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Probiotics for nutrition research in health and disease

Edited by
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Probiotics for nutrition research in health and disease

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Editorial: Probiotics for nutrition research in health and disease

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Editorial on the Research Topic
Probiotics for nutrition research in health and disease

Pathophysiological alteration in the gut microbiota has been linked with developing and maintaining non-communicable diseases. There has been increasing evidence that microorganisms with claimed probiotic properties could benefit human health in different age groups by modulating the host metabolism, physiology, nutrition, and immune functions (Sampaio et al., 2022). Several probiotic strains have been considered safe and commercially available for human use through delivery by foods, beverages, and supplements (de Brito Alves et al., 2020). Additionally, there has been encouraging clinical evidence that some probiotic strains effectively manage and prevent various metabolic disorders and illnesses in humans, characterizing probiotics as promising innovative therapeutic options for their treatment (Cavalcanti Neto et al., 2018; de Brito Alves et al., 2019).

Although several studies have reported that probiotic microorganisms with claimed probiotic properties exert host health benefits, there are current gaps in optimizing the use of probiotics for research nutrition and health that need to be clarified. These have included understanding the mechanisms of how probiotics can affect different biochemical and physiological functions and the optimum dose, frequency, and duration of treatment for different probiotic strains. Additionally, some species-level effects, such as enzymatic activity, bile salt metabolism, neutralization of carcinogen, and vitamin synthesis, or rare strain-specific effects, such as the production of specific bioactive, neurological, immunological, and endocrinological effects have been poorly explored in studies with probiotics. This Research Topic of Frontiers in Microbiology aimed at increasing knowledge on the administration of probiotic microorganisms in the prevention and treatment of chronic diseases, with a particular interest in the underlying cellular and molecular mechanisms. Within this topic, 14 articles have been published and fostered current knowledge regarding the use of probiotics in health and disease.

Several articles on the Research Topic addressed the potential antioxidant and anti-inflammatory effects of potential probiotics strains. The role of *Limosilactobacillus fermentum* HFY06 in preventing colitis was addressed by Liu et al.. The authors isolated the strain from yak yogurt and demonstrated that daily administration of *Limosilactobacillus fermentum* HFY06 at 1.0×10^9 CFU/mL for 2 weeks inhibited inflammation via the NF-kB signaling pathway and prevented the onset and development of colitis in mice. Administering other *Limosilactobacillus fermentum* strain isolated from the traditionally

fermented yogurt in China, Liu et al. showed that daily treatment with *Limosilactobacillus fermentum* ZS40 at a low (1.0×10^9 CFU) or high dose (1.0×10^{11} CFU) effectively inhibited inflammation via NF-κB signaling pathway and prevented colon cancer in mice. Zhou et al. isolated *Lactobacillus plantarum* GXL94, renamed as *Lactiplantibacillus planturum* GXL94, from fermented chili and showed *in vitro* that this strain has probiotic properties and displayed good tolerance to high concentrations of hydrogen peroxide and strong scavenging capacities to various free radicals, suggesting it to be a potential antioxidant probiotic. Chen et al. showed that daily administration of *Lactiplantibacillus planturum* KSFY, a strain isolated from fermented yak yogurt, at a dose of 1.0×10^{10} CFU/kg and 1.0×10^9 CFU/kg for 4 weeks alleviated exercise-induced fatigue and improved exercise capacity in aging mice by ameliorating the metabolite accumulation, glycogen storage, muscle and liver damage, and levels of oxidative stress. Plantaricin BM-1 is a class IIa bacteriocin produced by *Lactiplantibacillus planturum*. After isolating plantaricin BM-1 from *L. plantarum* BM-1, Wang et al. reported a promising anti-cancer effect of this bacteriocin in colon epithelial cells. Mechanistically, the authors showed that plantaricin BM-1 inhibited colorectal cancer by inducing apoptosis via the caspase-3 pathway in colon cancer cells. Lee et al. evaluated the potential anti-inflammatory effects of *Lactobacillus paracasei* ATG-E1, renamed as *Lacticaseibacillus paracasei* ATG-E1, in a mouse model of airway inflammation and respiratory disease. The authors showed that treatment with *Lacticaseibacillus paracasei* ATG-E1 for 12 days improved intestinal barrier function and reduced pro-inflammatory mediators in bronchoalveolar lavage fluid and lung, suggesting that *Lacticaseibacillus paracasei* ATG-E1 may exert preventive and protective effects against respiratory diseases. Considering that probiotic combinations may have superior functions in protecting the gut and regulating the immune system compared to single strains, Li et al. evaluated the effects of a multi-strain probiotic containing *Bifidobacterium lactis* XLTG11, *Lactobacillus casei*, *Lactiplantibacillus plantarum* CCFM8661, and *Lacticaseibacillus rhamnosus,* named Probio-M9, on ampicillin-induced antibiotic-associated diarrhea in mice. The authors showed that administration of Probio-M9 alleviated ampicillin-induced diarrhea by improving intestinal barrier function, reducing proinflammatory markers, and improving the diversity and composition of the gut microbiota. The production of metabolites or specific bioactive compounds is a strain-specific characteristic of probiotics. Sen et al. performed a comprehensive metabolomic analysis of a co-culture containing *Bifidobacterium breve* MCC1274 and small intestinal-like cells derived from induced pluripotent stem cells (iPS). This co-incubation increased the amount of the immunomodulatory metabolites indole-3-lactic acid and phenyllactic acid in intestinal epithelial cells, which was followed by an upregulation of the expression of genes involved in indole-3-lactic acid synthesis, such as transaminase and tryptophan synthesis-related genes. Postbiotics are bioactive molecules produced by the metabolism of microorganisms that may have health benefits for the host. Abdelazez et al. evaluated the effects of *Levilactobacillus brevis* KLDS, a postbiotic gamma-aminobutyric acid (GABA)-producing strain, against hyperglycemia and hyperlipidemia in streptozotocin-induced diabetes in mice. The authors showed that daily administration of *Levilactobacillus brevis* KLDS at a dose of $250\ \mu L \times 10^5$ CFU/ml for 4 weeks reduced hyperglycemia and hyperlipidemia in diabetic mice.

Developing technologies that provide certainty to assess the functional stability of a probiotic product is a challenge for the probiotic industry and companies. The study of Visciglia et al. showed that flow cytometry is a valid, reliable, and innovative tool to assess the numerosity and functional status of a probiotic population compared to the traditional plate count approach.

Han et al. evaluated the effects of the probiotic bacterium *Weissella cibaria* CMU in the treatment of halitosis through a randomized, double-blind, placebo-controlled trial. The authors showed that taking 800 mg probiotic tablets containing 1.0×10^8 CFU for 8 weeks reduced the production of volatile sulfur compounds, hydrogen sulfide, methyl mercaptan, and bad breath in patients with halitosis.

Ramirez-Olea et al. performed a systematic review to demonstrate the potential application of the probiotic *Bacillus licheniformis* as an adjuvant in treating diseases in humans and animals. The authors showed that *Bacillus licheniformis* exerts relevant anti-inflammatory, immunostimulatory, antimicrobial, and antioxidant effects. In addition, *Bacillus licheniformis* had great potential in preventing and treating gastrointestinal, liver, neurological, and cardiovascular diseases, as well as health benefits in growth, dental care, bone health, metabolic, and psychological disorders. Wan and Ma critically revised the efficacy of dietary supplements targeting gut microbiota in preventing and treating gestational diabetes mellitus. The authors showed that the administration of probiotics, prebiotics, and symbiotics is a promising strategy for preventing and treating gestational diabetes mellitus.

Although fermented foods containing live microbes do not fall under the probiotic construct, kefir consumption may have many metabolic health benefits. Bourrie et al. studying cell-free or heat-treated fractions of a pitched kefir showed that the metabolic benefits of consuming this kefir do not require whole kefir.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Postbiotic Gamma-Aminobutyric Acid and Camel Milk Intervention as Innovative Trends Against Hyperglycemia and Hyperlipidemia in Streptozotocin-Induced C_{57}BL/6J Diabetic Mice

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Diabetes is a serious disease that threatens human health worldwide. The study hypothesis is to investigate the novel trends that may aid in the prevention of diabetic complications. Camel milk was presented as traditional functional food, and *Lactobacillus brevis* $KLDS_{1.0727}$ and $KLDS_{1.0373}$ strains were shown to synthesize postbiotic Gamma-aminobutyric acid as a potential food additive, which can therapeutically intervene against hyperglycemia and hyperlipidemia in streptozotocin-induced C_{57}BL/6J mice. During a four-week timeframe, body weight and postprandial blood glucose levels were monitored. Post-euthanasia, blood plasma was obtained to investigate hyperlipidemia, insulin concentrations, liver, and renal functions. The liver, pancreas, kidney, and spleen underwent histopathological examinations. The results demonstrated that $KLDS_{1.0727}$ and $KLDS_{1.0373}$ (LAC_{S1}, LAC_{S2}) and camel milk treatments all had a significant influence on hypoglycemic activity, as evidenced by reduced postprandial blood glucose levels. LAC_{S1}, LAC_{S2}, and camel milk therapy significantly reduced blood hypolipidemic, and some liver enzymes such as (alanine aminotransferase and aspartate transaminase) levels. Therefore, we recommend consuming camel milk regularly and expanding its use with fermented foods containing *L. brevis*, one of the probiotics capable of producing gamma-aminobutyric acid (GABA) as future food additives that can improve human health and reduce the prevalence of several diseases disorders.

Keywords: postbiotic, gamma-aminobutyric acid, *Lactobacillus brevis*, diabetes type 1, camel milk, C_{57}BL/6 mice

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INTRODUCTION

Diabetes is rapidly becoming a global epidemic. It is also classified as a lifestyle disease and a critical metabolic disorder that developed with postprandial hyperglycemia which is mostly caused by insulin resistance (Patil et al., 2015). Organ failure, hypertension, hyperlipidemia, cardiovascular disease, as well as pancreatic oxidative stress, all lead to β-cell destruction and reduced insulin sensitivity. Therefore, all of these inevitable factors may lead to mortality for diabetic patients (Di Martino et al., 2021).

Currently, scientists are looking for innovative ways to manage diabetes complications, particularly from natural sources such as functional foods. Non-pharmacological daily activities (e.g., diet, exercise, and weight loss) and pharmaceutical alternatives (e.g., insulin stimulators, insulin inhibitors, and glucosidase inhibitors) are two strategies for lowering diabetes prevalence. However, Complementary and alternative medications may have some negative side effects (Verhaegen and Van Gaal, 2021).

The interaction between microbiota and human health disorders has become increasingly emerging (Suzzi and Corsetti, 2020). The gut–brain axis provides new insight into the interactions between the intestine and the brain across different pathways and molecules, such as the intestinal nervous system, the vagus nerve, microbial metabolites, and the immune system *via* gut microbiota functions that regulate the central nervous system (Jones et al., 2020).

Before using the word "Postbiotic" or using it as a food component, the United States Food and Drug Administration has required that it either go through pre-market clearance as an additive or be examined by experts to establish if it is generally recognized as safe (Salminen et al., 2021). Several investigations have demonstrated postbiotics as bioactive molecules produced by microorganisms' metabolism and released into the microbial environment before or after they die. Enzymes, polysaccharides, organic acids, short-chain fatty acids, cell surface proteins, vitamins, and lipids are examples of these essential substances (Aguilar-Toalá et al., 2018).

Gamma-aminobutyric acid (GABA) has potential bio-vital postbiotic functions and is authorized as a dietary supplement with growing evidence of its influence on the gut–brain axis and systemic metabolic health (Mancini et al., 2019). Additionally, it has been shown to exhibit antihypertensive and antidepressive effects on the host after oral treatment (Wu and Shah, 2017) preventing epilepsy, depression, diabetic complications, asthma, and cancer, as well, it plays a crucial role in the treatment of neurological disorders (Mele et al., 2019). The glutamic acid decarboxylase (GAD) enzyme, which is greatly increased under certain conditions, enables various lactic acid bacteria (LAB) to produce abundant levels of GABA, and the food industry sector has capitalized on this ability to develop functional foods rich in GABA (Diez-Gutiérrez et al., 2020). *L. brevis* strains are talented LAB that generates bioactive GABA *via* the GAD enzyme. It also plays a critical part in food processing as generally recognized as safe (GRAS) organisms as well as health-promoting probiotics and postbiotics (Wu and Shah, 2017; Oleskin and Shenderov, 2019; Abdelazez et al., 2022).

Camel milk is a functional superfood with an average of 3.1% protein, 3.5% fat, 4.4% lactose, 0.79% ash, and 11.9% total solids (Al Haj Omar and Al Kanhal Hamad, 2010). It can be used as a substitute for fruits and vegetables in arid and semi-arid areas owing to its richness in minerals (magnesium, potassium, iron, copper, zinc, and sodium) and vitamins (C, B2, A, and E) content. In addition, essential nutrients such as amino acids and fatty acids are provided to the population to enhance immunity and prevent the development of a range of disorders (Izadi et al., 2019; Seligsohn et al., 2020).

