensity of contact with the external world *via* air and food (18, 19). Assuming the health-related consequences of the composition of the oral microbiota, the oral cavity would be an ideal place to analyze biomarkers since the samples will be easier to obtain than other organs (20). To date, 445 types of oral microbiota have been

TABLE 1 List of clinical evidence regarding the effectivity of oral microbiome in detecting type 2 diabetes mellitus (T2DM).

No	Studies	Outcomes	References
1	Characterization of the type of oral microbiome in T2DM patients (RNA sequencing method, $n = 46$); BMI or Body Mass Index (kg/m2) defined as normal weight, preobese, obese, and the obese category had a higher Shannon index for the abundance of amplicon sequence variants (ASVs), followed by the preobese category.	• Based on phylum, Oral microbiomes that are detected in T2DM: *Firmicutes* (45%) ↑, *Bacteroidetes* (22%)↑, *Proteobacteria* (16%)↑, *Actinobacteria* (9%)↑, and *Fusobacteria* (6%)↑. • Based on the genus, the oral microbiome is dominated by *Streptococcus* (29%)↑, *Prevotella* (14%)↑, and *Neisseria* (5%)↑. • There were no significant differences in the oral microbiome profiles in diabetic and non-diabetic patients. • There was no significant difference in alpha and beta diversity levels in diabetic and non-diabetic groups.	Almeida-Santos et al. (37)
2	Characterization of the type of oral microbiome in T2DM patients (DNA sequencing method, $n = 26$); BMI 25.0 ± 1.3 (Diabetes group; $n = 10$) and 24.6 ± 2.2 (Non-diabetes group; $n = 13$).	• Based on the phylum, the oral microbiome is dominated by *Firmicutes*↑, while *Streptococcus*↑ dominates the genus level. • There was no significant difference in alpha and beta diversity levels in diabetic and non-diabetic groups.	Lee et al. (38)
3	Characterization of the type of oral microbiome in T2DM patients (DNA sequencing method, $n = 24$); BMI in this study was not reported.	• Diabetes mellitus causes environmental changes and a shift in microbial homeostasis of the oral mucosa. • Oral microbiomes that are detected in DMT2 patients include: *Bacillus mojavensis*↑, *Enterobacter cloacae*, *Proteus mirabilis*↑, *Staphylococcus epidermidis*↑, *Staphylococcus hominis*↑, *Staphylococcus pasteuri*↑, *Streptococcus mutans*↑, and *Streptococcus pasteurianus*↑.	Ali et al. (39)
4	Changes in oral microbiome in periodontitis in T2DM and non-diabetic patients (RCT study, $n = 133$); BMI was observed for each group.	• The proportion of *Prevotella copri*↑, *Alloprevotella rava*↑, and *Ralstonia pickettii*↑ increased in patients with periodontitis in both T2DM and non-diabetic groups. • Moreover, the oral microbiome will decrease once glucose levels in the patient are controlled.	Sun et al. (40)
5	Characterization of the type of oral microbiome in T2DM patients (DNA sequencing method, $n = 128$); BMI was observed for each group, 24.9 ± 5.7 in Normoglycemic ($n = 32$) and >30.0 in Pre-DM and DM groups.	• *Fusobacteria* and *Actinobacteria* are significantly more abundant (↑) than *Proteobacteria* (↓) in diabetic subjects.	Matsha et al. (13)
6	Characterization of salivary microbiota in elderly patients with T2DM (RNA metagenomic analysis, $n = 84$); As a matched case–control study and BMI in this study were not reported.	• The phylum *Firmicutes*↑ was abundant in patients with T2DM, whereas the phylum *Bacteroidetes*↑ was abundant in controls.	Omori et al. (41)
7	Characterization of oral microbiome profile of Chinese patients with T2DM (RNA sequencing, $n = 442$); 20.1 ± 1.2 in Control/Health groups and 27.1 ± 0.8 in T2DM	• The *Firmicutes*/*Bacteroidetes*↑ ratio increased in T2DM. • T2DM patients presented significantly higher numbers of *Neisseria*↑, *Streptococcus*↑, *Haemophilus*↑, and *Pseudomonas*↑, and lower numbers of *Acinetobacteria*↓ compared with healthy controls.	Chen et al. (42)

↑ Indicates an increase in number or abundance, and ↓ indicates a decrease in the number of oral bacteria.

recognized in literature, which is ranked second after the intestine and 57% of them have been named and perfectly cultured (21, 22).

Due to its diverse and easily detectable natures, oral microbiota can be used as a biomarker of some diseases in humans (18). In diseases related to children's mouths, some fungal species such as *Candida dubliniensis* and *Candida tropicalis* in saliva are indicators that children are at risk of dental caries (23). Changes in the oral microbiota to acidogenic and acidic cariogenic bacteria can indicate tooth decay and enamel demineralization in children (24). In adults, the oral microbiota can be an indicator of the incidence of oral cancer since the bacterial composition changes due to a cluster of factors that cause oral cancer, including alcohol and cigarettes. The byproducts of oral bacteria can induce genetic changes in mucosal epithelial cells that are predictors of Squamous cell carcinoma in the mouth (25). Further findings found that the use of oral microbiota was able to detect the presence of oropharyngeal cancer and malignancy in the human gastrointestinal tract (7, 26).

In addition, oral microbiota can also be an important biomarker of systemic diseases in other organs (27). Oral microbiota has also been detected in the lungs in cystic fibrosis patients and can cause various soft tissue infections in the event of wound bites (28, 29). *Selenomonas* (*S. artemidis* and *S. infelix*) can be used as important biomarkers of lung infections associated with acute respiratory distress syndrome (ARDS) (30). The oral microbiota has also long been known as a reservoir of infections in various parts of the body. The appearance of abscesses in the brain can also be detected with the discovery of an increase in *Porphyromonas gingivalis* which is an etiological agent in periodontitis (31). The study with 228 subjects diagnosed with metabolic syndrome disease in Korea also showed significant differences where the group with metabolic syndrome had slightly more *Firmicutes* (37.9%) and slightly fewer *Proteobacteria* (29.2%) than the healthy group (21). In other studies, several dominant oral microbiotas were found such as *Fusobacterium nucleatum*, *Veillonella*, *Streptococcus anginosus*, *Streptococcus oralis*, and *Actinomyces meyeri* in pyogenic infectious diseases of the brain and spinal cord (30, 32). Looking at these findings drew attention to the question "Can the oral profile of the microbiota be used as a diagnostic biomarker or biodetector of T2DM?" Following the purpose of this opinion article, the author tries to summarize the findings regarding the oral microbiome as a biodetector of T2DM.

3. Is the oral microbiome effective in detecting T2DM?

3.1. *In vivo* or preclinical trial studies

The decline of the body's immune system in people with diabetes mellitus occurs due to polymorphonuclear cell dysfunction (PMN) and various other inflammatory cytokines, which causes a shift in the normal flora of the oral mucosa and triggers the growth and development of various pathogenic germs (33). Xiao et al. conducted *in vivo* experiments on rats treated to be hyperglycemic. This study found elevated levels of *Enterobacteriaceae*, *Aerococcus*, *Enterococcus*, and *Staphylococcus* which are often associated with T2DM (34). The human and mouse microbiomes have identical compositions at the phylum level, including *Firmicutes* and *Bacteroidetes*, followed by *Actinobacteria* and *Proteobacteria* In addition, at the genus level, *Lactobacillus* is the dominant genus at 8 weeks of age and remains dominant during the development of T2DM (35, 36).

3.2. Is there sufficient clinical evidence or clinical trials?

Table 1 showed that some of these studies showed contrasting findings. Almeida-Santos et al. and Lee et al. found no difference in oral microbiome profiles between diabetic and non-diabetic groups (37, 38). However, it can be concluded that several oral microbiomes are quite often found in T2DM, including the *Firmicutes* and *Bacteroidetes* phylum, followed by *Actinobacteria* and *Proteobacteria*.

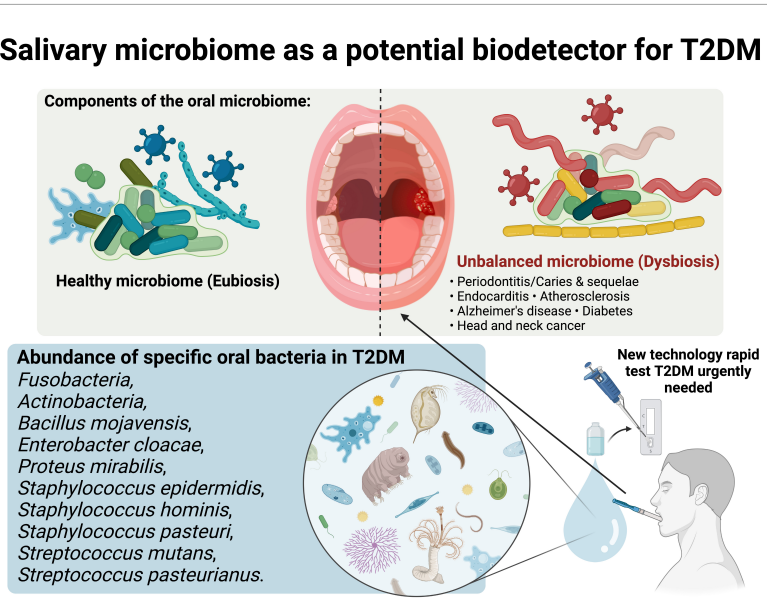

FIGURE 1
Salivary microbiome as a potential biodetector for type 2 diabetes mellitus (T2DM). Created with BioRender.com premium license by Fahrul Nurkolis.

Based on *in vivo* and clinical studies, the mechanism of identification and the correlation between the oral microbiome and the occurrence of T2DM still need to be further researched, especially considering the possible differences between ethnicities or populations (43). It is realized, the insignificant of studies in **Table 1** were likely underpowered because perhaps the number of samples was relatively small (<50 patients). Future clinical trial studies or RCTs should be conducted with more power based on sample size, may more than fifty patients or subject.

4. Discussion with future implications and strategies

Previous studies has highlighted the interesting bidirectional relationship between the salivary microbiome and T2DM (44). The abundance of *Firmicutes* and increased ratio of *Firmicutes*/*Bacteroidetes* were found in most studies. It has been suggested that the *Firmicutes* were more efficient than the *Bacteroidetes* at obtaining energy from food, resulting in more effective absorption of calories and the ensuing weight gain (45). On the other hand, dietary intake and obesity were correlated with the incidence of T2DM (46). A lower level of *Bacteroidetes* has also been linked with the incidence of inflammatory disease (47), while inflammation also initiates the pathophysiology of T2DM (48). However, even this fundamental theory was found to be contradicted as *Firmicutes* was argued to be health-promoting bacteria through the synthesis of butyrate (49). Lee et al., Chen et al. and Ali et al. also mentioned that *Streptococcus* was one of the abundant bacteria in the oral microbiome of T2DM subjects (**Figure 1**) (38, 39, 42). Lower *Prevotella* and higher *Streptococcus* may be explained by low salivary pH that is caused by prolonged elevated blood glucose (12). However, a clinical research report stated that salivary microbiome profiles were prone to change, as they might be influenced by different stages of oral diseases (13). This was confirmed by the study of Sun et al. (40) as the changes of bacteria between diabetic and non-diabetic groups were similar in the presence of periodontitis.

Even though oral microbial profiles exhibited wide metabolic implications that were similar but at a lower level compared to gut microbiome (50), they may contribute an important role as biodetector of diseases, especially T2DM. Learning from the studies reviewed in this opinion, it can be concluded that randomized controlled trials (RCTs) are needed to consolidate the evidence regarding the link between oral microbiome and T2DM. These studies presented us with insights regarding related bacteria that can be targeted during upcoming RCTs, as these bacteria have been studied for their correlation with impaired metabolical conditions such as T2DM. Profiling the salivary microbiome may become a feasible rapid test in detecting T2DM (**Figure 1**), considering the fact that bio samples of the oral cavity will be easier to procure compared to gut (fecal) microbiota for future use, compared to detection using blood which some people are afraid of needles.

Author contributions

HH and FN: conception and design of opinion studies. FN: figure visualization and BioRender license holder. All authors wrote, edited, and revised the manuscript, and approved the final version of the submitted manuscript.

Conflict of interest

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

Publisher's note

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EDITED BY
Carlos Gómez-Gallego,
University of Eastern Finland,
Finland

REVIEWED BY
Fei Pan,
The First Medical Center,
Chinese PLA General Hospital,
China
Qiulan Lv,
The Affiliated Hospital of Qingdao University,
China

*CORRESPONDENCE
Zhihong Ren
✉ renzhihong@icdc.cn

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Lactiplantibacillus pentosus P2020 protects the hyperuricemia and renal inflammation in mice

Zhihuan Wang[1], Liqiong Song[1], Xianping Li[1], Yuchun Xiao[1], Yuanming Huang[1], Yue Zhang[2], Jintong Li[3], Mingding Li[4] and Zhihong Ren[1]*

[1]State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Research Units of Discovery of Unknown Bacteria and Function (2018 RU010), Chinese Academy of Medical Sciences, Beijing, China, [2]Third Affiliated Hospital, Beijing University of Chinese Medicine, Beijing, China, [3]Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China, [4]Maiyata Institute for Beneficial Bacteria, Shaoxing, Zhejiang, China

Introduction: Hyperuricemia (HUA) is a common metabolic disease, and its prevalence has been increasing worldwide. Pharmaceutical drugs have been used for controlling HUA but they all have certain side effects, which thus calls for discovering alternative options including using treatment of probiotics to prevent the development of HUA.

Methods: We established HUA mice model induced by potassium oxonate and adenine and performed in vivo experiments to verify the ability to lower serum uric acid of *Lactiplantibacillus pentosus* P2020 (LPP), a probiotics stain extracted from Chinese pickle. We also tried to discussed the underlying mechanisms.

Results: Oral administration with LPP significantly decreased serum uric acid and reduced renal inflammatory response by downregulating multiple inflammation pathways including NK-kB, MAPK, and TNFα. We also found that LPP administration significantly promoted uric acid excretion by regulating expression of transporters in the kidney and ileum. In addition, LPP intake improved intestinal barrier function and modulated the composition of gut microbiota.

Discussion: These results suggest that probiotics LPP may have a promising potential to protect against development of HUA and HUA-related renal damage, and its working mechanisms involve regulation of inflammation pathways and expression of transporters in the kidney and ileum.