Furthermore, it contains bioactive peptides having therapeutic properties and significant benefits for human health including lactoperoxidase, hydrogen peroxide, lactoferrin, lysozyme, and immunoglobulin (El-Fakharany et al., 2012). Moreover, its anti-allergic and hypoglycemic benefits, notably among the elderly and diabetics, are attributed to its low cholesterol and sugar content (Khalesi et al., 2017). As a result, camel milk is at the forefront of more effective oral insulin administration strategies since it has higher quantities of the bioactive insulin-like protein as an anti-diabetic than other mammals milk (Agrawal et al., 2011; Khan et al., 2013; Kilari et al., 2021). It contains around 52.03 IU/ml (three times higher than cow milk) depending on the camel species, and lactation period (Abdulrahman et al., 2016; Ayoub et al., 2018).

Although there is a large body of suggestions demonstrating the ability of camel milk to balance glucose, increase insulin production, reduce insulin resistance, and improve lipid properties, as well as powerful anti-diabetes capabilities in clinical trials, *in vitro* and *in vivo* therapies (Shori, 2015). As a result, we undertook this study to investigate the current evidence on the use of camel milk for hyperglycemia and hyperlipidemia therapy, as well as GABA generation and therapeutic characteristics in regulating glucose levels in diabetic C_{57}BL/6J mice for commercial use in pharmaceutical and nutritional applications.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The Key Laboratory of Dairy Science provided bacterial strains *L. brevis* $KLDS_{1.0727}$ (LAC_{S1}) and $KLDS_{1.0373}$ (LAC_{S2}) strains, chemicals, and all reagents used in this study. As part of our prior investigation, 64 strains of lactic acid bacteria were screened and kept in the Key Laboratory of Dairy Science (KLDS) to determine the ideal strains that may carry the glutamic acid decarboxylase 65 (GAD_{65}) gene and express GABA. A single colony was randomly selected and subcultured three times in De Man Rogosa Sharpe (MRS, Oxide) agar plates before being transferred to MRS broth and incubated (Sheldon Manufacturing, Inc., Shel LAB, and Cornelius, OR, United States of America) at 37°C for 18 h. Finally, cells were cultivated in MRS broth and centrifuged at 8,000 rpm for 15 min (GL-21 M High-Speed Refrigerated Centrifuge, China) to yield cell pellets that were preserved in MRS broth supplemented with 30% (v/v) glycerol and frozen at −80°C (MDF4V; Panasonic, Tokyo, Japan).

PCR Amplification Analyses of *GAD*$_{65}$

Lactobacillus brevis KLDS$_{1.0727}$ (*LAC*$_{S1}$) and KLDS$_{1.0373}$ (*LAC*$_{S2}$) strains were inoculated (1% v/v) in MRS broth enhanced with 1% L-monosodium glutamate (Sigma, United States of America), and cultured anaerobically at 37°C overnight using a glove chamber (Sheldon, United States of America) provided incubation atmosphere with a gas mixture consisting of 90% nitrogen, 5% hydrogen, and 5% carbon dioxide to help strains to generate postbiotic GABA.

The TIANamp Bacteria DNA Kit (Tiangen Biotech Ltd., Beijing, China) was used to get the Genomic DNA under the manufacturer's extraction guidelines. Furthermore, DNA was extracted once the bacterial cell wall was ruptured with lysozyme. PCR amplification of the *gad* fragments was amplified by PCR (GeneAmp PCR System 9,700 thermal cycler Applied Biosystem, United States of America) using primers constructed with the oligo 6 software (Molecular Biology Insights, Inc. DBA Oligo, Inc.) as follows:

gadF:5′CCTCGAGAAGCCGATCGCTTAGTTCG-3′; *gadR*:5′TCATATTGACCGGTATAAGTGATGCCC-3.

The PCR technique stages have been previously described (Abdelazez et al., 2018). 50 μl of PCR mixture were divided as (1.0 μl of DNA template, two primer pairs (ComateBio Custom Primers, Jilin, Changchun, China), *gadF*/*gadR* (10 μmol/l); 2.0 μl, DNA Polymerase (2.5 U/μl); 0.5 μl, 10 × Taq Buffer DNA polymerase (Sigma, United States of America); 5.0 μl, 2′-deoxynucleoside 5′ triphosphate (dNTPs), (2.5 mM) 4.0 μl, and ddH_2O 35.5 μl). The PCR techniques were carried out at the denaturation stage (95°C for 5 min). Then came the annealing stage was performed (95°C for 30 s, 55°C for 1.30 min). Finally, the elongation stage was performed (1.30 min at 72°C). A further extension session of 10 min at 72°C was added after 30 cycles.

The amplification products were separated by electrophoresis on a 1.5% agarose gel (Sigma, United States of America) in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8). For 1 h, the gel was run at a constant voltage of 100 V then the gels were stained with 0.2 μg/ml ethidium bromide for 15 min. A UV light transilluminator was used to visualize the PCR products. To determine band sizes, a 100-base pair DNA ladder (Gibco-BRL, Grand Island, New York, NY, United States of America) was loaded into the first lane of each gel. A gel documentation system was used to photograph the gels under UV light (Bio-Rad, Hercules, CA, United States of America). KLDS$_{1.0727}$ and KLDS$_{1.0373}$ were compared using sequences taken from the NCBI genome database.[1]

Potential Probiotic Functions of KLDS$_{1.0727}$ and KLDS$_{1.0373}$ Strains

Before using *L. brevis* strains *in vivo*, several *in vitro* experiments were previously performed to examine their potential ability to survive in harsh GIT conditions such as (pH, bile tolerance, antagonism against pathogens, resistance to several types of antibiotics, viability in simulating gastrointestinal juice, etc.) as free pellets or freeze-dried strains were performed previously by Abdelazez et al. (2018, 2022).

In vivo Experiments

The Experiment Protocol

The *in vivo* experiment was carried out in accordance with the Northeast Agricultural University's institutional animal care and use committee guidelines, as well as the China Ministry of Science and Technology Guide for the Care and Use of Laboratory Animals, under the authorized protocol number specialized pathogen-free rodent management (SRM)-06.

Preparation of Oral Gavage Inoculums

For 4 weeks, *LAC*$_{S1}$ and *LAC*$_{S2}$ were subcultured in MRS broth (1% v/v) at 37°C. Strain pellets were harvested after 18 h, particularly in the third generation, by centrifuging at 2,500 rpm for 10 min at 4°C. The bacterial pellets were resuspended in sterile PBS after three rinses in phosphate buffer saline (PBS) at a concentration of 5×10^8 CFU/ml, and the vials inoculum comprised 250 μl/mouse. Raw camel milk samples were collected from a bulk tank containing milk from several animals in Taif, Saudi Arabia. The samples were delivered to the animal house lab and stored frozen in a sterile icebox until the study began. One vial of camel milk inoculum containing 100 μl/mouse was used in each of the control and diabetic mice therapies.

The Experimental Design

The experiments were designed on seven groups ($n = 8$ mice/group; C_{57}BL/6 J, 16–25 g/mice, Vital River Laboratory Animal Technology Company, Beijing, China, approval number SCXK, JING, 2012-0001). Mice were adapted for 1 week in a well-ventilated cage with access to potable water and standard pathogen-free rodent chow in a regular animal house (23 ± 2°C, relative humidity 50 ± 20%, 12 h light/dark). Except for the control group (*Cont*), which was given 250 μl sterile PBS/day, and the camel milk control (*Ca*$_{Cont}$), which was fed 100 μl of raw camel milk. The other five treated groups were injected with streptozotocin (*STZ*, 180 mg/kg; Sigma, United States of America) that was newly formulated in 50 mM sodium citrate buffer (pH 4.5) and delivered subcutaneously within 10–15 min after disintegrating for 1 day (Wu et al., 2011).

Three days later after receiving streptozotocin, animals having glucose concentrations of more than 7 mmol/l were deemed diabetic, while mice with low glucose levels were eliminated. There were five groups of diabetic mice in the study. Streptozotocin control (*STZ*) group that injected intraperitoneally with 180 μl streptozotocin only. (*INS*$_{STZ}$) was daily given insulin subcutaneously injection (Sigma, United States of America) and fed rodent chow for 4 weeks at a dosage of (0.5 unit/kg body weight). Two groups of *L. brevis* strains (*LAC*$_{S1}$ and *LAC*$_{S2}$) were dissolved in buffered saline and orally gavage at a dose intake of 250 μl × 10^5 CFU/ml/day, and one group was gavage orally 100 μl/day of camel milk (*Ca*$_{STZ}$), using a stainless oral needle.

[1]https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/lactobacillus%20brevis

Evaluation of Body Weight and Hyperglycemia in *STZ*-Induced Diabetic Mice Weekly

Weekly body weight was conducted using an electronic balance (Hogentogler & Co. Inc. United States of America). Furthermore, fasting blood glucose after 12 h and postprandial blood glucose after 2 h were determined using a glucometer (Contour H Meter, Bayer HealthCare LLC, United States of America) in blood samples taken from an ophthalmic vein. The postprandial average of glucose levels was calculated using the equation suggested by Abdelazez et al. (2018).

$$\text{Average of glucose levels} = \text{Postprandial 2 h} - \text{Fasting 12 h}$$

Evaluation of Hyperlipidemia and Glucose Levels in Blood Plasma

All blood plasma parameters were performed after being humanely sacrificed under diethyl anesthesia and all parameters were assessed in triplicate using the Beckman Coulter UniCel D × C 800 analyzer (Beckman Coulter, Miami, FL, United States of America) which is available at the Center of Drug Safety Evaluation, Heilongjiang University of Chinese Medicine, Harbin, China. Overnight fasting blood samples were collected from all groups and allowed to coagulate at 4°C before being centrifuged at 12,000 rpm/10 min. Triglyceride (TG), total cholesterol (CHOL), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) levels were measured to detect hyperlipidemia. Also, glucose (GLU) and Mg^{+2} were evaluated.

Evaluation of Liver and Renal Functions

To investigate liver functions, serum alanine aminotransferase (ALT), aspartate transaminase (AST), total bile acid (TBA), albumin (ALB), globulin (GLUB), and total protein (TP) levels were assessed. As well, uric nitrogen (BUN), creatinine (CREA), and uric acid (URIC) levels were measured to assess renal function.

Assessment of Blood Serum Insulin

ELISA kits (Meimian Biotech Co., Ltd., Yancheng, China) were used to detect serum insulin following the insulin kit manufacturer's guidelines.

Histopathological Analysis

The liver, pancreas, kidney, and spleen of euthanized mice had been aseptically removed from all test groups (Chen et al., 2012). After being placed in a 10% formalin solution, the organs were washed in PBS, followed by rinses with graduated alcohol concentrations of (75–100%), as well as xylene 100%. The specimen was then paraffin-fixed and sliced into 5–10 µm thickness before being stained with hematoxylin and eosin (Solarbio, China). The slices were inspected at 100 magnifications using light microscopy (Olympus, Japan). Three photos were taken of different organ sections then the average of the affected area/mouse was determined.

Statistical Analysis

All experiments were performed in triplicate using independent tests. The obtained data were analyzed using one-way analysis of variance (ANOVA) using GraphPad Prism 8.1 (GraphPad Software Inc, San Diego, CA, USA) to create graphs and calculate statistics, and the results were represented as Mean ± SD. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Evaluation of *GAD*$_{65}$ 16S rRNA Sequences

The quantity of GABA generated by selected strains was prior determined using PCR and HPLC. The results of the HPLC quantitative analysis revealed that two ($KLDS_{1.0727}$ and $KLDS_{1.0373}$) LAC_{S1} and LAC_{S2} had the best GABA-producing ability with concentrations of 1.98 ± 0.07 and 0.05 ± 0.05 g/l, respectively, and a long band of 1.407 bp (Abdelazez et al., 2018, 2022).