KEYWORDS

hyperuricemia, probiotics, *Lactiplantibacillus pentosus*, gut microbiota, kidney inflammation, urate reabsorption transporter, NF-kB, intestine barrier function

1. Introduction

Hyperuricemia (HUA), one of the common metabolic diseases, is caused by abnormal purine metabolism and/or decreased uric acid excretion. People with serum uric acid (SUA) levels higher than 420 μmol/l in men and 360 μmol/l in women can be diagnosed as HUA. Globally, the prevalence of HUA and gout both have increased over the last few decades. The prevalence of HUA in Chinese adults was 8.4%, accounting for approximately 92.9 million adults with HUA (1). The consequence of long-term HUA can lead to chronic kidney disease (CKD), known as hyperuricemic nephropathy (2). Moreover, asymptomatic HUA, which has drawn less attention from researchers and the public, is related to in the creased prevalence of multiple diseases including hypertension, acute and chronic kidney disease, metabolic syndrome, obesity, and diabetes mellitus fatty liver (3, 4). Several mechanisms

responsible for HUA development and urate-induced renal inflammation have been described. The presence of urate crystal can promote inflammasome-independent mechanisms, including releasing pro-inflammatory cytokines and neutrophil extracellular trap inflammation. Urate crystal also has pro-oxidative effect in multiple cell types and it activates inflammatory signaling through multiple mechanisms including activation of MAPK pathway and AKT–mTOR, and inhibition of AMPK (5–7).

Several methods are currently available to control HUA, such as diet, sport, drugs, and biotherapy, aiming to suppress HUA production or increase uric acid excretion (8). For many patients with HUA, dietary therapy for reducing the intake of high-purine is a low-cost and side effect-free or low side effect strategy with poor patient compliance (9, 10). Allopurinol, the representative of the HUA-lowering drug, can compete with the xanthine oxidase (XOD) enzyme to inhibit production of uric acid. Uricosuric agents such as benzbromarone, sulphinpyrazone, and probenecid can promote excretion of uric acid. Although the safety of XOD inhibitors and uricosuric agents has been improved, their adverse effects, such as headaches, diarrhea, rashes, severe allergic reactions, and nephrotoxicity, often limit their clinical use (11, 12). Therefore, it is necessary to find an effective intervention with better patient compliance and fewer side effects.

Gut microbiota is associated with multiple diseases and also plays an important role in the metabolism of purine and uric acid. Gout patients had a different gut microbiome pattern characterized by a significant decrease in bacteria expressing the uricase gene. In addition, the altered gut microbiota influences expression of UA transporters (e.g., ABCG2 and GLUT9) in the intestine and the systematic inflammation reaction, both of which contribute to the development of HUA (6, 13, 14). Thus, it is possible that probiotics can be used to ameliorate HUA and relieve the inflammatory damage caused by urate in urine. Researchers have found that several strains of *Lactobacillus* can reduce the levels of serum uric acid. For example, *Limosilactobacillus fermentum* JL-3 isolated from the Chinese traditional food "Jiangshui" was capable of degrading uric acid *ex vivo* and alleviating HUA in the mouse model (15). *Lactobacillus gasseri* PA-3 can directly utilize the purine compounds including adenosine monophosphate (AMP), inosine monophosphate (IMP), and guanosine monophosphate (GMP) for growth, and decrease the purine absorption in rats (16, 17). In the present study we isolated a strain named *Lactiplantibacillus pentosus* P2020 (LPP) from the Chinese pickle and evaluated its anti-HUA function using a mouse model. Our results showed that administration of LPP significantly downgraded the inflammation in kidney. We also observed the altered expression of transporters related to urate transportation in both kidney and ileum. It has been reported that several probiotics can improve epithelial barrier function and rebuild the disrupted intestinal flora (15, 17, 18). We found that LPP treatment upregulated expression of tight junction protein and reversed the changes in gut microbiota induced by HUA. Therefore, LPP can be considered a potential probiotic candidate to lower HUA, which warrants it to be further evaluated in human trials.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Review Committee of the National Institute for Communicable Disease Control and Prevention at the Chinese Center for Disease Control and Prevention (Beijing, China).

2.2. Bacteria strain culturing

LPP was isolated from a Chinese pickle. The strain was grown anaerobically in Man-Rogosa-Sharpe (MRS) medium in a CO_2 incubator (Forma CO_2, Thermo Fisher Scientific, Waltham, MA, United States) at 37°C for 24 h before use. The culture of LPP in the logarithmic phase was collected and washed twice with sterile phosphate-buffered saline (PBS) by centrifugation (2000xg, 10 min, 4°C) and then resuspended with sterile PBS for oral inoculation to mice.

2.3. *Lactiplantibacillus pentosus* P2020 strain identification based on the 16S rDNA sequence and genome sequence

The 16S rDNA sequence and genome sequence were used to identify LPP at the species level. The genomic DNA was extracted using FastPure DNA isolation mini kit (Vazyme Biotech Co., Ltd., Nanjing, China). The 16S rDNA sequence was amplified using two universal primers, 27F and 1492R, and the draft genome was sequenced by the Illumina HiSeq TM2000 platform.

The nearly complete 16S rDNA sequence (1,567 bp) was obtained and blasted using the Ezbiocloud database (ChunLab Inc., Seoul, Korea) to search for similar sequences. LPP had the highest similarity to *Lactiplantibacillus pentosus* DSM 20314 T (= ATCC 8041 T, NCDO 363 T, NCIB 8026 T) at 99.93%.

For further identification, genome comparative analysis was carried out. As a reference strain, the whole-genome sequence of *Lactiplantibacillus pentosus* DSM 20314 T (CP032757) was obtained from the NCBI GenBank database. The digital DNA–DNA hybridization (dDDH) was assessed using the DDH web software[1]. The average nucleotide identity (ANI) was estimated by an online ANI calculator[2]. The dDDH and ANI values of genome sequences between strain P2020 (3,876,975 bp) and *Lactiplantibacillus pentosus* DSM 20314 T (CP032757) were 98.00 and 99.74%, respectively, which were much higher than the 70.0% and 95.0–96.0% threshold of dividing into species (19, 20). Thus, LPP was considered to be a strain of *Lactiplantibacillus pentosus*.

2.4. Animal experiment

Male Kunming mice (6-week-old, 25 ± 2 g) were purchased from the Chinese Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were housed in the Animal Center of China CDC Mice with temperature at 24–26°C, humidity at 40–60%, and a 12 h light–dark cycle. Mice had free access to the standard commercial mouse food and water. After one-week adaption, mice were randomly divided into three groups ($n = 8$/group): negative control group (NC), hyperuricemic group (HUA),

1 https://ggdc.dsmz.de/
2 https://www.ezbiocloud.net/tools/ani

and LPP-treated group (LPP). The HUA mouse model was established according to literature with slight modification (18, 21–26). Briefly, potassium oxonate (Sigma-Aldrich, MA, United States,) and adenine (Sigma-Aldrich, MA, United States) were suspended in 0.5% Carboxymethylcellulose sodium (CMC-Na) solution. Mice in HUA and LPP groups were daily administered with 0.3 ml solution of potassium oxonate (PO, 250 mg/kg) and adenine (75 mg/kg) by gavage, and NC group mice were given the equal volume of 0.5% CMC-Na solution for 14 days. Four hours after the treatment of PO and adenine, mice in LPP group were orally administered with 1×10^9 CFU LPP in 0.3 ml PBS and mice in NC and HUA groups were given the equal volume of sterile PBS. After 14-day of daily administration of LPP or PBS, mice were sacrificed and body weight was recorded. Blood was collected from the eye vein. To acquire the serum, the collected blood samples were left undisturbed for 30 min then centrifuged at 5000 x*g* for 20 min. The kidney, liver, ileum, colon, and cecal content were also collected. All samples were stored at −80°C until analysis, except for the samples used for histopathology and RNA sequencing, which were kept in 4% paraformaldehyde and RNAlater (Thermo Fisher Scientific, Waltham, MA, United States) respectively.

2.5. Detection of biochemical indicators of HUA

SUA, serum creatinine (CRE), and blood urea nitrogen (BUN) concentrations were determined with commercial kits from the Jiancheng Biotech (Nanjing, China), and Xanthine oxidase (XOD) and adenosine deaminase (ADA) activities in livers were determined with commercial kits form the Jiancheng Biotech (Nanjing, China), following the manufacturer's instructions.

2.6. Histopathological examination

Kidney tissues were collected and fixed in 4% paraformaldehyde solution for 24 h and the fixed tissues were embedded in paraffin, sliced into 4 μm sections, then stained with Hematoxylin and Eosin by Wuhan Servicebio Technology Co., Ltd. The HE sections were pictured and analyzed by CaseViewer software.

2.7. Transcriptome analysis

Transcriptome analysis for kidney and liver samples was performed by the Beijing Genomics Institute Co., Ltd., (Beijing, China). Briefly, the Total RNA of kidney and liver tissues was extracted using Trizol according to the manufacturer's instruction. The qualified RNA sample, determined by Fragment Analyzer, was constructed data library. After enrichment and specific amplification of mRNA, the single-strand circular DNA library obtained was sequenced on the BGISEQ-500 platform by the Beijing Genomics Institute Co., Ltd. The clean data obtained from raw data by removing adapter-containing and low-quality reads were mapped to the reference genome using HISAT software for the second quality control of alignment. The matched clean data were normalized to FPKM by RSEM software with a threshold at $|\log2FC| \geq 1$, $p \leq 0.01$. KEGG pathway enrichment of differential genes was performed on the Dr. Tom analysis platform of Beijing Genomics Institute Co., Ltd. The heatmap of differential genes was exerted by online tools[3].

2.8. Quantitative real-time PCR analysis

RNA of kidney, colon and ileum tissue was extracted using Trizol and was reverse-transcribed using a Primescript RT master mix kit (Takara, Shiga, Japan). The cDNA samples were amplified in duplicate. Quantitative real-time PCR (qPCR) was conducted on Bioer Fast 9,600 (Bioer Technology, Hangzhou, China) with TB Green Premix Ex Taq. qPCR was run under the following conditions: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s followed by 40 cycles. The primer sequences of genes were designed using BLAST and listed as supporting information in Supplementary materials (Supplementary Table S1). Gene expression was normalized with β-actin, and $2-\Delta\Delta Ct$ was used to calculate the result.

2.9. Western blot analysis

Colon and kidney tissues were homogenized in iced RIPA (Solarbio Science & Technology, Beijing, China) and proteins were extracted following the manufacturer's instructions. The concentration of proteins was quantified using BCA protein assay kit (Thermo Scientific, MA, United States). Twenty-five micrograms of denatured protein were separated by 12% SDS-PAGE gel and transferred to an Immobilon-P Transfer Membrane (Millipore, Burlington, MA, United States). The membranes were blocked with 5% non-fat milk and incubated with primary antibodies (1:1000) overnight at 4°C. Then the membranes were incubated with horseradish peroxidase- (HRP-) conjugated secondary antibodies (1,5,000). All primary antibodies were purchased from Abcam plc., (Cambridge, United Kingdom), and HRP-secondary antibodies were purchased from Lablead Biotech Co., Ltd. (Beijing, China). The bands were visualized using ECL with Amersham Imager 680R (General Electric, Connecticut, United States). The grey values of the bands were calculated and normalized to those of β-actin using Image J software.

2.10. 16S rRNA sequencing and data analysis of cecal content

At the end of the experiment, fresh cecal content was collected and immediately stored at −80°C. 16S rRNA sequencing and data analysis were performed by the Beijing Genomics Institute Co., Ltd., (hBeijing, China). Briefly, V3 and V4 hypervariable regions of 16S rRNA were selected and amplified using the primer pairs: Forward primer (5′-CCTACGGGNGGCWGCAG-3′) and Reverse primer (5′-GACTACHVGGGTATCTAATCC-3′), and the amplicons and quality control of the raw data were conducted on the Illumina

3 https://software.broadinstitute.org/morpheus/

Hiseq 2,500 platform. The consensus sequence was generated by FLASH. The high-quality paired-end reads were combined into tags based on overlaps, then tags were clustered into Operational Taxonomic Unit (OTU) by scripts of software USEARCH with a 97% threshold. The α adversity and principal coordinates analysis (PCoA) was calculated and visualized by R software. Abundance prediction results of KEGG functions in bacterial communities were obtained by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) (27). The functions are named by KO ID, which represents the specific functional genes, and then the information on 3 levels of metabolic pathways is obtained from the KEGG database. The abundance table of each level is obtained separately.

2.11. FITC-dextran permeability assay

The intestinal permeability was assessed using FITC-dextran (FD4, Sigma) method. Briefly, after being deprived of water and food for 12 h, mice were subjected to FITC-dextran gavage at the dose of 400 mg/kg body weight *via* a gastric gavage tube. Four hours later, blood was collected from inner canthus, and serum was collected by centrifugation (4°C 5000 × g 5 min). The FD-4 level was detected with multifunctional enzyme marker at 485 nm (excitation) and 528 nm (emission).

2.12. Statistical analysis

Statistical analysis was performed with GraphPad Prism (v9.1.0). The normal distribution data, which passed Levene test, were analyzed using one-way analysis of variance (ANOVA) followed by multiple comparisons (Tukey's test) as post-hoc analysis to determine whether there was a statistical difference between every two sets of data. Results are presented as means ± standard deviation (SD). The nonparametric Kruska Wallis rank-sum test was used followed by Duncan's test for multiple comparisons for alpha diversity analysis of the gut microbiota. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of LPP on body weight and biochemical indicators related to HUA

We first examined the body weight of the different groups of mice (Figure 1A), as body weight is a visual indicator of the overall health status of mice. Compared with NC mice, the HUA mice had significantly lower body weight, indicating their worse health condition. LPP treatment prevented the weight loss observed in HUA mice, suggesting the beneficial effect of LPP on the overall health status of mice. Then we measured the SUA concentration in mice as well as the levels of some important indicators related to the development of HUA. The concentration of SUA was significantly increased in the HUA mice compared with NC mice (107.19 ± 25.48 μmol/l vs. 37.375 ± 4.26 μmol/l, $p < 0.0001$), which confirmed the success of establishing a HUA mouse model. Orally administration of LPP significantly decreased the SUA level compared with the untreated HUA mice (60.33 ± 12.60 μmol/l vs. 107.19 ± 25.48 μmol/l, $p < 0.001$), which thus confirmed the SUA-lowering ability of LPP (Figure 1B). BUN and serum CRE are important indicators of renal injury, and dysfunction of the kidney occurs commonly in HUA mice. We observed that HUA mice had significantly higher levels of BUN and CRE compared with NC mice, and oral administration of LPP effectively reversed the elevated levels of BUN and CRE caused by HUA (Figures 1C,D). These results indicate that LPP is protective against renal injure. Since UA is mainly synthesized in the liver, we next examined the activity of ADA and XOD in liver, the two essential enzymes for the synthesis of uric acid. We found that ADA and XOD activities were remarkably elevated in the HUA mice compared to the NC mice, and the enhancements of both enzymes were restored by LPP intervention (Figures 1E,F). All these results indicated that oral administration of LPP could effectively lower the SUA level and relieve the symptoms of HUA.

3.2. Protective effect of LPP on HUA-induced renal inflammation and pathology

We next examined the inflammatory damage of kidney caused by HUA in different groups. Histopathological examination showed necrotic cell fragments in the lumen of both renal tubules and collecting ducts, and multiple interstitial cell fibrosis of the tissue together with inflammatory cell infiltration in the kidney of HUA mice (Figure 2A). While LPP treatment reversed the damages induced by HUA, inflammatory cell infiltration could still be found in kidney tissues. We then examined the levels of inflammation-related cytokines and proteins. Quantitative PCR results showed that kidney expression of *Il6, Tnfa,* and *Myd88* was significantly increased in HUA mice compared with NC mice, which was reversed by LPP treatment (Figures 2B–D). These results suggest that NF-kB pathway may be involved in renal inflammation in HUA mice and LPP administration may protect the kidney from the damage by inhibiting NF-kB pathway. To confirm the inhibitory effect of LPP on NF-kB pathway at protein level, protein levels of NF-kB p65 and Ikkβ, the most important proteins in NF-kB pathway, were examined by Western blotting. The results were consistent with those found at mRNA level (Figure 2E). Together, our data provided evidence supporting an anti-inflammatory role of LPP in alleviating HUA-related renal inflammation by downregulating NF-kB pathway.

3.3. *Lactiplantibacillus pentosus* P2020 altered expression of uric acid transport-related proteins in renal tissue

Several renal transporters are involved in the process of UA extraction, including both excretion and reabsorption of uric acid. It has been known that SLC2a6 (GLUT9) and SLC22a12 (URAT1) contribute to UA reabsorption, while ABCG2 and SLC22a6/7/8 (OAT1/2/3) are related to UA excretion. The results of RNA sequencing showed that expression of *Abcg2* and *Slc22a6/7/8* was significantly down-regulated and expression of *Slc2a6* was up-regulated in the HUA mice compared with the NC mice, which indicated less UA excreted into renal tubules and more UA reabsorbed into the blood in HUA mice compared with NC mice (Figure 3A). However, we also found that in the HUA group, *Slc22a12* expression was downgraded, implying that ABCG2 might have a stronger influence on UA reabsorption. We further observed that administration of LPP significantly reversed altered expression of *Abcg2, Slc22a6/7/8/12,* and *Slc2a6* caused by HUA, indicating an improvement

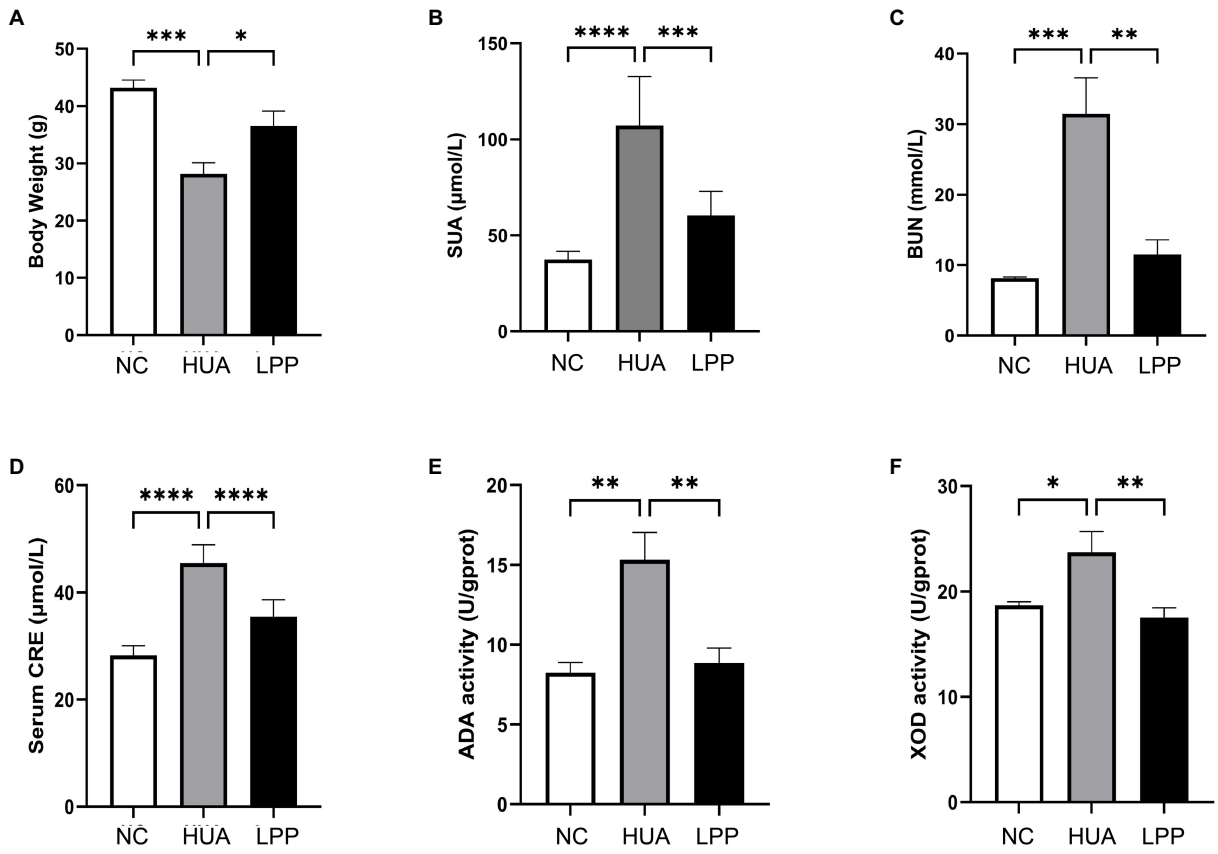

FIGURE 1
Effect of LPP on body weight **(A)**, levels of SUA **(B)**, BUN **(C)**, and CRE **(D)**, and activities of ADA **(E)** and XOD **(F)** in liver. NC, negtive control group; HUA, hyperuricemic group; LPP, hyperuricemic mice treated with LPP orally. Bars are means ± SD (n=6/group), p<0.05 was set as the threshold for significance by one-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

of UA excretion in renal and UA reabsorption into blood mediated by LPP (Figure 3A). Selecting *Abcg2*, *Slc2a6*, and *Slc22a6* genes for quantitative PCR validation, we confirmed the results of RNA-sequencing (Figures 3B–D). Furthermore, we evaluated expression of the transporters at protein level by Western blotting and observed that the level of ABCG2 was dramatically decreased while the level of SLC2a6 was significantly increased in the renal tissue of HUA mice compared with NC mice. The LPP administration partially reversed these alterations observed in HUA mice, suggesting that oral administration of LPP might prevent HUA development by both promoting UA excretion *via* urine and reducing UA reabsorption into blood (Figure 3E).

3.4. Effect of LPP on intestinal barrier and transporters in the intestine

About one-third of uric acid excretion takes place in the intestine, and illeum is the main site where intestinal excretion of uric acid occurs (28). It has been shown that uric acid transporters synthesized by intestinal microbes appear to integrate into the intestinal wall to regulate uric acid excretion (29). Therefore, we examined expression of two important UA transporters, ABCG2 and SLC2a6, to determine whether LPP affects the UA transporters in the intestine. The qPCR and Western blot results showed that expression of ABCG2 and SLC2a6 was enhanced in the ileum of HUA mice in contrast to NC mice. We also observed that administration of LPP further increased the levels of ABCG2 and SLC2a6 compared with HUA mice, suggesting that administration of LPP might reduce the serum level of UA *via* influencing expression of UA transporters in the ileum (Figures 4A,B).

Since it has been reported that destruction of the intestine barrier correlates with the hyperuricemic condition (30), we wondered whether LPP could ameliorate the impaired intestinal barrier caused by HUA. The quantitative PCR and Western blot (Figures 4C–F) results showed that expression of tight junction (TJ) proteins Occludin and Claudin-1 in colon tissue was decreased under the high SUA condition, which reflects the disruption of intestinal barrier function due to high SUA level. However, we did not observe a significant decrease in ZO-1 protein expression (statistical data not shown), although a decrease was observed at its mRNA level. The finding that LPP treatment partially reversed the reduction of TJ protein expression provides evidence supporting the idea that oral intake of probiotics can improve intestinal barrier function. To further determine whether LPP gavage could improve the disturbed intestinal barrier induced by HUA, intestinal permeability was monitored uisng FITC-dextran (4 kD) method *in vivo*. As shown in Figure 4G, FITC-dextran concentration in serum was significantly reduced in LPP group, which confirmed the ability of LPP to attenuate the damage of intestinal barrier. With all these results together, we conclude that oral administration of LPP may have a protective effect on HUA development and the mechanisms for this effect involve increased expression of tight junction proteins and transporter proteins in the intestine.

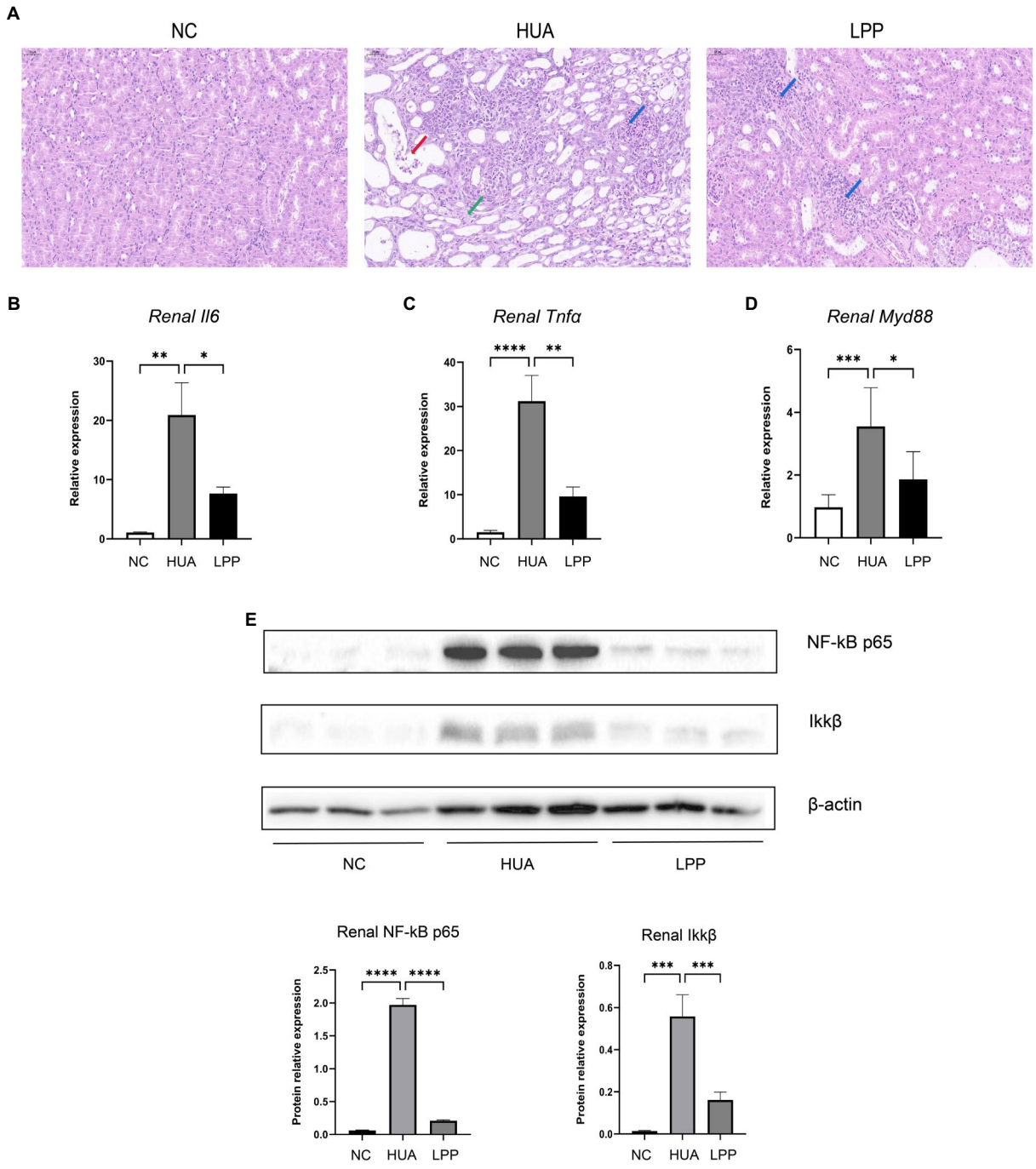

FIGURE 2
Protective effect of LPP on HUA-induced renal inflammation and pathology. **(A)** H&E stain of renal tissue (200×), red arrow: necrotic cell fragments; green arrow: interstitial fibrosis; blue arrow: inflammatory cell infiltration. **(B–D)** Quantitative PCR results of *Il-6* **(B)**, *Tnfa* **(C)**, and *Myd88* **(D)** relative expression standardized by β-actin (n=6 per group). **(E)** Representative western blotting images of NF-kB p65 and Ikkβ in renal tissue. The protein expressions were quantitated by Image J software and normalized by β-actin (n=3 per group). Bars are means ± SD, p<0.05 was set as the threshold for significance by one-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.5. Effect of LPP on gut microbiota in HUA mice

Since both HUA status and oral administration of probiotics may change the composition of intestinal flora, we investigated how LPP impacts the gut microbiota of HUA mice using 16S rRNA sequencing. The Shannon Diversity Index and the Simpson Diversity Index were evaluated to determine the alpha diversity of gut microbiota. As shown in Figures 5A,B, there was no significant difference in the alpha diversity of gut flora among the three groups. To analyze the beta diversity, the distance for each sample was calculated using the presence or absence of microbial flora (unweighted) and displayed in the coordinate space. The PCoA analysis showed a significant difference in beta diversity of gut microbiota between HUA and NC mice, and LPP treatment partially reversed this alteration in HUA mice (Figure 5C). Furthermore, at the phylum level, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*,