Glucose Determination During 4 Weeks

The results demonstrated the significant differences ($p < 0.05$) between treatments during the first week, the INS_{STZ} group showed severe glucose concentration reduction, and this trend continued to drop during the third and fourth weeks. **Figure 1** shows the glucose values of INS_{STZ} (−5.12 ± 2.12, and −7.67 ± 2.40 mmol/l) respectively and it exhibited the lowest postprandial glucose concentrations when compared to *STZ* (6.75 ± 3.75 and 6.77 ± 0.97 mmol/l) in the first and the 4th weeks, respectively. However, Ca_{Cont} and Ca_{STZ} had (1.97 ± 1.31 and 1.87 ± 0.59 mmol/l) in the first week and (5.17 ± 0.18 and 5.82 ± 1.07 mmol/l) respectively in the fourth week exhibited the lowest GLU concentrations in the first week of feeding camel milk when compared to *Cont* (3.55 ± 1.44 mmol/l). While, LAC_{S1} and LAC_{S2} showed (4.25 ± 0.83

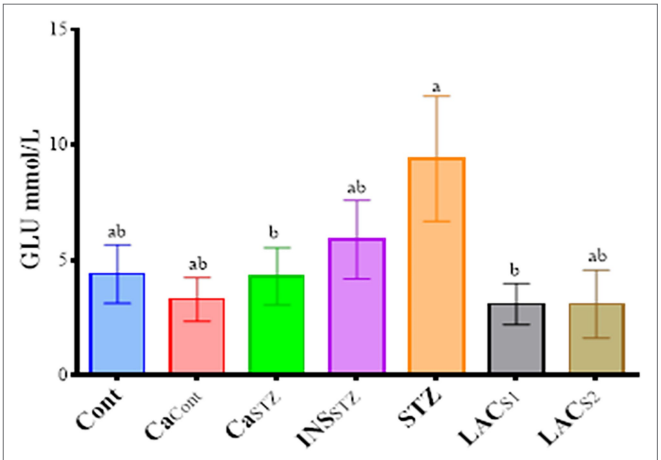

FIGURE 1 | Glucose levels (mmol/l) during 4 weeks. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{S1}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. Result are given in triplicate as mean ± SD. Different letters indicate significant differences among groups ($p < 0.05$).

and 5.66 ± 2.84 mmol/l) respectively. All treatments had higher postprandial glucose concentrations than *Cont* (4.15 ± 0.14 mmol/l) after 4 weeks of therapy.

Average of Bodyweight During 4 Weeks

Significant differences ($p < 0.05$) were seen across groups during 4 weeks of treatment. **Figure 2** shows that *Cont*, Ca_{STZ}, and INS_{STZ} had the highest average body weights (21.13 ± 0.68, 21.22 ± 0.50, and 21.21 ± 1.07 g) respectively. While *STZ* showed the lowest average body weight 18.30 ± 0.85 g.

Hyperlipidemia Parameters, Glucose, and Mg^{+2} Levels Analysis in Blood Plasma

Figure 3 displays hyperlipidemia, glucose, and Mg^{+2} in blood plasma, which includes TG, CHOL, HDL, and LDL. In all treatment groups, the results revealed a high significance ($p < 0.05$). The greatest TG content was found in *STZ* (8.05 ± 0.92 mmol/l), whereas LAC_{S1} had the lowest TG level (0.96 ± 0.27 mmol/l). In addition, the greatest concentration of CHOL was found in *STZ* (7.89 ± 1.41 mmol/l), while, *Cont* showed the lowest CHOL concentration (3.25 ± 0.66 mmol/l). Furthermore, *STZ* had the highest HDL and LDL values (4.2 ± 0.63, and 1.14 ± 0.32 mmol/l) respectively, whereas Ca_{STZ} and Ca_{Cont} had the lowest HDL (1.54 ± 0.44 and 1.6 ± 0.5 mmol/l) respectively. Furthermore, LAC_{S1} had the lowest LDL concentration (0.38 ± 0.10 mmol/l). Also, there was a significant difference in GLU levels ($p < 0.05$), *STZ* had the greatest (9.4 ± 2.71 mmol/l) and LAC_{S1} and LAC_{S2} showed the lowest GLU levels (3.1 ± 0.89 and 3.1 ± 1.47 mmol/l), while Ca_{Cont} showed the highest Mg^{+2} level (3.58 ± 1.03 mmol/l) and INS_{STZ} showed the lowest level (1.48 ± 0.42 mmol/l).

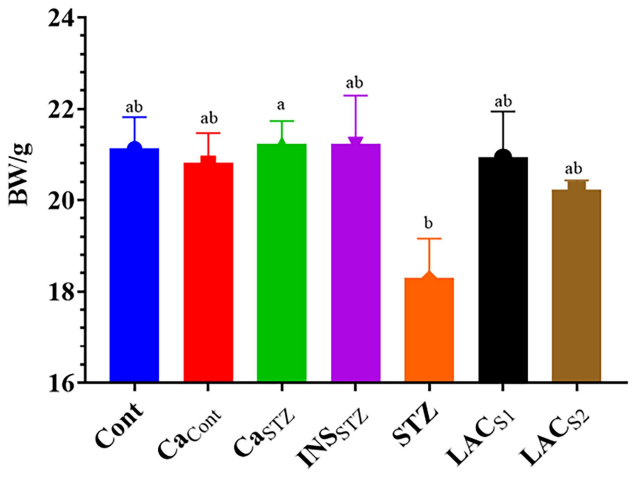

FIGURE 2 | Average of bodyweight/g during 4 weeks. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{Sl}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. Result are given in triplicate as mean ± SD. Different letters indicate significant differences among groups ($p < 0.05$).

Determination of Insulin Blood Plasma

Figure 4 depicts the insulin blood plasma concentrations, which showed no significant difference ($p < 0.05$). Ca_{Cont} and Ca_{STZ} had the highest insulin concentrations (14.14 ± 0.11 and 13.52 ± 0.13 IU/ml) respectively, while *Cont* had the lowest level (10.27 ± 0.43 IU/ml).

Assessment of Liver Functions

Figure 5 indicates that INS_{STZ} resembled *Cont*, which had the lowest TP concentrations (58.1 ± 16.77 and 60.3 ± 9.63 g/l) respectively compared to *STZ*, which had the greatest TP level (172.1 ± 19.68 g/l). Also, it was significant differences at ($p < 0.05$). Conversely, Ca_{Cont} had the lowest ALB concentration (33.7 ± 9.72 g/l), whereas INS_{STZ} had the highest ALB level (58.8 ± 16.97 g/l). The highest GLUB concentration was found in *STZ* (113.3 ± 32.70 g/l), whereas the lowest was found in LAC_{S1} (24.47.04 g/l). *Cont* showed the Lowest TBA value (1.8 ± 0.41 mmol/l), while the greatest concentration was observed in *STZ* (8.5 ± 0.63 mmol/l). Furthermore, *STZ* showed the highest levels of ALT (551 ± 45.66 IU/l). While LAC_{S1} had the lowest level (36.0 ± 10.39 IU/l). Also, *STZ* displayed the highest concentration of AST (785 ± 51.28 IU/l) while *Cont* and LAC_{S2} showed the lowest concentration (176 ± 50.80 and 174 ± 50.56 IU/l) respectively.

Assessment of Renal Functions

The findings in **Figure 6** showed that *STZ* had the greatest concentrations of BUN, CREA, and URIC (37.8 ± 10.91, 121.5 ± 35.07, and 180.6 ± 52.13 mmol/l) respectively, whereas *Cont* had the lowest concentrations of BUN, CREA, and URIC (8.6 ± 2.48, 39.5 ± 11.40 and 76.5 ± 22.08 mmol/l) respectively. There was no abnormal elevation in URIC at ($p < 0.05$). However, BUN and CREA indicated a significant difference at ($p < 0.05$).

Briefly, using *Lactobacillus brevis* strains and camel milk had a clear influence on fasting and postprandial glucose levels, as well as body weight, in diabetic mice for 4 weeks. Furthermore, the impact of GABA and camel milk was more visible in blood plasma by comparing insulin concentration, liver and kidney function, as well as cholesterol compounds and glucose levels in streptozotocin-treated mice to control groups.

Histological Evaluation

Figures 7A–D depicts a histological examination of (the liver; kidney; spleen, and pancreas). *Cont* shows that the liver had no aberrant shape, cytoplasmic nuclei was clean borders, and there was no congestion, denatured fat, or inflammatory cell infiltration. Also, the renal glomeruli were normal, with no shrinkage or atrophy, and the renal tubules were also normal. The spleen was a normal proportion of red pulp and white marrow cells, normal splenic trabecula structure, and lymphocytes with no hyperemia or fibrosis. Pancreatic cells and vessels, which were typical islet cells, did not shrink and showed no signs of fibrosis or expansion.

Ca_{Cont} reveals no apparent abnormalities or sporadic liver nuclei pyknosis and no inflammatory cell infiltration. Mild

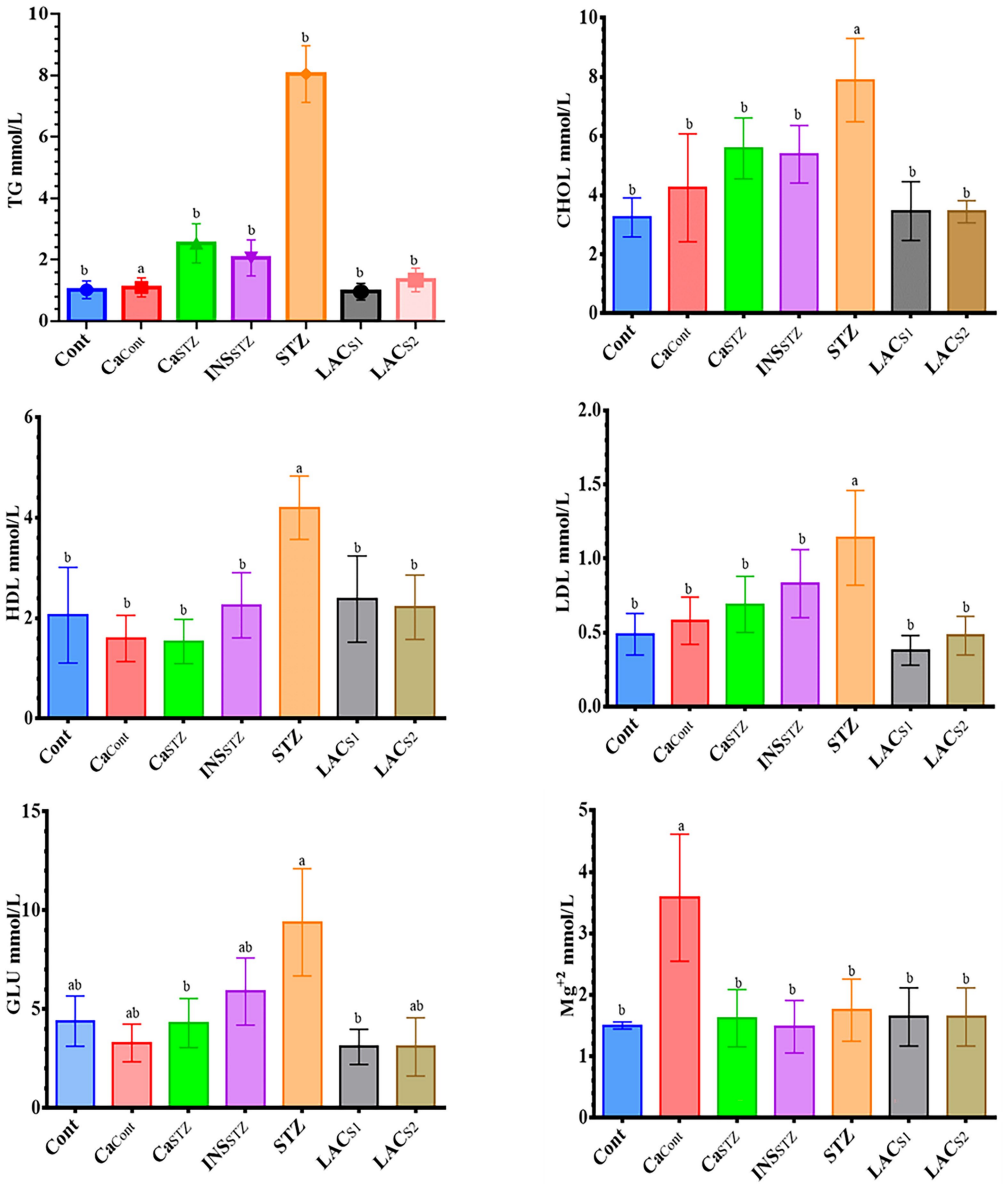

FIGURE 3 | Hyperlipidemia parameters, glucose and Mg^{+2} level analysis in blood plasma. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{S1}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. Result are given in triplicate as mean ± SD. Different letters indicate significant differences among groups ($p < 0.05$). TG, triglycerides; CHOL, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; GLU, glucose.

fatty deterioration was seen. Meanwhile, there was some glomerular atrophy and renal tubular fusion in the kidney. The spleen had enhanced phagocytic activity as well as lymphopenia. On the other hand, the pancreas shows no abnormalities.

Ca_{STZ} indicates no apparent abnormalities or sporadic hepatic nuclei pyknosis and no inflammatory cell infiltration in the liver. Meanwhile, the kidney was atrophied and glomerular shrinkage was visible in the renal tube. The spleen displayed enhanced phagocytic and lymphopenia, whereas the pancreas was normal.

Streptozotocin (*STZ*) showed hepatic steatosis, and inflammatory cells collected and spread in heaps. While renal glomeruli shrunk or atrophied, renal tubules were deemed normal, and no inflammatory cell infiltration or fibrosis was observed. Meanwhile, the spleen indicated that the number of lymphocytes had reduced and their structure had been disrupted. Pancreatic cells show vacuolar degeneration and islet atrophy.

Insulin with Streptozotocin (INS_{STZ}) demonstrated that the liver had moderate fatty degeneration and was slightly denatured. Renal glomeruli shrunk or atrophied, and epithelial cells fell off

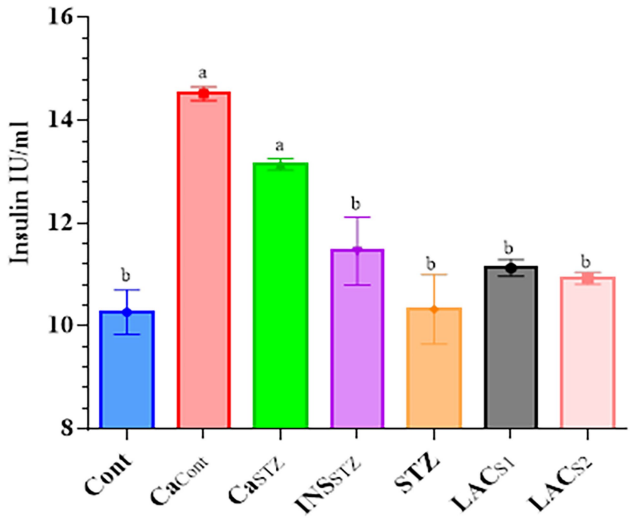

FIGURE 4 | Determination of insulin blood plasma. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{SI}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. Result are given in triplicate as mean ± SD. Different letters indicate significant differences among groups ($p < 0.05$).

renal tubules. The spleen showed increased trabecular and lymphatic decrease, as well as structural loss. Pancreatic islet cells shrank significantly, and there was extensive vacuolar degeneration.

LAC_{S1} and LAC_{S2} indicate that the liver has normal morphology. There were fewer lymphocytes in the spleen tissue. There had been no abnormalities in pancreatic cells it had not been altered. The glomerulus was similarly normal, and the renal tubes were slightly fused but generally normal.

DISCUSSION

A dysbiosis of the gut microbiota can lead to a variety of disorders, including type 1 diabetes and cancer (Carding et al., 2015). Diabetes type 1 can be diagnosed by taking measurements of both body weight and blood glucose levels. As a result, it is now recognized as a serious metabolic disorder defined by chronic hyperglycemia caused by insufficient insulin secretion (Di Martino et al., 2021).

Postbiotics are substrates that are created or synthesized by microbial metabolic activities and have a direct or indirect beneficial impact on the host (Żółkiewicz et al., 2020; Salminen et al., 2021). The capability of LAB to synthesize GABA differs between species and even within species, and it may be related to glucose metabolism and growth rate (Cataldo et al., 2020). For several lactic acid bacteria such as *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, and *Bifidobacterium infantis* postbiotic supplementation increased GABA activity and improved oxidative balance (Bagheri et al., 2019). Also, *Lactobacillus brevis* can generate GABA and control the increase of glucose levels in diabetic mice (Abdelazez et al., 2022). Furthermore (Tian et al., 2004), showed that administering the GABA molecule as a medicinal agent can reduce inflammatory responses and the development of pre-diabetes. Wan et al. (2015) discovered that the GABA molecule has a regulatory effect on human diabetic islands, namely inhibiting insulitis and systemic inflammatory cytokine production.

A blood test reference range is a set of numbers that a health practitioner uses to evaluate a range of medical test results from clinical specimens. Several factors, including age, gender, fitness, and ethnicity, as well as analytical procedures and measuring units, all influence the reference range of the results achieved. Individual results should always be interpreted in light of the test laboratory's testing facility (Virani et al., 2021). Notably, we used these standard references to evaluate the results and compared the other groups with the control group.

Glucose levels are lower before the first meal and rise for 1–2 h after eating. Extra blood glucose levels may suggest the existence of chronic diseases. The findings are similar to those of (Rees et al., 2016), who stated that diabetes was considered as fasting blood glucose levels of greater than 7 mmol/dl to 11.1 mmol/dl or higher with hyperglycemia symptoms. Sacks et al. (2011), reported the GLU varied between 3.57 and 6.12 mmol/lb. As a result, INS_{STZ} had the lowest glucose level due to the quick insulin injection, while *STZ* had the highest glucose concentration. Whereas the remainder of the test groups remained within the standard range of glucose over the 4 weeks of treatment.

The results are consistent with the findings of (Graham et al., 2007) who revealed that insulin is the most important factor in improving plasma glucose homeostasis and it ranges between 3 and 19 IU/ml in blood plasma.

Changes in ALT and AST levels are likely the most commonly employed in clinical diagnosis and research concerning liver damage and tissue activities. According to Contreras-Zentella and Hernández-Muñoz (2016), a rise or reduction in AST and ALT levels might occur as a result of liver injury, which causes changes in cell membrane permeability. The streptozotocin injunction may explain the dramatic increase in AST and ALT levels in the *STZ* group therefore the results were consistent with Saravanakumar et al. (2021), who found that (ALT and AST), and TBA levels were (1.0–40.0 IU/lb) and 0.01–20.0 mmol/lb, respectively, and who discovered that streptozotocin raises AST, ALT, and ALP levels in blood plasma. Also, the findings were in agreement with those of Newsome et al. (2018) who found that the normal ranges for TP, ALB, and GLUB were 60.0–80.0 g/dl, 35.0–55.0 g/dl, and 25.0–40.0 g/dl, respectively.

Renal dysfunction is caused by hyperuricemia, hyperglycemia, and hypertension. Also, diabetes kidney failure is one of the leading causes of mortality. Therefore, kidney function tests are the most important indicators of renal activity, vitality, and general health (Stevens and Levin, 2013).

Ibraheem et al. (2016) displayed that the reference range for BUN was 1.07–7.14 mmol/lb, CREA was 53.0–132.0 mmol/lb, and the URIC was 142.0–401.0 mmol/l. The results demonstrate that CREA levels in the therapy groups were within the safe range of CREA, which is one of the most significant kidney function tests whose results are used to evaluate renal functioning. Furthermore, serum creatinine measurement is a straightforward

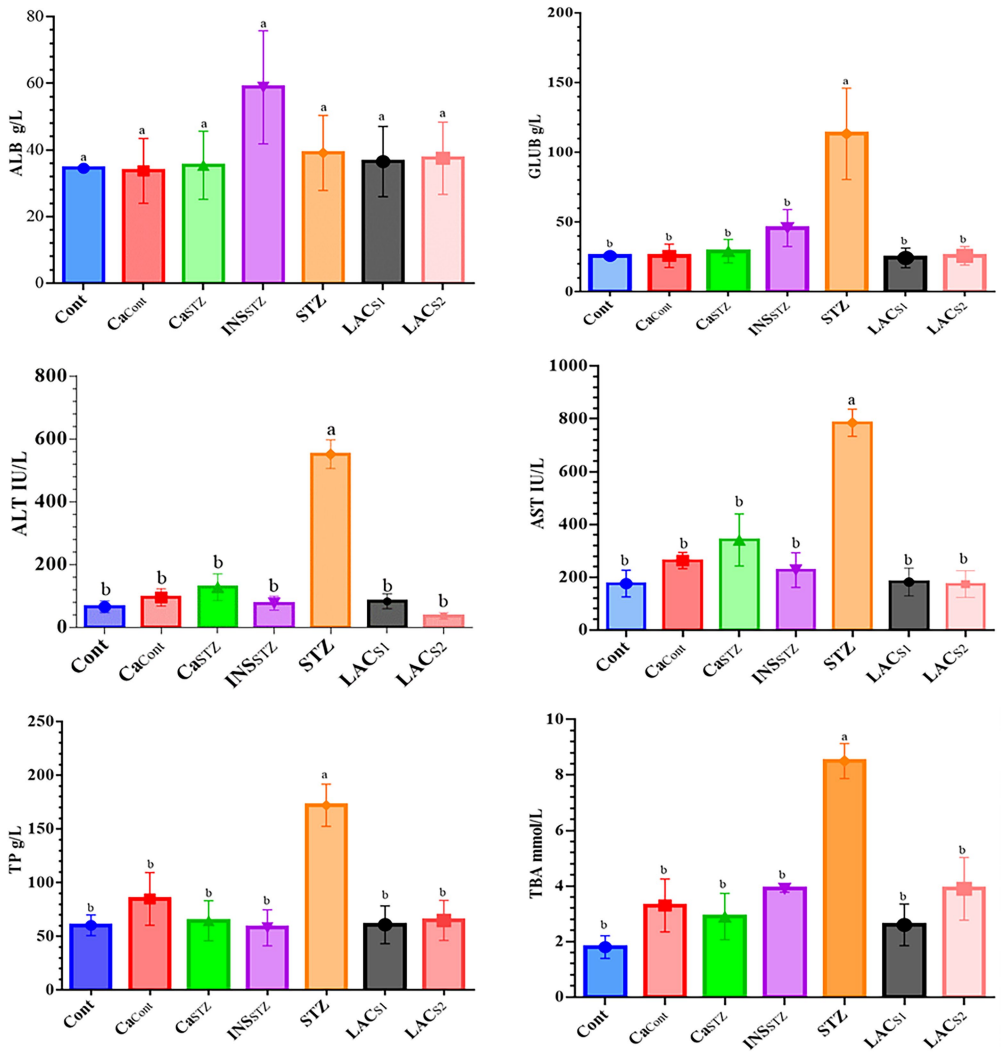

FIGURE 5 | Assessment of liver functions. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{S1}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. Result are given in triplicate as mean ± SD. Different letters indicate significant differences among groups ($p < 0.05$). ALB, albumin; GLUB, globulin; ALT, serum alanine aminotransferase; AST, aspartate transaminase; TP, total protein; TBA, total bile acid.

test that may be performed to diagnose acute renal damage or dehydration, and it is the most frequent indication of kidney function (Harloff et al., 2021).

Camel milk has an insulin-like protein that mimics the interaction of insulin with its receptors, is resistant to proteolysis, and has higher storage buffering capacity than other ruminants milk (El-Sayed et al., 2011; Swelum et al., 2021). It is also encapsulated in nanoparticles such as lipid vesicles and rapidly absorbed into the circulation. As a consequence, innovative oral insulin treatment strategies might be investigated (Ashraf et al., 2021). Camel milk has a high concentration of zinc, which is essential for the activity of insulin production in pancreatic β-cells (Deeba et al., 2020). Camel milk's functional purpose is not limited to stimulating, increasing, and boosting insulin production; it also aids in the development and improvement of pancreatic β-cells efficiency (Ayoub et al., 2018).

The findings were compared to those of investigations that evaluated the effect of camel milk intake on type 1 diabetes mellitus (Dikhanbayeva et al., 2021; Manaer et al., 2021), regardless of whether the clinical treatment period was 3 or 6 months. Patients who consumed camel milk for a short or long period exhibited significant decreases in blood glucose levels as well as insulin requirements. Furthermore, they evaluated insulin sensitivity and glycemic control in individuals with type 1 and 2 diabetes, revealing that fasting and postprandial blood glucose levels and insulin needs decreased considerably (Mudgil et al., 2018).

Camel milk supports decreased LDL cholesterol levels, hypoglycemia, a boost in immunostimulants, anticancer, and antibacterial agents, and improved consumer overall health (Esraa et al., 2016).