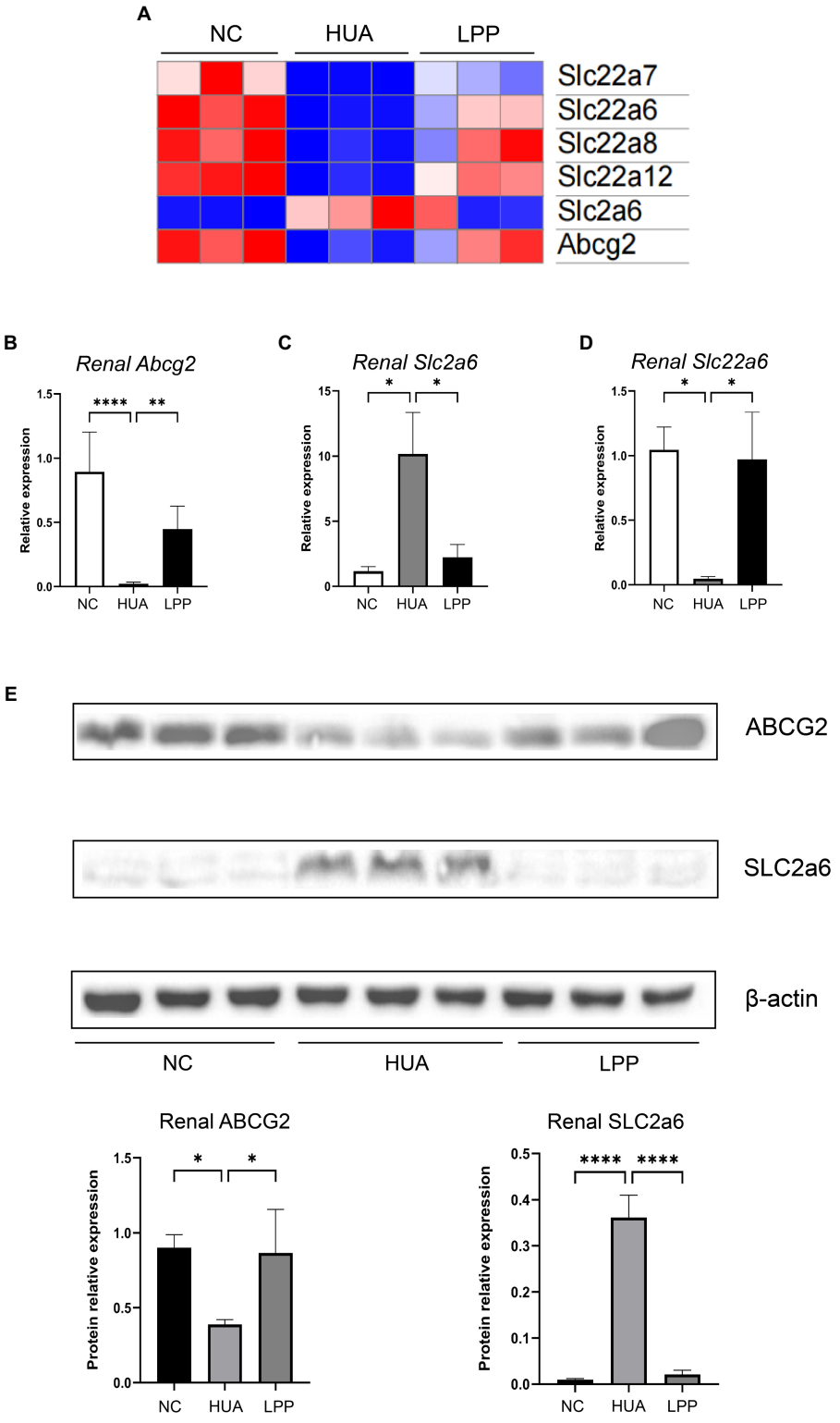

FIGURE 3
LPP altered the expression of uric acid transport-related proteins in renal tissue. **(A)** Heatmap of expression of UA transporters in renal measured by RNA-seq. (**B–D**) Relative expression of renal *Abcg2* **(B)**, *Slc2a6* **(C)** and *Slc22a6* **(D)** mRNA normalized with β-actin (n=6 per group). **(E)** Representative western blotting image of ABCG2 and SLC2a6 in renal tissue. The protein expressions were quantitated by Image J software and normalized by β-actin (n=3 per group). Bars are means±SD, p<0.05 was set as the threshold for significance by one-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Verrucomicrobiota, and *Deferribacteres* constituted the main microbiota in all three groups with *Bacteroidetes* and *Firmicutes* being dominant (Figure 5D). The *Firmicutes/Bacteroidetes* ratio (F/B ratio) in HUA mice was significantly higher than that in NC mice, while the F/B ratio was reversed after LPP treatment (Figure 5E). At the genus level, the gut microbiota was mainly composed of *Clostridium XlVa*, *Barnesiella*,

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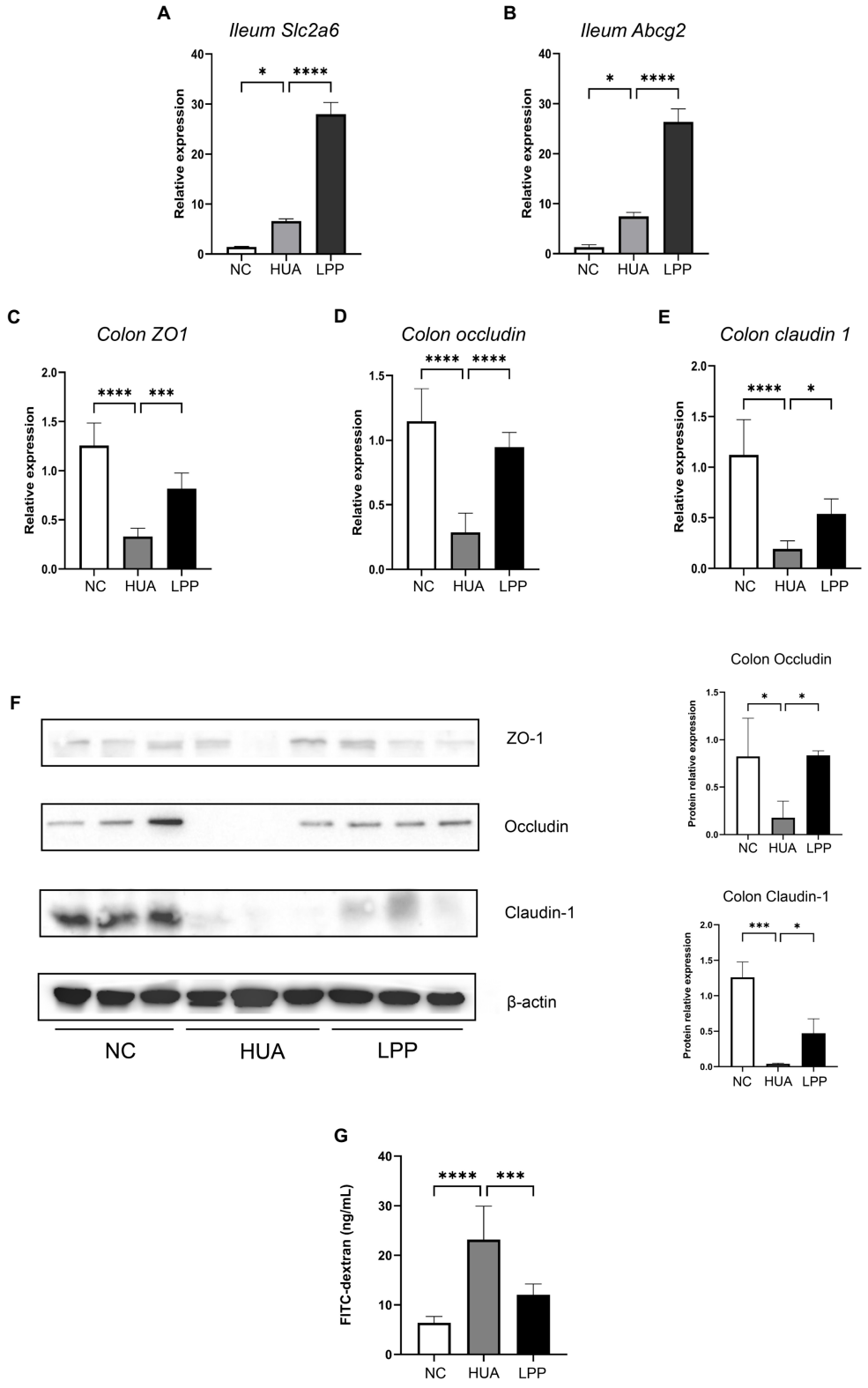

FIGURE 4
Effect of LPP on expression of tight junction protein and transporters in intestine. **(A,B)** Relative expression of *Slc2a6* and *Abcg2* mRNA in ileum tissue normalized by β-actin (n=6 pre group). **(C–E)** Relative expression of TJ proteins (*ZO1, occludin, claudin1*) mRNA in colon tissue normalized by β-actin (n=6 per group). **(F)** Representative western blotting images of ZO-1, Occludin and Claudin-1 in renal tissue. The protein expressions were quantitated by Image J software and normalized by β-actin (n=3 per group). **(G)** FITC-dextran detected in serum of mice from NC, HUA and LPP groups after administration with oral gavage. Bars are means±SD, p<0.05 was set as the threshold for significance by one-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

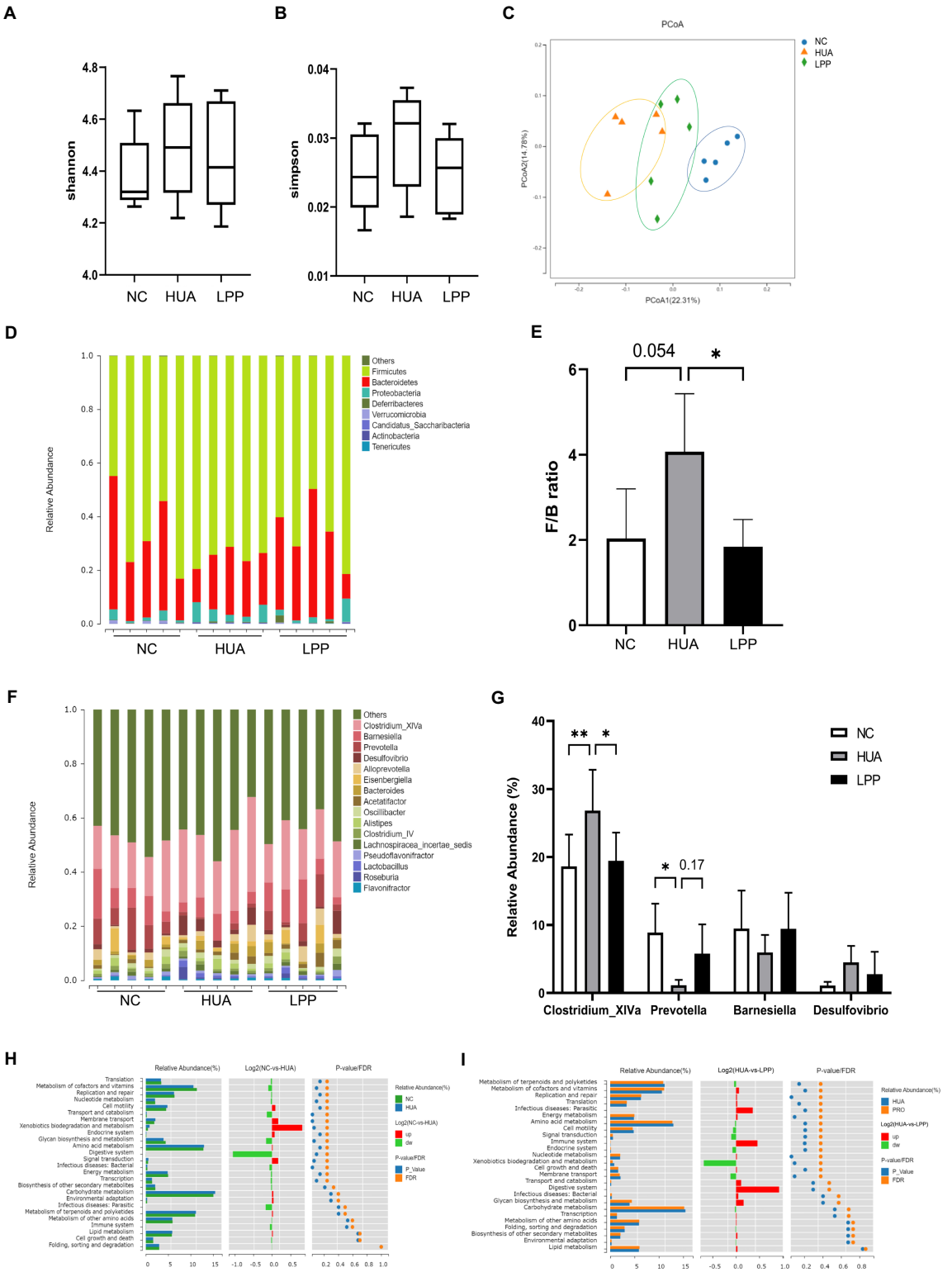

FIGURE 5
LPP restored alteration in gut microbiota of HUA mice. **(A,B)** Shannon Index and Simpson Index indicated α diversity in different groups. **(C)** Principal coordinate analysis (PCoA) plot based on unweighted UniFrac distance. **(D)** Comparison of phylum relative abundance in different groups. **(E)** The ratio of relative abundance of Firmicutes and Bacteroidetes (F/B ratio). **(F)** Comparison of genus relative abundance in different groups. **(G)** Relative abundance of the key differential genus in different groups. **(H,I)** Predicted microbial function comparisons. Gene functional categories were from level 2 of KEGG pathways. FDR, false discovery rate. **(H)** Comparing microbial function between the NC mice and HUA mice, the enriched functions in the NC mice were presented with green color and the enriched functions in the HUA mice were presented with blue color. **(I)** Comparing microbial function between the HUA mice and LPP-treated mice, the enriched functions in the HUA mice were presented with blue color and the enriched functions in the LPP-treated mice were presented with orange color. The nonparametric Kruska Wallis rank-sum test was used followed by Duncan's test for multiple comparisons for alpha diversity analysis of the gut microbiota. Other data were analyzed by one-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Prevotella, *Eisenbergiella*, *Alloprevotella*, *Alistipes*, *Bacteroides*, *Oscillibacter* and *Clostridium IV* (Figure 5F). The relative abundance of *Clostridium XlVa* was significantly higher and *Prevotella* was lower in HUA group than NC group (*Clostridium XlVa:* 26.97% in HUA vs. 18.67% in NC and *Prevotella:* 1.15% in HUA vs. 8.79% in NC; Figure 5F). Administration of LPP resulted in an increased abundance of *Prevotella* (5.75% in LPP vs. 1.15% in HUA) and decreased abundance of *Clostridium XlVa* (19.54% in LPP vs. 26.97% in HUA), suggesting that LPP may partially reverse HUA-induced alteration in gut microbiota (Figure 5G). Since alterations in bacterial taxa may also potentially affect the metabolic functions of the gut microbiota, we analyzed and compared the metabolic functions of the gut microbiota among different groups. As shown in Figure 5H, KEGG pathways in level 2 were different between NC and HUA groups. Compared with NC group, upregulated pathways in HUA mice were mainly related to xenobiotics biodegradation and metabolism, membrane transport, and cell motility. We noticed that these up-regulated pathways by HUA were downregulated by LPP treatment (Figure 5I). Together, these results indicate that treatment of LPP may not only attenuate HUA symptoms but also restore the composition and the function of gut microbiota toward the microbiota seen in normal mice.