Importantly, the obtained results were consistent with earlier investigations in diabetic rabbits, where regular camel milk intake

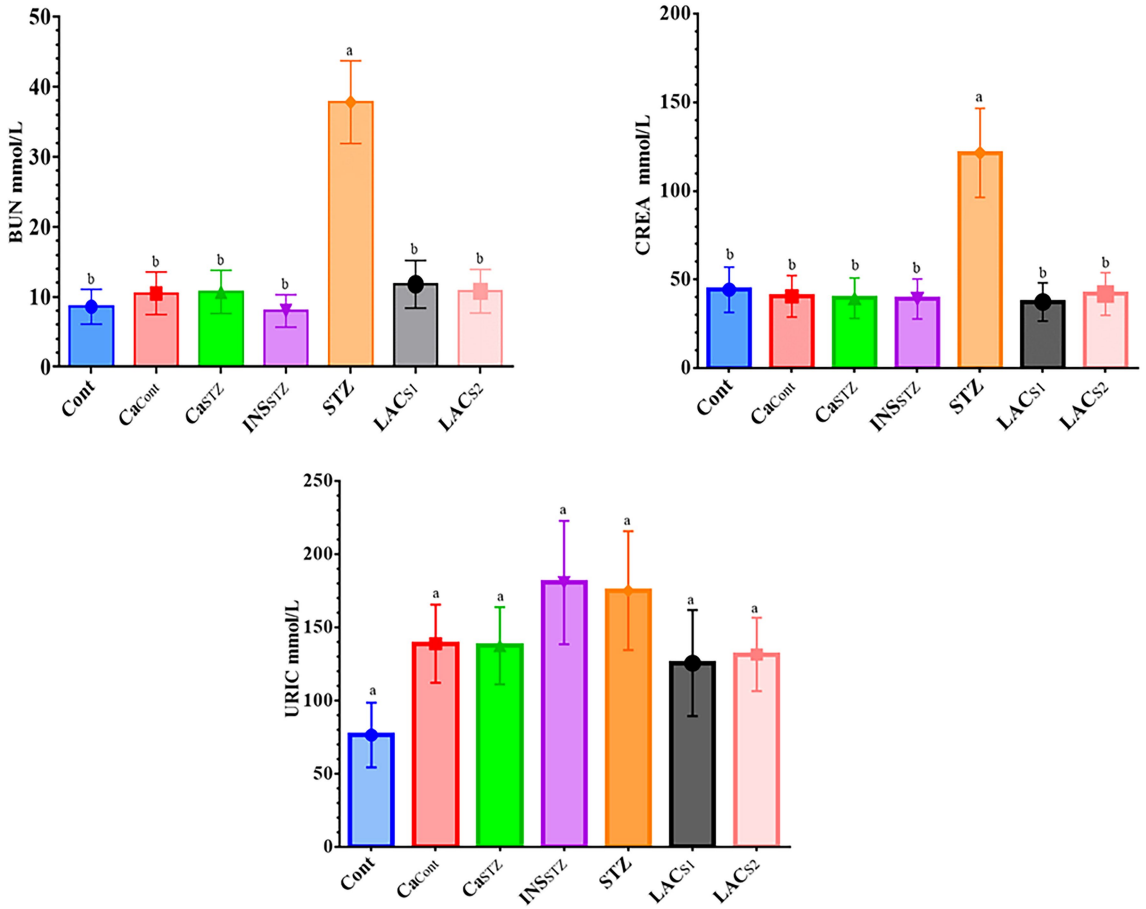

FIGURE 6 | Assessment of renal functions. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{SI}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. Result are given in triplicate as mean ± SD. Different letters indicate significant differences among groups ($p < 0.05$). BUN, uric nitrogen; CREA, creatinine; URIC, uric acid.

reduced oxidative damage (Aqib et al., 2019), whereas, raw camel milk reduces blood glucose levels in diabetic rats by 55% compared to 43% for raw cow milk (El-Zahar et al., 2021). Khan et al. (2013) reported an almost 30% decrease in blood glucose levels in *STZ*-induced rats given camel milk for 6 weeks, from 560 to 235 mg/dl compared to rats given buffalo milk or cow's milk.

Camel milk feeding lowers the percentage of lipid peroxide (malondialdehyde levels) and catalase activity while raising glutathione and superoxide dismutase (SOD) levels in *STZ*-induced diabetic rabbits. Furthermore, camel milk has a significant influence on insulin receptor function and glucose transfer in insulin-sensitive organs such as the liver and pancreas, both of which play key roles in the maintenance of blood glucose homeostasis (Al-Hashem et al., 2009). On the contrary, Fallah et al. (2020) discovered that the hypoglycemic benefits of camel milk on type 2 diabetes patients can only be sustained when combined with diabetic medicines. Furthermore, Ejtahed et al. (2015) discovered no change in glucose, lipids, or blood pressure parameters after consuming camel or cow's milk. Therefore, we conducted these experiments to demonstrate the effect of regularly consuming camel milk as a type of traditional functional food, as well as expanding its use with fermented foods that contain *L. brevis* as one of the probiotics capable of producing GABA as future food additives that can improve human health and reduce the prevalence of some diseases disorders such as diabetes.

CONCLUSION

Postbiotics, such as Gamma-aminobutyric acid, were shown to promote human health and to be effective as food additives. Furthermore, camel milk was conventionally regarded as a traditional function food. As a result, the current study proposes to evaluate the effects of $KLDS_{1.0727}$ and $KLDS_{1.0373}$ strains on hyperglycemia and hyperlipidemia in *STZ*-induced $C_{57}BL/6$ mice. Our findings indicate that GABA and camel milk can regulate blood glucose levels in mice without causing serious injury to the organs and can improve the overall composition of blood plasma. The study's findings suggested that using camel milk to manage type 1 diabetes has extremely promising results due to the high concentration of insulin-like protein. However, further research is needed before camel milk may be used as

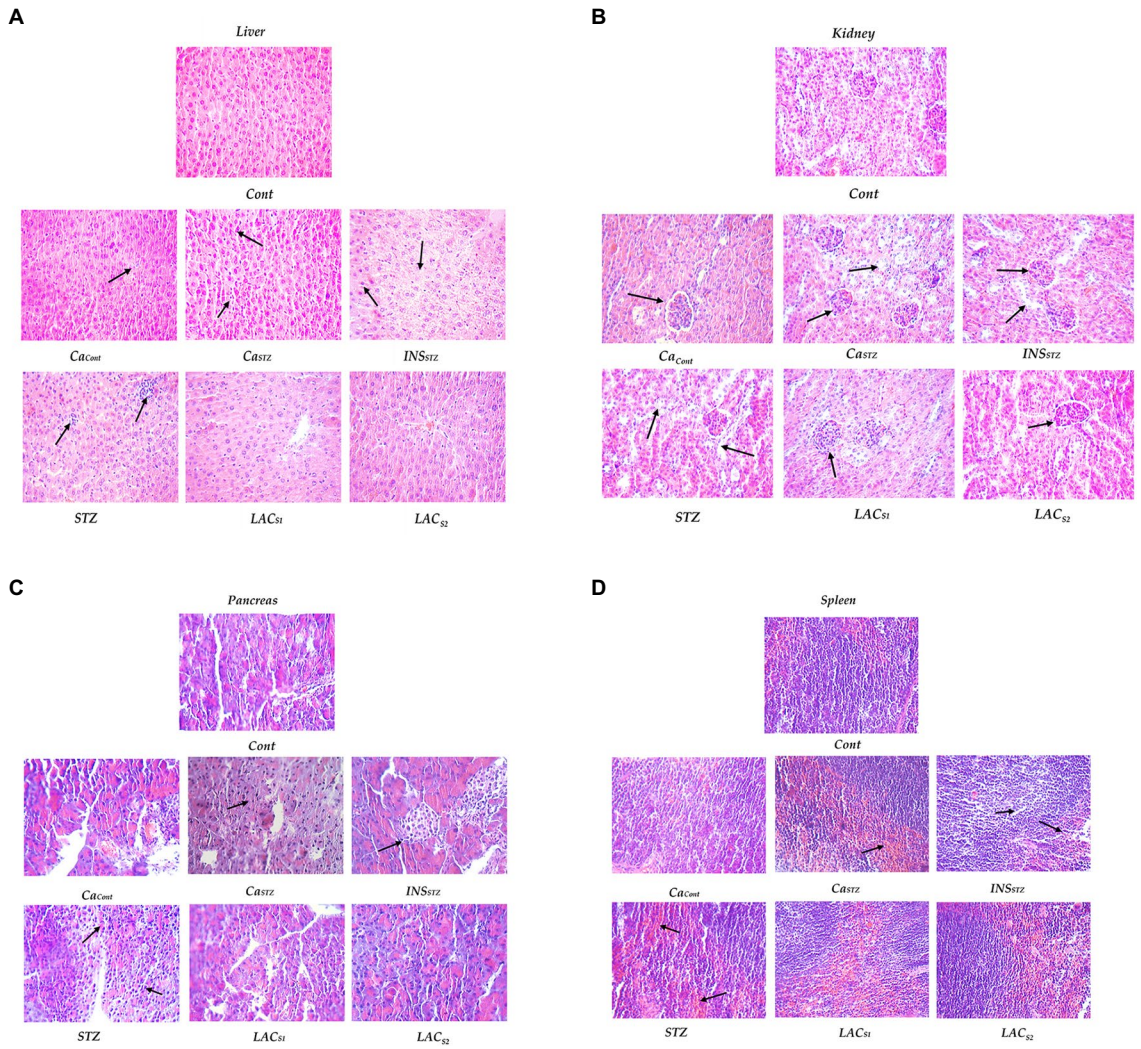

FIGURE 7 | Histopathological evaluation. *Cont*, control; Ca_{Cont}, camel milk control; Ca_{STZ}, camel milk with streptozotocin; INS_{STZ}, insulin with streptozotocin; *STZ*, streptozotocin; LAC_{Si}, $KLDS_{1.0727}$ with streptozotocin; LAC_{S2}, $KLDS_{1.0373}$ with streptozotocin. **(A)** Lever; **(B)**) Kidney; **(C)** Pancreas and **(D)** Spleen.

an alternative bio-resource for some medications like oral diabetes therapy. Furthermore, we recommended a greater emphasis on the creation of dietary items enriched with postbiotics GABA, as well as innovative pharmabiotics applications.

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 *in vivo* experiment was carried out in accordance with the Northeast Agricultural University's institutional animal care and use committee guidelines, as well as the China Ministry of Science and Technology Guide for the Care and Use of Laboratory Animals, under the authorized protocol number specialized pathogen-free rodent management (SRM)-06.

AUTHOR CONTRIBUTIONS

AA and HA-M performed conceptualization, methodology, writing review, and editing. AA, GA, EA, HAJ, and HA-M performed investigation, and formal analysis. XCM performed supervision. All authors contributed to the study's conception and design. All authors contributed to the article and approved the submitted version.

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Efficacy of dietary supplements targeting gut microbiota in the prevention and treatment of gestational diabetes mellitus

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Gestational diabetes mellitus (GDM) is a kind of metabolic disease occurring during gestation period, which often leads to adverse pregnancy outcomes and seriously harms the health of mothers and infants. The pathogenesis of GDM may be bound up with the abnormal gut microbiota composition in pregnant women. Previous studies have clarified that dietary supplements can regulate the gut microbiota to play a role. Therefore, using dietary supplements, such as probiotics, prebiotics, and synbiotics to target the gut microbiota to regulate the disordered gut microbiota would become a potential method that benefits for preventing and treating GDM. This paper reviews a series of clinical trials in recent years, expounds on the clinical effects of dietary supplements such as probiotics on GDM, and discusses the intervention effects of dietary supplements on GDM related risk factors, including overweight, obesity, and type 2 diabetes mellitus (T2DM). In addition, the relationship of GDM and gut microbiota is also discussed, and the possible mechanisms of dietary supplements are summarized. This review will help to promote the further development of dietary supplements targeting gut microbiota and provide more knowledge support for clinical application in the prevention and treatment of various diseases.

KEYWORDS

probiotics, prebiotics, gut microbiota, gestational diabetes mellitus, obesity, type 2 diabetes mellitus

Introduction

Gestational diabetes mellitus (GDM) is discovered in pregnancy firstly, which is a metabolic disorder during pregnancy with impaired glucose tolerance (Baz et al., 2016). The imbalance of gut microbiota is related to the formation of GDM (Hasain et al., 2020). In the past few decades, the prevalence of GDM has been increasing, the prevalence of GDM in the United States is estimated to be 4.6–9.2% (DeSisto et al., 2014), the reported prevalence of GDM in China is 9.3–18.9% (Gao et al., 2019). The global prevalence of hyperglycaemia in pregnancy is 15.8%, and the global prevalence of GDM is 12.8% (Yuen et al., 2019). GDM also leads to long-term risk in pregnant women and infants such as type

2 diabetes mellitus (T2DM; Song et al., 2018), affecting the health of mothers and offspring. A large meta-analysis and systematic review showed that women with a history of GDM had a 10-fold higher risk of developing T2DM, especially in the 5 years after delivery (Vounzoulaki et al., 2020). The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) follow-up studies found that more than 50% of women diagnosed with GDM had impaired glucose tolerance after pregnancy (Lowe et al., 2018). Therefore, with the increasing incidence of GDM and the consequent occurrence of T2DM, GDM needs more attention. GDM has multiple risk factors. Maternal obesity and overweight are essential adverse factors for developing GDM (Poston et al., 2016). Pregnant women's weight gain during pregnancy and pre-pregnancy weight gain are closely related to GDM (Najafi et al., 2019; Lan et al., 2020). Gestational women with a family history of T2DM were more likely to have a higher incidence of GDM (Solomon et al., 1997; Chen et al., 2021). Therefore, it is necessary to find effective interventions to prevent and treat GDM and its related risk factors.

As a pregnancy metabolic disease, GDM is associated with insulin resistance (Baz et al., 2016). The imbalance of gut microbiota is related to the pathogenesis of GDM (Wang et al., 2020). In recent years, the imbalance of gut microbiota is considered a vital reason leading to the occurrence of metabolic diseases (Fan and Pedersen, 2021). Gut microbiota homeostasis can be considered an important part of the balance of the whole metabolic system. Regulating beneficial bacteria and conditional pathogens in the intestine is helpful to improve metabolic function (Lin and Zhang, 2017). Consequently, targeted regulation of gut microbiota to improve metabolism is a possible way (Guevara-Cruz et al., 2019). At present, the main dietary supplements interventions include probiotics, prebiotics, and synbiotics, which can target gut microbiota through various mechanisms, such as improving intestinal barrier function (La Fata et al., 2018) and regulating immunity (Vulevic et al., 2015). Commonly used probiotics include *Lactobacillus* and *Bifidobacterium* (Azad et al., 2018), which are used in various dosage forms. Common prebiotics include galactooligosaccharides (GOS), fructooligosaccharides (FOS), inulin, and some dietary fibers (Gibson et al., 2017).

Recently, the intake of dietary supplements to interfere with diseases by targeting gut microbiota has become a popular research direction. This paper reviews some research status of the efficacy of the above intervention measures in the prevention and treatment of GDM and related diseases, providing more evidence for the clinical application of dietary supplements.

GDM and gut microbiota

In the first and second trimesters, as gestational weeks increase, the fetal demand for nutrients increases, and glucose acquisition from the mother through the placenta becomes the primary source of energy for the fetus (Di Cianni et al., 2003). Maternal plasma glucose levels decrease as pregnancy progresses, with approximately a 10% reduction in fasting plasma glucose (FPG). By mid to late gestation, there is an increase in antagonistic insulin-like substances, such as tumor necrosis factor (TNF), leptin, placental lactogen, estrogen, progesterone, and cortisol (Powe et al., 2019, 2022). Insulin resistance exacerbates β-cell dysfunction, reduced glucose uptake further leads to hyperglycemia, and β-cell overburden requires the production of additional insulin as feedback. As a result of impaired metabolism, glycogen accumulation contributes to this "glucotoxicity" by dysregulating the biochemical pathways that promote β-cell dysfunction (Ashcroft et al., 2017). The sensitivity of pregnant women to insulin decreases as gestational age increases. In order to maintain the normal level of glucose metabolism, insulin requirements must increase accordingly (Di Cianni et al., 2003). However, for women with restricted insulin secretion, the gestation status does not alleviate this metabolic change, so GDM occurs, or the degree of preexisting diabetes increases.

Some studies indicate that gut microbiota participates in the pathogenesis of GDM (Kuang et al., 2017; Wang et al., 2018). The human microbiota plays a crucial role in health. In general, the gut microbiota remains relatively stable during normal human pregnancy (DiGiulio et al., 2015). But it has also been shown that the composition of gut microbiota changes significantly at different stages of pregnancy, such as the decrease of butyrate-producing bacteria, the overall increase of *Proteobacteria* and *Actinobacteria*, the reduction of flora richness and α diversity, and β diversity increasing at the late pregnancy (Koren et al., 2012). A small number of studies looked at the microbiota of GDM patients and showed opposite results, with no differences (Koren et al., 2012). Kuang et al. (2017) found that the abundance of bacteria in GDM patients and healthy controls was different at the genus level. Compared with average blood glucose in pregnant women, the gut microbiota of GDM was abnormal at phylum and genus levels. In the GDM cohort, the abundance of *Actinomycetes* at the phylum level and *Collinella*, *Rothia*, and *Desulfovibrio* at the genus level were higher. These results show that gut microbiota is dysregulated in GDM patients (Crusell et al., 2018). Higher bacterial richness and association with metabolic and inflammatory variables were detected throughout pregnancy in GDM patients (Ferrocino et al., 2018). Compared with normal pregnancies, GDM patients did have an altered gut microbiota composition. This also suggests that the approaches to improving the gut microbiota are potential directions to influence the metabolic-related health of the mother.

With the change of gestational age, on the one hand, the gut microbiota is involved in the physiological adaptation of pregnant women's metabolism; on the other hand, the abnormal composition of the gut microbiota of pregnant women is related to the higher possibility of GDM and is related to macrosomia, premature birth, and other adverse pregnancy outcomes. Therefore, it has been proposed to modify the gut microbiota for adjuvant treatment or prevention of GDM.

Dietary supplements mechanisms of action

Probiotics are defined as, "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Probiotics can directly act on the intestinal mucosal barrier, regulate immune response, thereby increasing glucose tolerance, and may restore the gut microbiota imbalance caused by obesity and diabetes (Gibson et al., 2017). Prebiotics are not digested and absorbed by the host but can promote the metabolism and proliferation of beneficial bacteria in the body selectively and selectively utilized by host microorganisms that confer a health benefit (Marco et al., 2021). They promote the value-added of target flora and improve intestinal microecology by increasing the abundance of probiotics in the intestine (Gibson et al., 2017). Synbiotics are mixture comprising live microorganisms and substrates selectively utilized by host microorganisms that confers a health benefit on the host (Swanson et al., 2020). And postbiotics are defined as, "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021; Figure 1). Most mechanism research on probiotics or prebiotics is based on studies using animals, cell cultures, or *in vitro* human models, some of which are unproven in humans.

Mechanisms of *Lactobacillus* and *Bifidobacterium* specific probiotics are to enhance intestinal epithelial barrier, modulate mucus production, and improve tight junction protein expression (La Fata et al., 2018). Some probiotics may enhance intestinal barrier function by increasing mucus-secreting genes that reduce pathogen-epithelial cell binding (Shen et al., 2018); while factors that improve barrier function also include the downregulation of inflammation (Sanders et al., 2018).

Probiotics also work by promoting the production of short-chain fatty acids (SCFAs) and other small molecular acids. For example, probiotics including *Lactobacillus* and *Bifidobacterium* produce lactic acid and acetic acid as major products of metabolism. SCFAs have particular utility in helping to improve the gut environment. When these SCFAs are made, they lower the pH of the lumen and prevent the pathogen growth (Ríos-Covián et al., 2016). SCFAs can activate cells through the surface of cells G protein–coupled receptors (GPR). For example, SCFAs activate GPR41 and GPR43 on intestinal epithelial cells (Brown et al., 2003). SCFAs and GPR41/43 promote acute inflammatory responses in the intestine for tissue inflammation and protective immunity (Kim et al., 2013). SCFAs also regulate glucose and lipid metabolism through GPR41/43. Most SCFAs are absorbed by colon cells and liver as their energy source, while others are metabolized by muscle and adipose tissue (van der Beek et al., 2015). Prebiotics is commonly consumed to improve health by stimulating the growth of beneficial bacteria and the production of SCFAs. One study analyzed the effects of five widely used prebiotic fibers on beneficial bacteria and SCFAs, and found intaking prebiotic fibers promoted the formation of beneficial SCFAs (Carlson et al., 2017).

Some probiotics have been shown to interact directly with dendritic cells, and increase the activity of phagocytic or natural killer cells (Klaenhammer et al., 2012). Levels of anti-inflammatory cytokines can be increased by supplementation with probiotics (Klaenhammer et al., 2012). After prebiotics supplementation, pro-inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) decreased, and anti-inflammatory cytokines interleukin-10 (IL-10) raised (Vulevic et al., 2015). In addition, a randomized placebo-controlled trial of *Lactobacillus rhamnosus* HN001, found it had a significant protective effect against eczema development at 2–6 years of age while preventing allergic sensitization when children were 6 years of age (Wickens et al., 2018).

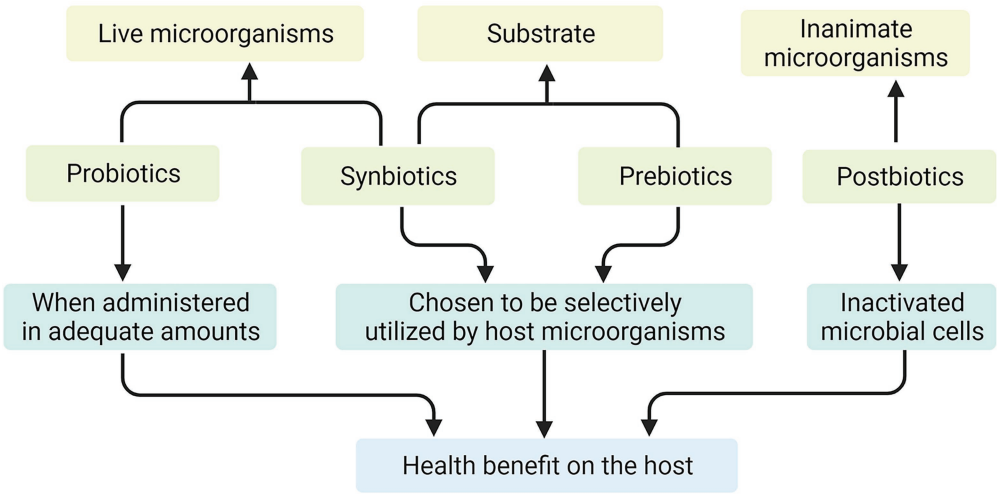

FIGURE 1
The mechanisms of action of probiotics, prebiotics, synbiotics, and postbiotics.

Prebiotics may also affect satiety healthy. Some SCFAs produced by gut fermentation influence free fatty acid receptor 2 (FFAR2). In addition, SCFAs also regulate the lipolysis and the release of incretin glucagon-like peptide-1 (GLP-1; Bolognini et al., 2019). According to an animal study, SCFAs acid acetate formed by prebiotics goes through the blood–brain barrier and into the hypothalamus, stimulating anorexia signaling and playing a direct role in central appetite regulation (Frost et al., 2014). Several studies have shown that SCFAs acted on colonic epithelial cells to induce the production of anorexia hormones peptide-YY (PYY) and GLP-1 (Christiansen et al., 2018).

Since prebiotics are often indigestible dietary fiber, they are also beneficial for improving gut function. Improvements in fecal characteristics, including defecation frequency and fecal viscosity, have also been observed with prebiotics or probiotics in several randomized controlled trials (Ford et al., 2014; Micka et al., 2017). It has also been observed in animal experiments that SCFAs produced by prebiotic fermentation, such as butyrate and propionate regulate intestinal peristalsis and contraction at different speeds (Hurst et al., 2014). The prebiotic inulin also improves constipation and softens stools (Micka et al., 2017).

Probiotics work through a variety of means, including improving the functional integrity of the gut barrier, producing SCFAs, and regulating the immune function (Figure 2). In addition, they also increase satiety and reduce weight. The improvement of intestinal function and constipation also has certain benefits. The use of dietary supplements as an intervention may be a promising approach. The possible mechanisms of action of probiotics in GDM are summarized in Figure 3.

Prevention and treatment effects of dietary supplements on GDM

Prevention effects on GDM

Dietary supplements play a role in the prevention and treatment of GDM by regulating the gut microbiota of pregnant women. Probiotics are still controversial in preventing GDM and reducing the incidence of GDM in pregnant women. Wickens et al. (2017) reported in the clinical trial of normal-weight pregnant women that daily supplementation of *L. rhamnosus* HN001 (6×10^9 CFU) during 14–16 weeks of gestation reduced the incidence and recurrence rate of GDM, especially in elderly pregnant women and people who have a history of GDM.

However, the effect of probiotics intervention was inconsistent among high-risk pregnant women, such as overweight and obesity. Shahriari et al. (2021) finally analyzed 507 elderly obese pregnant women taking probiotics between 14 and 24 weeks of gestation. The results showed that the incidence of GDM was 41.9% in the probiotic group and 40.2% in the placebo group, and the difference was not statistically significant ($p = 0.780$). Supplementation of probiotics for a certain period in the second trimester did not decrease the incidence of GDM (Shahriari et al., 2021). A study by Callaway et al. (2019) compared the results of probiotics and placebo groups in obese and overweight pregnant women revealed probiotics containing *Bifidobacterium lactis* and *L. rhamnosus* were not very good at preventing GDM. Similarly, Pellonperä et al. (2019) evaluated whether daily use of fish oil and probiotic supplements in overweight and obese pregnant reduced the risk of GDM and improved the glucose metabolism. It turned out that there was no positive reduction (Pellonperä et al., 2019; Table 1). Therefore, weight and other high-risk pregnancy conditions are the critical factors affecting the preventive effect of probiotics on GDM. Meanwhile, some studies have found changes in the relative abundance of bacterial species during pregnancy in the overweight and obese pregnant women without GDM, suggesting that this population gain more benefit from the dietary regulation of gut microbiota (Mokkala et al., 2021). The presence of GDM may interfere with the flexibility of mother gut microbiota, therefore limiting the ability of GDM patients' feedback to dietary regulation. Thus, the clinical application of probiotics in high-risk pregnant women needs to be further studied.