3.6. LPP exerted anti-inflammatory potential mechanism underlying UA-induced inflammation and the protective effect of LPP transcriptome

To explore the mechanism of how oral administration of LPP protects the renal injury caused by HUA, transcriptome analysis for the kidneys from all groups was conducted. The volcano diagram showed that 2,535 differential expressed genes (DEGs) including1372-upregulated and 1,163-downregulated genes between HUA and LPP groups were identified (Supplementary Figure S1). The bubble diagram showed the top 20 KEGG pathways upregulated in the LPP group compared with the HUA group, in which TNF, NF-kB, and MAPK signaling pathways were closely associated with tissue inflammation and necrosis (Figure 6A). The heatmap of gene expression enriched in MAPK, TNF and NF-kB signal pathways showed that expression of genes such as *Tnf, Fos, Jun, Junb, Ccl2/5/12/20, Cx3cl1* was dramatically upregulated in HUA mice. Administration of LPP could significantly downregulate these genes involved in MAPK, TNF, and NF-kB signal pathways, suggesting that LPP could attenuate HUA-induced inflammatory pathological processes in renal tissues (Figure 6B). Literature has demonstrated that UA can activate inflammatory pathways by binding Toll-like receptor (TLR) 2 and TLR4 (31). To understand whether LPP administration affects expression of TLR-related genes, we examined expression of *Tlr4*, *Lbp* and *Cd14*, and *Tlr2* and found higher expression of these gene in the kidney of HUA mice compared with NC mice and a reversal of these changes by LPP administration. The results of q-PCR confirmed the transcriptomic results (Figure 6C). Besides, expression levels of other TLRs (*Tlr1/6/7/8/9*) were also changed in HUA mice, which indicates that other TLRs other than TLR4 and TLR2 may also be involved in kidney injury caused by UA.

The liver is an important organ for UA metabolism, and liver transcriptome results may reveal some clues regarding how LPP affects SUA levels. We identified 1,200 DEGs (868 upregulated, 332 downregulated) between HUA and LPP groups (Supplementary Figure S2). A bubble diagram of the top 20 KEGG pathways downregulated in the LPP group indicated that retinol metabolism may be affected by LPP intake (Supplementary Figure S2). Many clinical and animal studies have shown that development of HUA is associated with upregulation of the retinol metabolic pathway or high dose of vitamin A (one of the products of retinol metabolism) intake (32–34). Retinol dehydrogenase (RDH) is the key enzyme for vitamin A production. Consistent with other studies, our transcriptomic data showed that the expressions of *Rdh5*, *Rdh9,* and *Rdh16f2* were significantly increased in HUA mice and LPP intake could partially reverse the changes in these genes (Figure 6D). Retinol-binding protein 4 (RBP4) plays a key role in retinol and vitamin A metabolism (35). RBP4 belongs to the apolipoprotein family and transports retinol from the liver to peripheral tissues as the retinol-specific carrier in the blood. Therefore, we verified the transcriptome data on *Rbp4* by q-PCR and confirmed that *Rbp4* mRNA level was significantly elevated under HUA status, and this upregulation was suppressed by oral LPP administration.

4. Discussion

Primary HUA induced by genetic factors is uncommon in the population, while secondary HUA driven by a high purine diet is mainstream (36). The use of UA-lowering drugs is not the first choice for patients with asymptomatic HUA (37). However, patient compliance and outcomes are often unsatisfactory with simple diet control therapy. Thus, probiotics administration is an alternative adjunctive treatment of HUA. Among the probiotic strains, *Lactobacillus* showed diverse biological functions (38, 39). Only a few *Lactobacillus* strains have been reported to alleviate HUA in the *in vivo* studies. In this study, we isolated and evaluated a candidate probiotic strain LPP for its UA-lowering function. Our results showed that LPP could significantly lower SUA levels and relieve symptoms of HUA.

The liver is the main site of uric acid synthesis, and XOD and ADA play a key role in this process (40). Our results showed that activity of ADA and XOD in the liver of HUA mice was significantly higher than that in NC mice, and oral administration of LPP could suppress the activity of ADA and XOD. Previous studies have demonstrated that anti-inflammation bioactivity and short-chain fatty acids (SCFAs) induced by probiotics may suppress XOD activity in the liver since the pro-inflammation factors such as LPS and IL-1β can increase XOD activity (22, 41, 42). In this study, we observed that oral administration of LPP displayed anti-inflammatory activity in the kidney, which may contribute to suppressing XOD activity in the liver through an unidentified mechanism. Although the importance of the liver for uric acid synthesis is well recognized, the transcriptomics of the liver has rarely been studied. To our surprise, the liver transcriptome revealed a possible relationship between the retinol metabolic pathway and HUA, suggesting that LPP may reduce SUA by affecting this pathway. Although the interaction between UA synthesis, retinol metabolism, and intestinal flora remains unclear, we speculate that influencing retinol metabolism by regulating gut microbiota may be a possible solution to prevent HUA.

Both crystallized and soluble UA can induce inflammation in multiple organs and tissues, and the kidney is one of the main organs affected during the long-term HUA state. The main objectives of the current clinical treatment of HUA are (1) to inhibit XOD activity, (2) to affect the transport of UA in the kidney to enhance UA excretion, and (3) to alkalize urine to improve UA excretion (43, 44). These drugs do not have an anti-inflammatory effect and cannot relieve the HUA-related inflammatory damage in the kidney. In this study, we identified the

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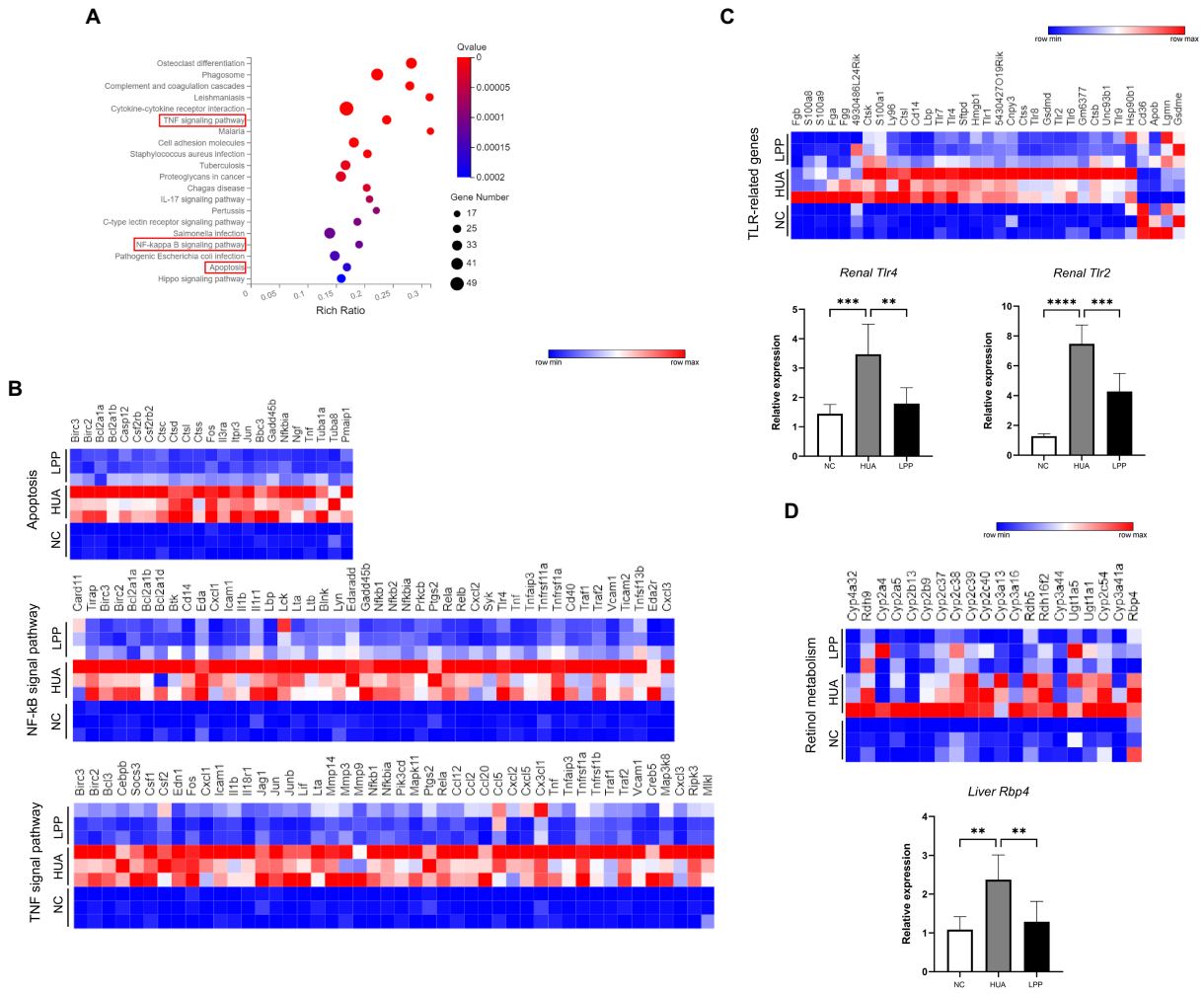

FIGURE 6
Transcriptome data revealed the potential mechanisms for UA-induced renal inflammation and LPP protective effect. **(A)** Bubble diagram of the top 20 KEGG pathways downregulated in the LPP group compared with the HUA group in renal tissue. **(B)** Heatmap depiction of the expression of apoptosis, TNF, and NF-kB signal pathway in renal tissue from different groups. **(C)** Heatmap depiction of the expression of TLR-related genes and qPCR results of *Tlr2/4* in renal tissue from different groups. **(D)** Heatmap depiction of retinol metabolism signal pathway and qPCR results of *Rbp4* in liver tissue from different groups. Bars are means±SD, $p<0.05$ was set as the threshold for significance by one-way ANOVA followed by *post hoc* comparisons using Tukey's test for multiple groups' comparisons, *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

anti-inflammation effect of LPP on HUA-induced renal inflammatory lesions by inhibiting NF-kB pathway at both transcriptional and translational levels. Based on our result, LPP has the potential value as adjuvant medicine to alleviate renal injury caused by HUA. Additionally, our transcriptomic data suggest that UA-induced renal inflammation may be initiated by extensive activation of TLRs (*Tlr2/4/1/6/7/8/9*). NF-kB pathway is triggered by TLRs' activation. It has been reported that secretions from some specific strains can suppress inflammatory reactions in a TLR4-dependent way (45, 46). Similarly, we speculate that certain small molecules produced by LPP may enter the circulation from the intestinal lumen and inhibit inflammatory pathways by interacting with various TLRs, resulting in inhibition of HUA-induced renal inflammation. However, the underlying mechanism as to how LPP interacts with TLRs remains to be elucidated.

About two-thirds of uric acid excretion is accomplished through the kidney, and members of solute carrier (SLC) family and ATP-binding cassette (ABC) superfamily play a significant role in this process (36). Several probiotics and prebiotics have been reported to promote uric acid excretion in animal studies by altering expression of uric acid transporters in the kidney, which in turn reduces SUA levels (22, 41, 47–49). For example, *Lacticaseibacillus paracasei* MJM60396 intake increased expression of OAT1 and OAT3 and decreased expression of URAT1 and GLUT9 in the HUA mouse model (47). To examine whether LPP would affect expression of uric acid transporters in the kidney, we found that administration of LPP significantly reversed the alteration in expression of genes related to UA excretion and reabsorption. Recently, studies on the interaction between gut microbiota and kidney (also known as "gut-kidney axis") have intensified. Metabolites of intestinal flora are thought to enter the kidneys through circulation to perform their biological activities and to influence renal function (42). More studies are needed to specifically elucidate how the intestinal flora interacts with SLC and ABC family proteins.

Oral administration of probiotics can affect intestinal function directly. In addition to the excretion of UA *via* kidney, another important route of UA excretion is *via* the intestinal tract, which is responsible for about one-third of the total UA elimination. ABCG2 and SLC2a6

expressed in the intestinal epithelium translocate uric acid from the blood to the intestinal tract. When the renal excretion pathway of UA is disturbed, UA is compensated for by excretion from the intestine, resulting in elevated levels of uric acid in the patient's stool (50, 51). As we expected, the levels of ABCG2 and SLC2a6 were both upregulated in the ileum tissue of mice in the HUA group. However, the expression of these two transporters elevated even higher after treatment with LPP. We speculate that oral intake of probiotics could enhance the intestinal excretion of uric acid by upregulating the expression of ABCG2 and SLC2a6, thereby reducing SUA concentration. Intestinal barrier function is related to systemic inflammation. A weakened intestinal barrier, also known as "gut leak," can increase serum levels of pro-inflammatory substances such as lipopolysaccharides (LPS), which in turn can trigger a systemic inflammatory response (52, 53). Many prebiotics or probiotics have been reported to enhance intestinal barrier function. We observed impaired intestinal barrier function in HUA mice, which is consistent with some previous findings (30). The recovery of intestinal wall function may decrease serum LPS concentrations, lessen systemic inflammation, reduce XOD activity, and inhibit uric acid synthesis as previously described (30, 54, 55).