In brief, whether probiotics prevent GDM in pregnant women remains controversial. For normal-weight pregnant women, certain dietary supplements effectively reduce the risk of GDM. Still, the intervention effect is limited for overweight and obese pregnant women, and body weight is a factor affecting the prevention effect of probiotics. The presence of GDM also interferes with the gut microbiota, reducing the sensitivity of dietary supplements to intervention. The research about the intervention of dietary supplements on intestinal microecology during pregnancy is still in the initial stage and need further improved.

Treatment of GDM

Treatment effects of probiotics on GDM

Some studies suggest that probiotics supplementation in pregnant women with GDM during the second and third trimesters reduces FPG and increases insulin sensitivity, which may be an adjunct therapy to control GDM. Fifty-seven pregnant women with GDM were enrolled at the second trimester of gestation and randomized to intake probiotic supplements containing *Bifidobacteria* and *Lactobacillus* or placebo, the probiotic group showed the benefits of glucose metabolism, including FPG, fasting insulin, and insulin resistance (Kijmanawat et al., 2019). Babadi et al. (2019) conducted a trial investigating the effect of probiotic supplementation on cytokine expression and metabolism in 48 GDM patients. After 6 weeks of continuous intervention, the results showed beneficial effects on the expression of insulin and inflammatory factors, glucose control, lipid metabolism indicators, inflammatory markers, and oxidative stress (Babadi et al., 2019). Similarly, a clinical trial was completed among 60 subjects with GDM. Subjects were randomly allocated to intake probiotic capsules or placebo. And they found probiotic supplements had beneficial effects on FPG,

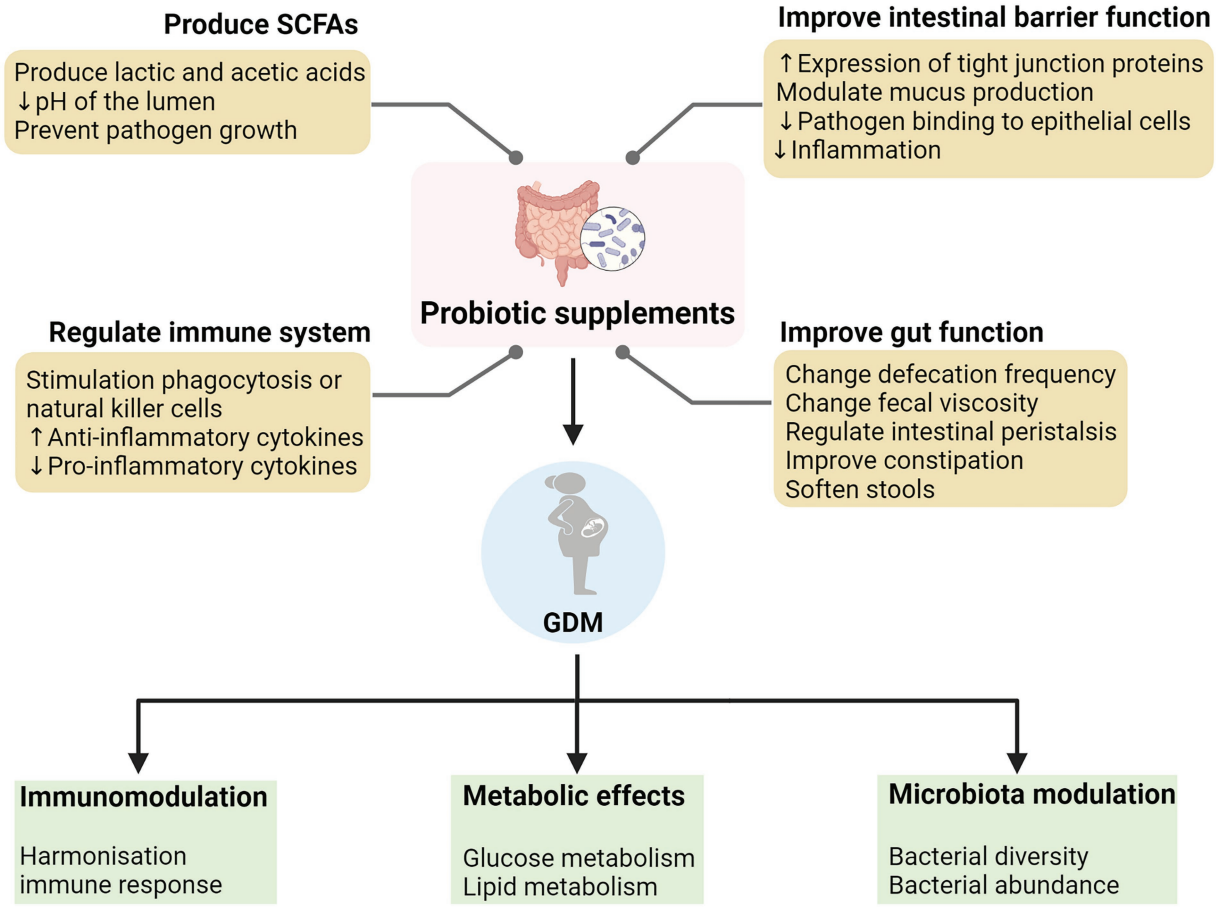

FIGURE 2
The mechanisms of action of probiotic supplements and its role in alleviating the pathology of gestational diabetes mellitus (GDM).

high-sensitivity C-reactive protein (hs-CRP), total antioxidant capacity (TAC), malondialdehyde (MDA), and oxidative stress index (Badehnoosh et al., 2018). Karamali et al. (2016) studied the effects of probiotics intake on blood glucose control and blood lipid levels in GDM women. The results showed that probiotics have beneficial effects on glycaemic control, triglycerides, and very low-density lipoprotein cholesterol (VLDL-C) concentrations (Karamali et al., 2016). A study also examined the effects of probiotics on weight gain and glucose metabolism in GDM. Compared with the placebo group, the probiotics intervention group reduced the fasting blood glucose, weight gain also decreased during the last 2 weeks of the study (Dolatkhah et al., 2015). However, a clinical trial involving 149 GDM patients had no effect on glycemic control after probiotics intervention (Lindsay et al., 2015). Probiotics play a role in immune response and inflammatory regulation. About 82 women diagnosed with GDM were randomly assigned to receive probiotics or placebo for 8 weeks. Women with GDM who received probiotics had significantly reduced levels of TNF-α, hs-CRP, and IL-6 (Jafarnejad et al., 2016; Table 1).

In summary, probiotics improve glucose metabolism, lipid metabolism, and inflammatory markers in GDM. There are also several differences between different studies. Most studies used probiotics containing two or more strains. However, Lindsay et al. used a single strain (*Lactobacillus salivarius* UCC118; Lindsay et al., 2015). The dosage of probiotics was also different, 2×10^9 CFU/g was a commonly used dose. The populations included in these studies ranged from a few dozen to more than 100. The length of intervention time also affects the intervention effects of probiotics. The duration of intervention in these studies was at least 6–8 weeks. Some confounding factors, such as diet patterns and physical activity, also influence the results. In the future, more clinical trials are needed to investigate the efficacy of probiotics in the treatment of GDM.

Treatment effects of synbiotics on GDM

Synbiotics have some disputes on insulin metabolism or lipid metabolism in GDM pregnant women. In a study, 90 pregnant women with GDM were supplemented with synbiotics containing *Lactobacillus* and fructooligosaccharides. Compared with the placebo group, FPG, insulin resistance index, insulin sensitivity, lipid mass spectrometry, and TAC in the synbiotics supplemented group had no significant changes ($p > 0.05$; Nabhani et al., 2018). The difference is that Ahmadi et al. and

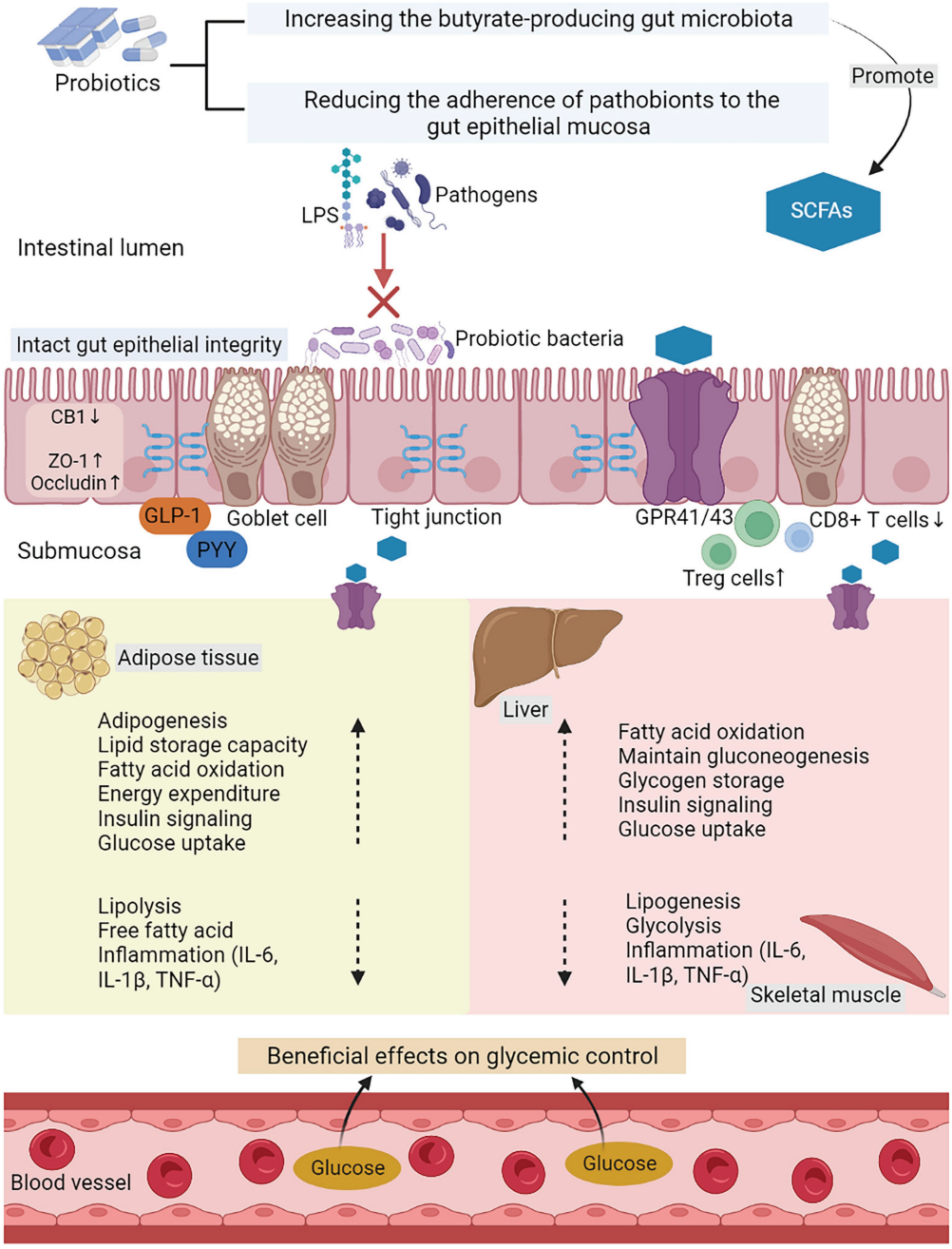

FIGURE 3
The possible mechanisms of action of probiotics in GDM. LPS, lipopolysaccharide; SCFAs, short-chain fatty acids; GPR 41/43, G-protein-linked receptor 41/43; CB1, cannabinoid receptor 1; ZO-1, zona occludens 1; GLP-1, glucagon like peptide-1; PYY, peptide YY; Treg cells, regulatory T cells; IL-6, interleukin-6; IL-1β, interleukin-1β; and TNF-α, tumor necrosis factor-α.

others took synbiotics supplements containing multiple strains and inulin in patients with GDM for 6 weeks, which had beneficial effects on insulin metabolism and VLDL-C (Ahmadi et al., 2016; Table 1).

In short, there are different conclusions in the studies on the intervention of synbiotics on GDM, and different strain combinations and doses may be the possible reasons for the difference. Clinical studies on different combination preparations are expected. The main improvement effects of dietary supplements on GDM are summarized in Figure 4.

Dietary supplements and existing treatment strategies for GDM

The current treatment for GDM is to reverse hyperglycemia and reduce the risk of adverse pregnancy outcomes. An important part of gestational diabetes management is lifestyle interventions

TABLE 1 Clinical efficacy of dietary supplements on GDM.