Similar to many metabolic diseases, HUA can also alter the intestinal microbiota. We found altered composition in intestinal flora of HUA mice at the phylum and genus levels, as evidenced by an increase in F/B ratio, the relative abundance of *Clostridium XIVa*, and a decrease in *Prevotella*. *Clostridium XIVa,* a butyrate-producing genus, has been reported to increase the number of Treg cells and ameliorate the symptoms of IBD (56). Nevertheless, some researchers reported that *Clostridium XIVa* contributed to the discrimination of obese patients with high UA, serum lipid, or blood pressure (57). *Prevotella* spp. is a member of the most dominant genera in the oral cavity. However, the function of *Prevotella* spp. and its contribution to host–microbiome crosstalk remains unclear (58), and conclusions regarding the relationship between *Prevotella* and human health are controversial. In a HUA rat model, genera level of *Prevotella* in fecal is decreased (14), suggesting that *Prevotella* may be negatively correlated with the systemic inflammatory response. In contrast, a study showed that increased *Prevotella* abundance is associated with augmented T helper type 17 (Th17) -mediated mucosal inflammation, which is in line with the marked capacity of *Prevotella* in driving Th17 immune responses *in vitro* (59). The interpretation and significance of these changes in *Clostridium XIVa* and *Prevotella* caused by LPP depend on further investigation.

5. Conclusion

In the present study, we isolated, identified, and evaluated a probiotic candidate strain LPP with HUA-lowering character. We found that LPP treatment suppressed inflammation in renal by inhibiting multiple pathways including NF-kB, TNF, MAPK signaling pathways. In addition, LPP treatment also regulated expression of UA transporters, protected the intestinal barrier, and restored the disrupted gut microbiota. This study has provided evidence supporting the application of LP*P* as an effective intervention to ameliorate HUA, which needs to be verified in humans in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA896723, https://www.ncbi.nlm.nih.gov/, PRJNA897871, https://www.ncbi.nlm.nih.gov/, PRJNA898670, https://www.ncbi.nlm.nih.gov/, PRJNA899144.

Ethics statement

The animal study was reviewed and approved by the Ethics Review Committee of the National Institute for Communicable Disease Control and Prevention at the Chinese Center for Disease Control and Prevention (Beijing, China).

Author contributions

ZW and ZR conceptualized the experiments. ZW, LS, XL, YH, YX, YZ, JL, and ML conducted the experiments. ZW and YH analyzed the data. ZH and ZR wrote the paper. ZW, XL, and ZR discussed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1094483/full#supplementary-material

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EDITED BY
Hani El-Nezami,
The University of Hong Kong,
Hong Kong SAR, China

REVIEWED BY
Alexandre Lamas,
University of Santiago de Compostela, Spain
Ancuta Lupu,
Grigore T. Popa University of Medicine and Pharmacy, Romania

*CORRESPONDENCE
Linlin Wang
✉ linlinwang@bjmu.edu.cn
Peng Liu
✉ liu_peng@pkuph.edu.cn

†These authors have contributed equally to this work and share first authorship

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Characteristics of intestinal microbiota in infants with late-onset breast milk jaundice

Qianying Guo[1†], Xinran Liu[1†], Mingxuan Cui[1], Xuening Li[2], Chen Yang[3], Shilong Zhao[1], Lina Pan[4], Xiaoyu Peng[4], Linlin Wang[3*] and Peng Liu[1*]

[1]Department of Clinical Nutrition, Peking University People's Hospital, Beijing, China, [2]Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China, [3]National Health Commission Key Laboratory of Reproductive Health, School of Public Health, Institute of Reproductive and Child Health, Peking University, Beijing, China, [4]Ausnutria Dairy (China) Co., Ltd., Changsha, China

Introduction: In this paper, microbiota analysis was determined to analyze the structure and difference of intestinal microbiota between LBMJ (late-onset breast milk jaundice) infants and healthy individuals.

Methods: We collected fresh fecal samples from 13 infants with LBMJ and 13 healthy individuals, then determined the intestinal microbiota by 16s rRNA sequencing. The differences of microbiota structure, diversity and functional characteristics between the two groups were analyzed, and the correlation between dominant genus and TcB (transcutaneous bilirubin) value was calculated.

Results: In this study, there were no significant differences in maternal demographic characteristics, neonatal status and macronutrients in breast milk between the two groups ($p>0.05$). There are differences in the structure of intestinal microbiota between LBMJ and the control group. At the genus level, the relative abundance of *Klebsiella* in the case group is high ($p<0.05$). At the same time, correlation analysis indicates that the abundance of *Klebsiella* is positively correlated with TcB value. The intestinal microbiota richness and diversity (Alpha diversity and Beta diversity) of the two groups were significantly different ($p<0.05$). LEfSe analysis showed that 25 genera including *Klebsiella* was significantly enriched in the LBMJ infants, and the other 17 species are enriched in the control group. Functional prediction analysis indicated that 42 metabolic pathways may be related to the occurrence of LBMJ.

Conclusion: In conclusion, characteristic changes are seen in intestinal microbiota compositions between LBMJ infants and the healthy controls. *Klebsiella* is closely associated with the severity of the disease, which may be due to enhanced β-glucuronidase activity.

KEYWORDS

late-onset breast milk jaundice, intestinal microbiota, 16S rRNA, breastfed infants, *Klebsiella*

1. Introduction

Hyperbilirubinemia is one of the most common conditions in newborns (1). Although it is generally considered to have a good prognosis, a large proportion of newborn patients maintain high bilirubin levels, which may lead to serious complications, such as growth retardation, encephalopathy, autism and hearing impairment.

LBMJ (late-onset breast milk jaundice) is a common type of hyperbilirubinemia, which occurs 1–2 weeks after the birth of breastfed infants, lasting 4–6 weeks or even 2–3 months. Breastfeeding is the biggest risk factor for LBMJ, but the etiology and mechanism are still unclear, and the diagnosis mainly depends on clinical exclusivity (2). Under these circumstances, interruption of breastfeeding to treat LBMJ is controversial and may increase the risk of early termination of breastfeeding which also contradicts the WHO (World Health Organization) recommendation of exclusive breastfeeding for 6 months (3).

Few studies have been reported on the effect of dietary factors of lactating mothers on LBMJ. A study on the effect of a Mediterranean diet on serum bilirubin levels showed that long-term adherence to this dietary pattern reduced serum bilirubin concentrations, but this study did not analyze the effect of specific food or nutrients in the diet on bilirubin metabolism (4). Loprinzi and Mahoney studied the effect of consumption of flavonoid-rich fruits and vegetables on serum bilirubin levels and found a positive correlation between them (5). In addition to the direct effect of diet on bilirubin metabolism, it is unclear whether dietary factors can contribute to the development of LBMJ by affecting the composition of breast milk. Gut microbiota is critical for bilirubin metabolism. Studies have shown that the composition of intestinal microbiota is closely related to the serum bilirubin level (6). Intestinal microbiota participates in bilirubin metabolism, while the disorder of intestinal microbiota interferes with the transformation of bilirubin, which may lead to the increase of bilirubin intestinal and liver circulation, leading to hyperbilirubinemia. Previous studies have found a relationship between *Clostridium*, *Bifidobacterium*, and bilirubin metabolism, but the role of microbiota induced neonatal bilirubin is not fully understood (7). Using germ-free multidrug resistance 2 knockout mice model, the researchers found that the absence of intestinal microbiota would aggravate the hepatobiliary disease of mouse models, proving the importance of symbiotic microbiota and its metabolites in preventing biliary injury (8). A randomized controlled trial has found that prebiotic oligosaccharides can reduce the level of bilirubin in premature infants and thus treat neonatal hyper-bilirubinemia (9). These results hint that infants with LBMJ may have abnormal intestinal microbiota, but its role in the development of LBMJ remains unclear.

We investigated the structure, diversity and difference of intestinal microbiota between LBMJ infants and controls on the 42nd day of birth to understand the possible mechanisms by which intestinal microbiota induces jaundice.

2. Materials and methods

2.1. Subjects

This study is based on a follow-up mother-infant cohort from the Peking University People's Hospital.

Infants and their mother were screened and enrolled in the study based on the following inclusion criteria: Full-term infants born at Peking University People's Hospital; The mother is 20–45 years old; Exclusive breastfeeding or mainly (breast feeding ac-counts for more than 80%); presenting with skin or sclera jaundice and do not subside for more than 3 weeks; Transdermal bilirubin value was greater than 7.87 mg/dL on the 42nd day of birth.

Infants were excluded for the following reasons: with risk factors including hemolytic disease, blood type incompatibilities, reticulocytosis, abnormal blood smear, polycythemia, Coombs' test positive, glucose-6-phosphate dehydrogenase deficiency, skull hematoma, hypothermia, intracranial hemorrhage, cholestasis, neonatal hypothyroidism, phenylketonuria screening positive and used antibiotics and probiotics.

Finally, 13 infants with LBMJ were selected in case group and 13 healthy infants were included in control group. The study was approved by the Ethics Committee of the Peking University People's Hospital (Approval Number: 2020PHB113-01), and all participating mothers signed an informed consent form.

2.2. Questionnaire survey

The questionnaire survey process by the training of qualified investigators to take a "face-to-face" questioning method to collect information, the collection of content includes mother's maternal age, BMI, gestational week, delivery mode, parity, pregnancy complications, infant's gender, birth length, birth weight, 1-min Apgar score and 5-min Apgar score.

The Apgar score is the standard method of assessing a child's physical condition immediately after birth. It includes: muscle tone (Activity), pulse (Pulse), frowning movements response to stimuli (Grimace), appearance skin color (Appearance), and respiration (Respiration).

2.3. Composition detection of breast milk

On the morning of the 42nd day after delivery, 5 mL of breast milk from the single breast of the mother was collected with an electric breast pump or manual milking. The breast milk collected is the mid-stream breast milk (5–7 min after lactation). After recording the collection time, freeze it immediately and transfer it to the refrigerator at −80°C as soon as possible for further inspection.

Lactose, fat, protein, energy, minerals and water in breast milk were detected by infrared spectral analysis technology with an automatic breast milk composition analyzer (HKANGYU KY-9003).

2.4. Fecal sample collecting and 16S rRNA gene sequencing

Within postnatal days 35–42, the participants provided samples of infant feces using sterile stool collection tubes and transferred to the laboratory as soon as possible for storage in a −80°C refrigerator.

Stool microbiome DNA was extracted according to the instructions of the Omega E.Z.N.A. Stool DNA Kit (MoBio Laboratories, Carlsbad, CA). The extracted DNA was tested for DNA quality and concentration by 1% agarose gel electrophoresis and NanoDrop 2000 spectrophotometry (Thermo Scientific Inc., United States). The quality-checked samples were stored at −20°C for subsequent experiments. V3-V4 regions of the 16S rRNA gene were amplified with the primers

338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and then sequenced using the Illumina MiSeq sequencing platform (Illumina, San Diego, CA) at Beijing Ovison Gene Technology Co. Raw data was filtered to remove sequences less than 230 bp in length, and chimeric sequences were removed by comparing with the Gold Database database using the uchime method (10). OTU clustering (operational taxonomic units) of high-quality sequences was performed using the VSEARCH (v2.7.1) software uparse algorithm with a sequence similarity threshold of 97% (11). Comparison with the Silva128 database using the RDP Classifier algorithm was performed, and a confidence threshold of 70% was set to obtain the species classification information corresponding to each OTU.

2.5. Statistical analysis

SPSS (v23.0) was used for statistical analysis of infant characteristics, nutrient components of breast milk and clinical data. The α-diversity index analysis (including Shannon and Simpson indices) was performed using QIIME1 (v1.8.0) software. Based on species annotation and relative abundance results, species composition histogram analysis was performed using R Studio software (12). The beta diversity indices were also calculated using R Studio software. LEfSe (linear discriminant analysis effect size) was used to discover the features contributing to the most variation between control and case groups [LDA (linear discriminant analysis) > 2.0]. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the functional composition of the microbial community metagenome from its 16S profile. The KEGG database was used to obtain KO, pathway and EC information. STAMP (v2.1.3) software package was used for analyzing metabolic profiles (13).

3. Results

3.1. Characteristics of population

In this study, 26 mother-infant pairs participants (13 LBMJ infants in the case group and 13 healthy individuals in the control group) were enrolled for questionnaire survey, breast milk composition determination and 16S rRNA gene sequencing.

Demographic characteristic information of the infants and their mothers is shown in Table 1. There were no significant differences in mother's maternal age, BMI, gestational week, delivery mode, parity, pregnancy complications, infant's gender, birth length, birth weight, 1 min Apgar score and 5 min Apgar score between the two groups.

3.2. Composition of breast milk

The composition of mother's milk in case group and control group is shown in Table 2. In general, the nutritional components of breast milk in case group were lower than those in control group, but there were no significant statistical differences between the two groups.

3.3. Clusters in infant gut microbiota

On an average 57,179 ± 25,385 reads were generated per sample and we identified 169 OTUs (operational taxonomic units). 169 OTUs were obtained from the two groups of subjects through cluster analysis, including 129 shared OTU sequences among two groups, 15 unique OTU sequences in case group and 25 unique OTU sequences in control group.

TABLE 1 Demographic characteristic information of participants.

Variables		Case (*n*=13)	Control (*n*=13)	*p*-value
Maternal age (Mean ± SD, y)		31.62 ± 3.75	33.15 ± 3.13	0.268
BMI (Mean ± SD)		20.52 ± 2.39	21.64 ± 3.27	0.332
Gestational week (Mean ± SD, w)		38.98 ± 0.71	39.43 ± 0.57	0.089
Delivery mode (%)	Vaginal	10 (76.92)	12 (92.31)	0.277
	Cesarean	3 (23.08)	1 (7.69)	
Parity (%)	1	8 (61.54)	8 (61.54)	0.574
	2	5 (38.46)	4 (30.77)	
	3	0 (0.00)	1 (7.69)	
Pregnancy complications (%)	Yes	3 (23.08)	3 (23.08)	1
	No	10 (76.92)	10 (76.92)	
Gender (%)	Male	6 (46.15)	6 (46.15)	1
	Female	7 (53.85)	7 (53.85)	
Birth length (Mean ± SD, cm)		50.23 ± 2.09	50.54 ± 1.76	0.688
Birth weight (Mean ± SD, g)		3416.15 ± 447.07	3392.31 ± 400.96	0.887
1-min Apgar score (Mean ± SD)		9.92 ± 0.28	9.92 ± 0.29	1
5-min Apgar score (Mean ± SD)		9.92 ± 0.28	10.00 ± 0.00	0.337

TABLE 2 Nutrient components of breast milk.

Group	Lactose (g/100g)	Fat (g/100g)	Protein (g/100g)	Energy (kcal/100g)	Mineral (g/100g)	Moisture content (g/100g)
Case	7.38 ± 1.33	4.07 ± 1.44	0.90 ± 0.16	69.80 ± 9.31	0.22 ± 0.04	87.42 ± 1.04
Control	7.75 ± 1.38	4.26 ± 0.75	0.95 ± 0.17	73.10 ± 10.67	0.23 ± 0.04	86.81 ± 1.99

Mean ± SD, $n = 13$.

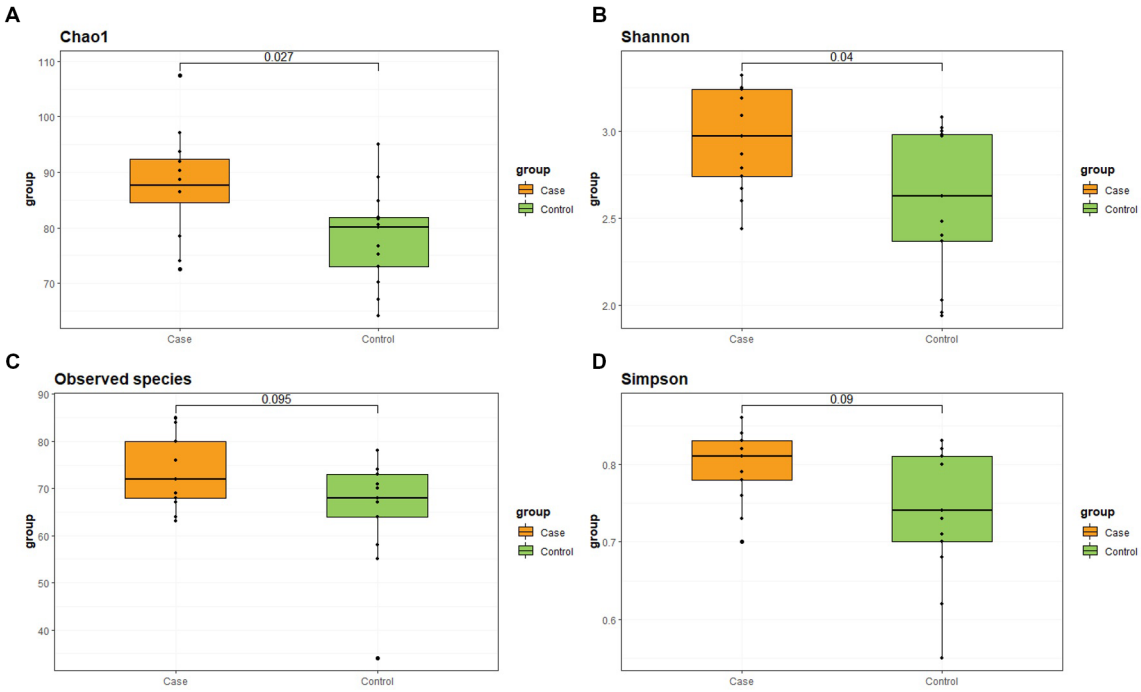

FIGURE 1
Differences of α-diversity between two groups. **(A)** Chao1 index. **(B)** Shannon index. **(C)** Observed species. **(D)** Simpson index.

3.4. Fecal alpha and beta diversity

The α-diversity indicators are shown in Figure 1 and Table 3. Chao1 and Shannon indexes have significant differences between the two groups, which are reflected in the higher α-diversity of case group.

To explore the diversity of clusters (β-diversity), the study calculated the community distance using Bray-Curtis distance based on the OTU counts. The Kruskal-Wallis's test revealed a significant difference between the two groups in the diversity of the microbiota (Figure 2).

Moreover, as shown in Figure 3, microbiota from the two groups could be completely separated and significantly different ($p < 0.05$).

3.5. Taxonomic analysis

Figure 4 shows the relative abundance of the top 4 phyla of infant gut microbiota. The most dominant phylum among them is Firmicutes, accounting for 39.4% (41.0 and 37.8% in case and control groups, respectively). Other relatively abundant bacteria are Proteobacteria (33.2 and 31.6% in case and control groups, respectively), Actinobacteria (12.1 and 23.1% in case and control groups, respectively), and Bacteroidota (13.7 and 7.5% in case and control groups, respectively). Figure 5 shows the abundance of the top 10 genera of infant gut microbiota. At the genus level, we observed that *Klebsiella* is the most abundant genus in the study population, accounting for 18.7% (25.4 and 12.0% in case and control groups, respectively). In addition, the relative abundance of *Klebsiella* is significantly different between two groups ($p < 0.05$), the infants in the case group have a higher relative abundance. No significant difference was found in other bacteria.

We take the demographic information of the mother and infant, the composition information of breast milk, the relative abundance of each genus of bacteria and α-diversity index is used as independent variable, and LBMJ is used as dependent variable for stepwise regression analysis. After automatic identification of the model, the relative abundance of *Klebsiella* was finally left in the model, with the R square value of 0.197, which means that the relative abundance of *Klebsiella* could explain 19.7% of the change between groups. Moreover, the model passed the F test ($F = 5.873$, $p = 0.023$), indicating that the model was effective (Table 4). The model formula is: LBMJ = 0.224 + 1.477 * *Klebsiella*. In addition, it is found by testing the multicollinearity of the model that all VIF values in the model are less than 5, which means that there is no collinearity; The D-W value is near 2, which means that the model has no autocorrelation and no

TABLE 3 Alpha diversity index of two groups.

Group	Chao1	Observed_species	Shannon	Simpson
Case	87.59 (12.78)	72 (14.5)	2.97 (0.54)	0.81 (0.06)
Control	80.12 (11.76)	68 (12.5)	2.63 (0.79)	0.74 (0.13)

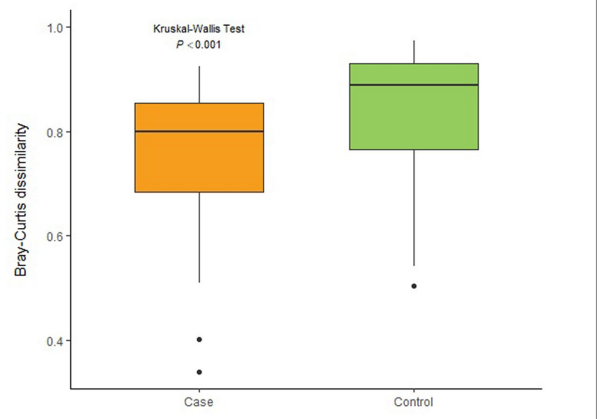

FIGURE 2
Differences of β-diversity between two groups.

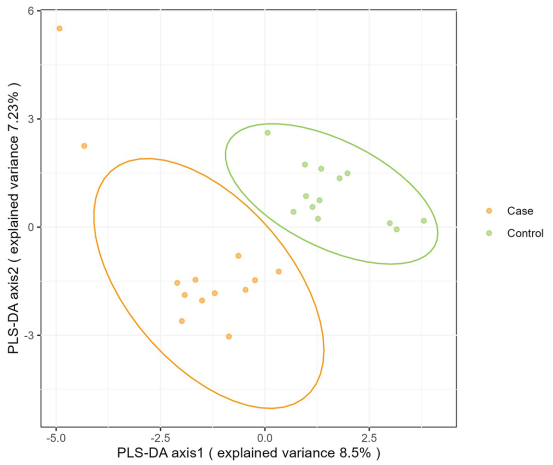

FIGURE 3
PLS-DA of two groups of bacteria based on OUT.

correlation between sample data, so the model is good. The value of *Klebsiella*'s regression coefficient is 1.477 ($t = 2.423$, $p = 0.023$), which means *Klebsiella* will have a significant positive relationship with jaundice.

Furthermore, Spearman correlation coefficient was used to analyze the strength of the correlation between each genus and TcB value of forehead. The results showed that the correlation coefficient between TcB value and *Klebsiella* relative abundance was 0.392 ($p < 0.05$), indicating that there was a significant positive correlation between TcB and *Klebsiella* relative abundance.

The proportion of species belonging to *Klebsiella* genus is shown in Figure 6, in which *Klebsiella_quasipneumoniae* is dominant.

3.6. LEfSe analysis

LDA Effect Size analysis (LEfSe) was employed to identify different features between control and case groups at all levels. According to the threshold LDA >2.0, 42 features were found to be significantly different between controls and cases. Twenty five features were more abundant in case group and 17 features was more abundant in the control group (Figure 7). The relative abundance of *Klebsiella*, which exhibited the highest LDA score, was significantly associated with jaundice.

3.7. PICRUSt 2 function prediction analysis

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) was used to predict microorganisms of the 16S rRNA data followed by function categorizing according to KEGG orthology. In the study, 42 metabolic pathways belonging to Metabolism, Genetic Information Processing and Environmental Information Processing were predicted to be associated with the presence of LBMJ (Figure 8).

4. Discussion

There are several different theories about the pathogenesis of LBMJ, which are still inconclusive. Jaundice is essentially due to an imbalance in the production and clearance of bilirubin. We collected breast milk and infant fecal sample for a comprehensive comparative analysis in an attempt to find a link with the development of LBMJ.

We compared the breast milk macronutrients between the case and control groups and no significant differences were found. However, a trend toward lower nutrient composition in the case group could be observed, a result similar to those found in other studies. It is suggested that ensuring the adequacy of nutrients in newborns may be beneficial in reducing their jaundice. This result is only a clue, and follow-up studies with larger sample sizes and more in-depth testing of breast milk composition are needed (14).

As an important part of the pathogenesis of neonatal jaundice, the enterohepatic circulation has attracted widespread attention. Changes in the gut microbiota may be one of the mechanisms leading to LBMJ. In this study, we found that the gut microbiota in LBMJ infants had unique features that can affect the occurrence and development of LBMJ. In total, 13 infants with LBMJ and 13 healthy infants were selected for intestinal microbiome composition analysis. High-throughput sequencing covered more than 99.84% of the genes, and the sequencing depth was sufficient to compare the richness of taxa.

Alpha-diversity provides the most fundamental statistic of microbial communities, higher α-diversity index means more species and more evenness (15). Chao1 index focuses on measuring richness, while Shannon index gives consideration to both rich-ness and evenness. There was found a higher richness/evenness in case

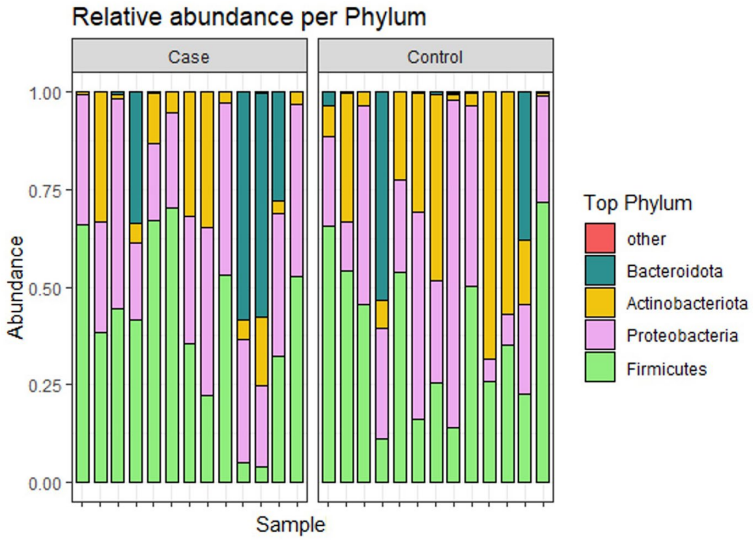

FIGURE 4
Relative abundance of microbiota at phylum level.

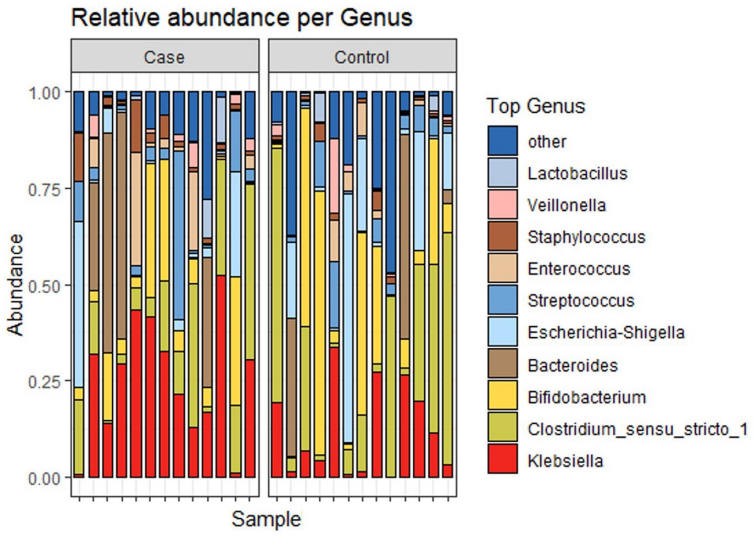

FIGURE 5
Relative abundance of microbiota at Genus level.

TABLE 4 Spearman correlation analysis results.

	Coefficient	*t*	*P*	VIF	R^2	*F*
Constant	0.224	1.531	0.139	–	0.197	5.873
Klebsiella	1.477	2.423	0.023*	1.000		

D-W value: 2.398; *$P < 0.05$.

group on Chao1 and Shannon index but no difference using another (Simpson). Generally speaking, higher diversity is considered to be more ecologically valuable and stable. The intestinal microbiota in LBMJ infants is characterized by a significant increase in species richness, possibly due to the proliferation of potentially pathogenic species (16). In this study, the case group showed higher diversity, which may be related to the presence of more pathogenic bacteria, but this requires more research to verify.

At the phylum level, no significant difference in the relative abundance of the two groups was observed. However, at the genus level, the dominant genus *Klebsiella* was significantly elevated in the case group, more than half of which were *Klebsiella quasipneumoniae* species (17). LEfSe analysis also showed that *Klebsiella* was the most significant bacteria in the case group. *Klebsiella* is widespread in the environment, and is frequently linked to an abnormal pattern of intestinal microbiota (18). *Klebsiella_quasipneumoniae* belongs to the *Klebsiella pneumoniae species* complex, which can pose a serious health threat to newborns and immunocompromised (19). In a study

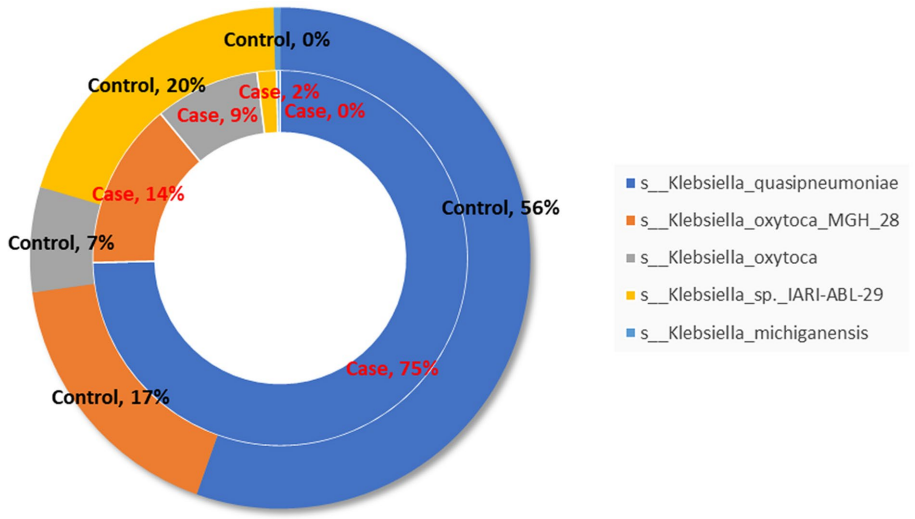

FIGURE 6
Species composition of *Klebsiella* genus of two groups.

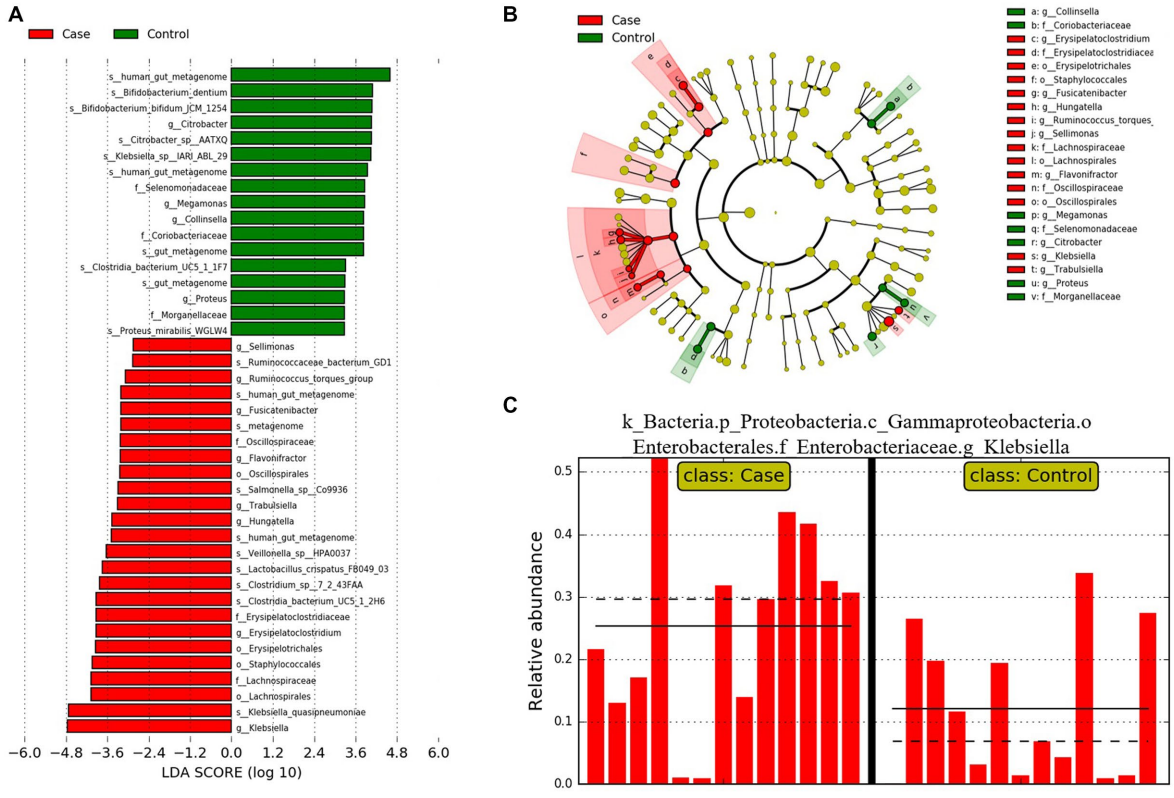

FIGURE 7
LEfSe analysis results of two groups. **(A)** Bar plot of the LDA Score between two groups. **(B)** Evolutionary branching diagram of LEfSe analysis based on taxonomic information between groups. **(C)** Histogram of the *Klebsiella* relative abundances between groups.

characterizing the gut microbiota of 29 healthy Chinese neonates and 2-month-old infants, *Escherichia/Shigella* and *Klebsiella* were the main genera of Enterobacteriaceae in Chinese neonates (20). And the enrichment of *Enterobacteriaceae* in the gut is usually associated with the pathogenesis of cholecystitis and IBD (inflammatory bowel disease) (21). Therefore, these abundant *Klebsiella* may pose a health threat to infants.

In the first few days after birth, the microbiota in the intestine has not been established, so the conjugated bilirubin that enters the intestine with bile cannot be reduced to fecal bilirubin; On the other

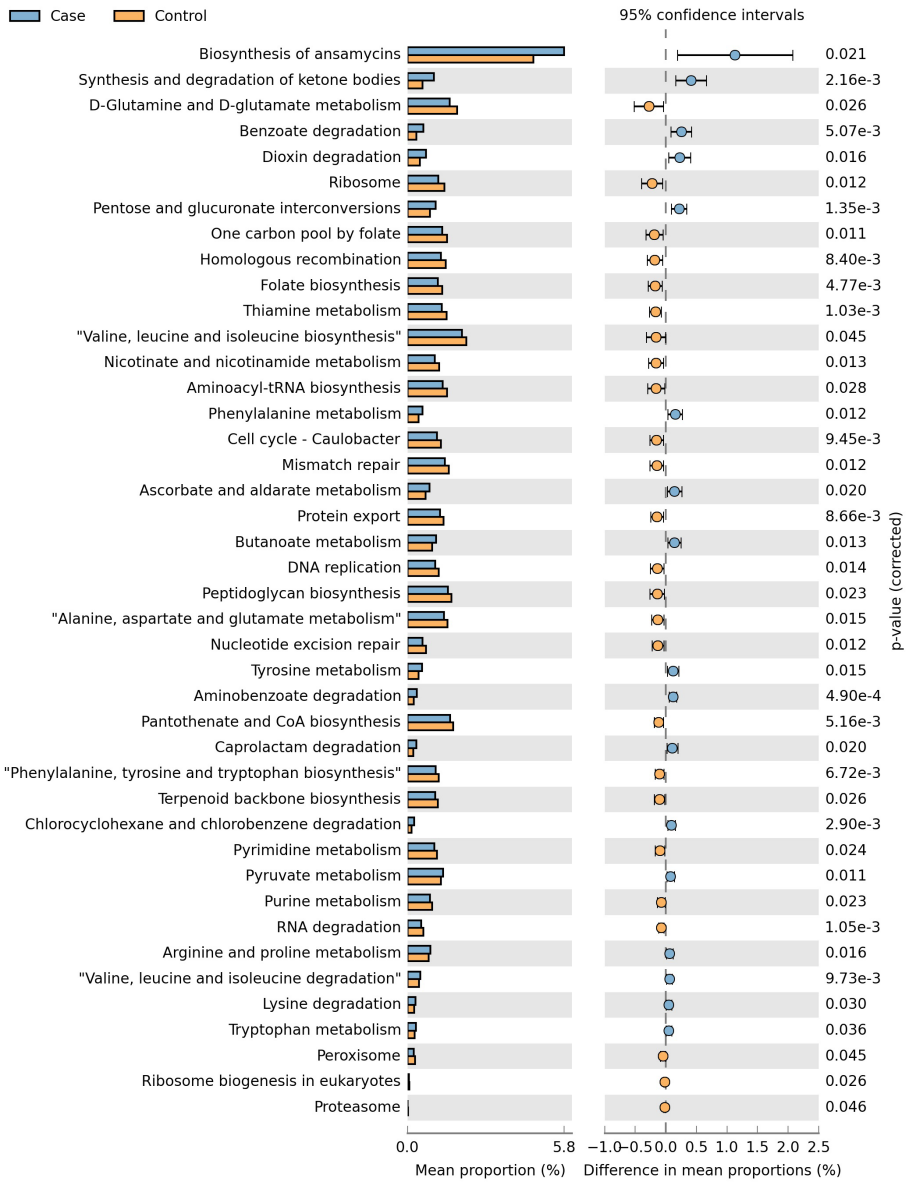

FIGURE 8
Metabolic pathways predicted to be related to the occurrence of breast milk jaundice.

hand, there are more β- glucuronidase in the intestine of new-born can hydrolyze conjugated bilirubin into unconjugated bilirubin, which is absorbed by intestinal mucosa and returned to liver through portal vein (22). This process of enterohepatic circulation in neonates cause the burden of liver metabolism of bilirubin increases, and more unconjugated bilirubin remains in the blood.

β-glucuronidase participates in the decomposition of bilirubin and plays a role in the development of hyperbilirubinemia in infants. Studies have found that the intestinal metagenome of infants at birth and 12 months old has a higher level of β-glucuronidases than that of their mothers (23). Later research found that, many bacteria, such as *E. coli*, *Klebsiella* sp., *Clostridium* can possess β-glucuronidase activity (24). Therefore, we have reason to speculate that *Klebsiella* in the study may be a key participant in the production of intestinal microbial β-glucuronidase, which further induces hyperbilirubinemia.

Beta-diversity can be used to measure between-group differences in microbiota communities. The Kruskal-Wallis's test showed a difference in the bacterial composition profiles between case and control groups. Moreover, PLS-DA analysis revealed that participants in case group cluster together in multidimensional space by their micro-biota, but separately from control group (25). These results all prove that the intestinal microbiota structure of infants in the case group is significantly different from that of normal infants.

Functional prediction analysis screened 42 metabolic pathways that may be related to the occurrence of LBMJ. Among them, Ansamycin synthesis has the greatest impact in case group. Ansamycin is a spiro piperidine derivative of rifamycin (26). It is mainly used for pulmonary infection of mycobacterium, and effective for rifampicin resistant *mycobacterium tuberculosis* strains. Its adverse reactions are similar to rifampicin, so the exposure is also

positively related to the concentration of bilirubin (27). However, such antibiotics should significantly inhibit *Klebsiella*, which is inconsistent with the results of this study and is worth for further exploration (28). Functional prediction results can also be used as a reference for future research.

This study has two strengths. First, breast milk composition has the potential to influence the development of jaundice and the composition of the intestinal microbiota. We determined the macronutrients composition of breast milk and obtained some preliminary trends that set the stage for further studies. Secondly, we analyzed the correlation between different bacteria and bilirubin levels, which also helps to determine the relationship between intestinal microbiota and jaundice. However, there are two shortcomings in this study. One is that the amino acid composition and fatty acid composition of breast milk were not examined and analyzed, so that more information on the nutritional composition of breast milk and its effect on the development of intestinal microbiota and jaundice could not be obtained. Second, our study was a characteristics analysis of intestinal microbiota profiles based on a cross-section study, so the conclusions drawn could not be further causally inferred. In the future, as the size of mother-infant cohort expands, we will test and refine these findings by expanding the sample size and analyzing infant gut microbiota at multiple points in time.

5. Conclusion

In conclusion, the richness and diversity of intestinal microbiota were significantly different in the case group compared to the control group. The important biomarker *Klebsiella* with significant difference was found through stepwise regression and LEfSe analysis. Combining the correlation analysis of *Klebsiella*' relative abundance and TcB value, it is found that *Klebsiella* is closely related to the disease severity of LBMJ patients. A total of 42 genera showed statistically significant differences between the two groups. Functional prediction analysis of 16S indicated that 42 metabolic pathways may be related to the occurrence of LBMJ.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA951195.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Peking University People's Hospital (Approval Number: 2020PHB113-01). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

QG and XRL: design and guidance of the trial. QG: methodology. XRL, MC, XNL, and SZ: sample collection. QG, XRL, LP, and XP: data curation. QG and XRL: writing—original draft preparation. LW and PL: writing—review and editing. PL: funding acquisition. All authors agreed to be accountable for the content of the work.

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Conflict of interest

LP and XP were employed by the Ausnutria Dairy (China) Co., Ltd.

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

Publisher's note

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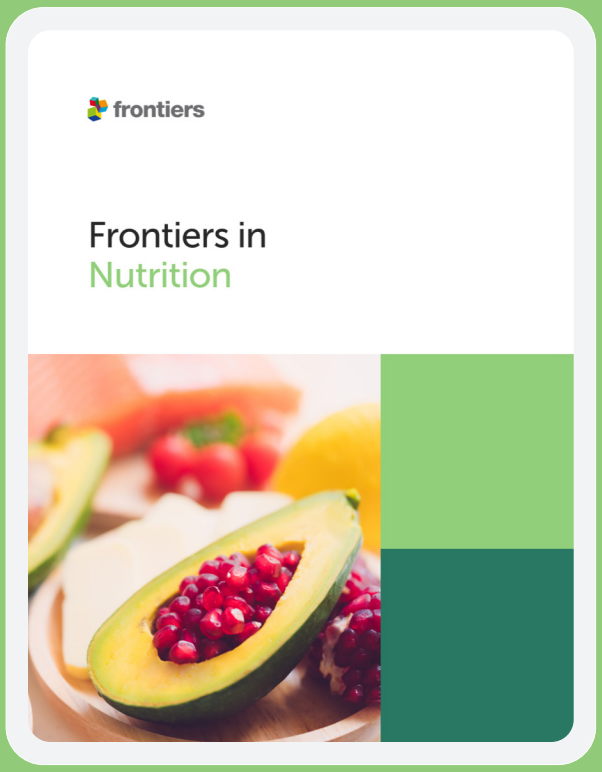

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