Supplements	Doses	Duration	Sample size	Target disease	Main effects	References
Probiotics						
Lactobacillus rhamnosus HN001	6×10^9 CFU	14–16 weeks' gestation	423	Gestational diabetes mellitus	Reduce the incidence and recurrence rate of GDM.	Wickens et al., 2017
Lactobacillus acidophilus LA1, *Bifidobacterium longum* sp54 cs, and *Bifidobacterium bifidum* sp9 cs	1.5×10^{10} CFU	14–24 weeks' gestation	542	Gestational diabetes mellitus	Could not reduce the incidence of GDM.	Shahriari et al., 2021
Bifidobacterium lactis and *Lactobacillus rhamnosus*	1×10^9 CFU	16–28 weeks' gestation	411	Gestational diabetes mellitus	GDM cannot be prevented.	Callaway et al., 2019
Lactobacillus rhamnosus HN001 and *Bifidobacterium animalis* ssp. lactis 420	1×10^{10} CFU	14 weeks' gestation~6 months postpartum	439	Gestational diabetes mellitus	It could not reduce the incidence of GDM or improve glucose metabolism.	Pellonperä et al., 2019
Bifidobacterium bifidum and *Lactobacillus*	2×10^9 CFU	24–28 weeks' gestation	57	Gestational diabetes mellitus	Significantly improved glucose metabolism, including FPG fasting insulin, and insulin resistance.	Kijmanawat et al., 2019
Lactobacillus acidophilus, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum*	2×10^9 CFU/g	6 weeks	48	Gestational diabetes mellitus	It has beneficial effects on the expression of insulin and inflammation-related factors, control of blood glucose, lipid metabolism, inflammatory markers, and oxidative stress.	Babadi et al., 2019
Lactobacillus acidophilus, *Lactobacillus casei*, and *Bifidobacterium bifidum*	2×10^9 CFU/g	6 weeks	60	Gestational diabetes mellitus	Beneficial effects on FPG, hs-CRP, TAC, MDA, and oxidative stress index.	Badehnoosh et al., 2018
Lactobacillus acidophilus, *Lactobacillus casei*, and *Bifidobacterium bifidum*	2×10^9 CFU/g	6 weeks	60	Gestational diabetes mellitus	Beneficial effects on glycaemic control, triglycerides, and VLDL cholesterol concentrations.	Karamali et al., 2016
Lactobacillus acidophilus LA-5, *Bifidobacterium* BB-12, *Streptococcus thermophilus* STY-31, and *Lactobacillus delbrueckii bulgaricus* LBY-27	4×10^9 CFU	8 weeks	64	Gestational diabetes mellitus	Affect glucose metabolism and weight gain.	Dolatkhah et al., 2015
Lactobacillus salivarius UCC118	1×10^9 CFU	From GDM diagnosis until delivery	149	Gestational diabetes mellitus	Had no impact on glycemic control.	Lindsay et al., 2015
Streptococcus thermophilus, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus delbrueckii* subsp. Bulgaricus	112.5×10^9 CFU	8 weeks	82	Gestational diabetes mellitus	May help to modulate inflammatory markers and may have benefits on glycemic control.	Jafarnejad et al., 2016
Synbiotics						
Lactobacillus probiotic strains consisting of *L. acidophilus, L. plantarum, L. fermentum,* and *L. gasseri* plus fructooligosaccharide	$1.5–7.0 \times 10^{9–10}$ CFU/g and 38.5 mg	6 weeks	90	Gestational diabetes mellitus	There were no significant changes in TAC, FPG, and insulin resistance/sensitivity index.	Nabhani et al., 2018
Lactobacillus acidophilus, *Lactobacillus casei*, and *Bifidobacterium bifidum* plus inulin	2×10^9 CFU/g and 800 mg	6 weeks	70	Gestational diabetes mellitus	Beneficial effects on insulin metabolism, TAG, and VLDL-C.	Ahmadi et al., 2016

including healthy eating, physical activity, weight management, and self-monitoring of blood sugar levels. A Cochrane study evaluated the effect of lifestyle interventions on women with GDM. Exposure to lifestyle interventions reduced the risk of large for gestational age infants (RR 0.60, 95% CI 0.50–0.71). Lifestyle interventions were associated with a reduced risk of postpartum depression in women (RR 0.49, 95% CI 0.31–0.78; Brown et al., 2017). Other studies have shown that specific dietary interventions have a positive impact on maternal blood glucose, fasting blood glucose, and postprandial blood glucose have varying degrees of decline (Yamamoto et al., 2018).

If the lifestyle intervention cannot achieve the goal of blood glucose control, pharmacotherapy is used. Insulin injection therapy is a common method. Metformin and glibenclamide are also used as oral treatment for GDM. A randomized controlled trial was conducted to study the effect of glibenclamide or insulin combined with metformin in the treatment of gestational diabetes. It was found that the combination of metformin and insulin was superior to the combination of metformin and glibenclamide in the control of blood glucose (Reynolds et al., 2017). Affres et al. (2021) showed that glibenclamide was an effective treatment for women with GDM to achieve blood glucose targets during pregnancy. When glibenclamide was not well controlled, blood glucose control was improved by switching to insulin (Affres et al., 2021). Studies of the long-term effects of pharmacological therapy on mothers and fetuses are less clear.

In recent years, the relationship between GDM and diet has promoted the study of dietary supplements as a potential prevention and treatment strategy for GDM. Dietary supplements are well tolerated, safe and easy to administer. Dietary supplements such as probiotics and prebiotics usually produce effect by targeting the gut microbiota. Eating food rich in prebiotic fiber along with fermented foods promotes the growth of bacteria that break down plant starches and fibers into SCFAs. SCFAs may impact the expression of appetite regulation hormones, such as GLP-1 (Tilg and Moschen, 2015). In the second and third trimester of pregnancy, the use of probiotic supplements in women with gestational diabetes decreased fasting blood glucose and increased insulin sensitivity (Kijmanawat et al., 2019). Larger, longer trials of different probiotic strains are needed.

Effects of dietary supplements on GDM related risk factors

Overweight and obesity

Pre-pregnancy overweight and obesity are linked to increased GDM risk (Li et al., 2020). A cohort study based on an Australian population also assessed the profound influence of overweight and obesity of GDM, and the results were consistent (Black et al., 2022). Moreover, the increase of BMI before pregnancy is also related to maternal and infant adverse outcomes, such as the risks of large for gestational age (LGA), macrosomia, and cesarean delivery (Zheng et al., 2022). It is meant to improve the overweight and obesity status through dietary supplements before pregnancy. Meanwhile, obesity is closely related to gut microbiota. Changes in gut microbiota are observed in obese people.

Regulating beneficial flora through specific probiotics may be a potential treatment option for obesity (Balakumar et al., 2018). An 8-week clinical trial of 101 obese adolescents showed

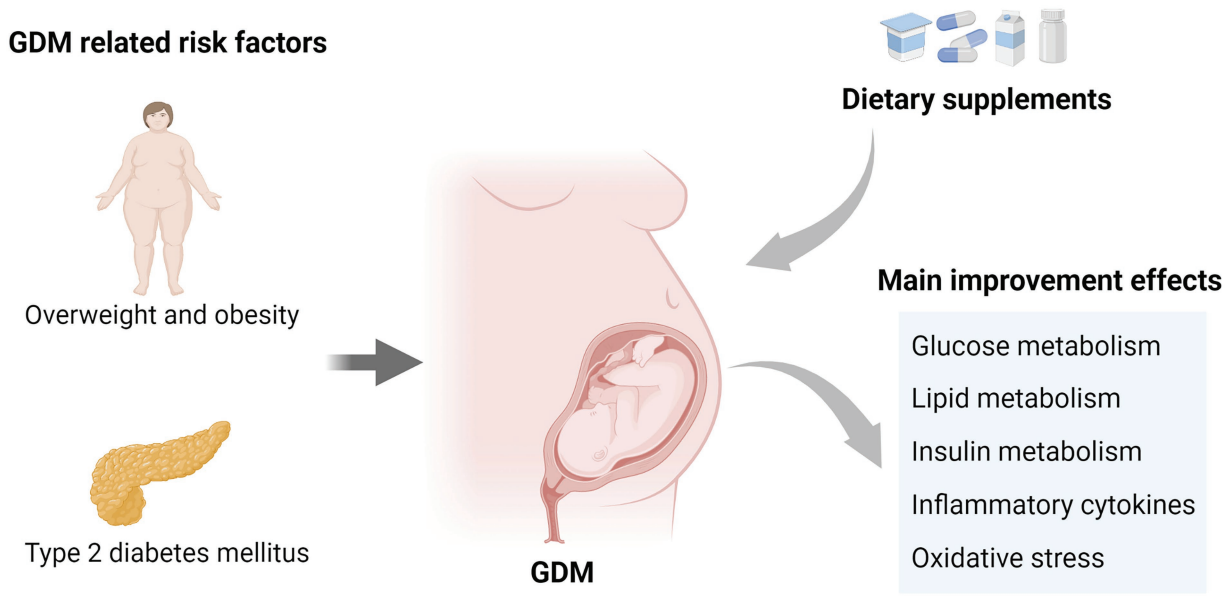

FIGURE 4
GDM related risk factors and the main improvement effects of dietary supplements on GDM.

that the daily intake contained 2×10^9 CFU/AFU *Bifidobacterium breve* BR03 and B632 improved metabolic indexes, insulin sensitivity, reduced body weight, and the number of *Escherichia coli* (Solito et al., 2021). In addition, in a study in Indonesia, 60 overweight adults were fed probiotic powder containing *Lactobacillus plantarum* Dad-13 for 90 days. The results revealed that compared with the placebo group, the weight and body mass index (BMI) of the intervention group significantly decreased, and amounts of *Bacteroidetes*, especially *Prevotella*, increased significantly, while the number of *Firmicutes* decreased significantly (Rahayu et al., 2021). The commonly used probiotics include *Lactobacillus* and *Bifidobacterium* strains. The combination of multiple strains may have an excellent clinical prospect in order to study the recovery of gut microbiota with probiotics, which is a possible clinical target for the treatment of flora-related diseases, such as obesity. Gomes et al. (2020) conducted cluster analysis on the subjects according to their body composition after taking probiotic mixture into 32 overweight or obese women. They found that the proportion of TM_7 in obese women was higher. At the same time, after the intervention, *Clostridiaceae* increased, and TM_7 bacteria tended to decrease. Intervention specific bacteria such as TM_7 bacteria are considered as a new target method for the treatment of obesity (Gomes et al., 2020).

Prebiotic intake also improves overweight and obesity and its adverse effects. A clinical trial of 150 obese people showed that the daily intake of 16 g inulin for 3 months reduced weight to a greater extent than the placebo group by regulating specific flora, and reducing diastolic blood pressure (DBP), aspartate aminotransferase (AST), and insulinemia (Hiel et al., 2020). Reimer et al. conducted a 12 weeks study on overweight or obese people. They found fructooligosaccharides inulin and whey protein was helpful to regulate appetite, but fructooligosaccharides increased the abundance of *Bifidobacterium* (Reimer et al., 2017). Obesity often leads to emotional disorders. Research scholars analyzed the changes in gut microbiota and their effects on emotion and cognition in 106 obese patients treated with prebiotic inulin and placebo. The results showed that inulin supplementation had moderate beneficial effects on emotional ability and cognitive flexibility. In particular, patients with higher levels of *Coprococcus* at baseline were more likely to benefit from prebiotic supplements in terms of mood (Leyrolle et al., 2021). Thus, people with specific microbial characteristics or intestinal types will be more sensitive to prebiotics. In addition, recent evidence suggests that increasing colonic and plasma concentrations of SCFAs protect against obesity and obesity-induced insulin resistance, which is a novel approach (Canfora et al., 2015). Gut microbiota ferment prebiotics, such as inulin into acetate, propionate, and butyrate. In one study, 14 overweight and obese men were given inulin preparation, and the acute metabolic effects after a single dose of 24 g were tested. The results showed that the intake of prebiotic inulin improved fat oxidation, increased the level of metabolites of gut microbiota, and promoted the production of SCFAs (van der Beek et al., 2018).

The use of prebiotics or probiotics alone probably has a better improvement effect, but the synergistic effect of combined use of synbiotics is not clear. Some researchers think that synbiotics are more effective than probiotics alone in correcting the destruction of gut microbiota caused by obesity. Sergeev et al. (2020) supplemented synbiotics to 20 obese patients participating in the weight loss program for 3 consecutive months. As a result, the abundance of intestinal bacteria that have a positive impact on health was increased, especially *Bifidobacteria* and *Lactobacillus*. Synbiotics supplements can regulate human intestinal microorganisms by increasing the abundance of potentially beneficial bacteria (Sergeev et al., 2020). However, Krumbeck et al. compared the effects of prebiotic GOS and probiotic *Bifidobacterium* IVS-1 and *B. lactis* BB-12 when used alone and as synbiotics. The results showed that single-use improved colonic permeability, but there was no synergistic effect when used together (Krumbeck et al., 2018).

In summary, regulating the microbiota through the ingestion of specific probiotics is a new target for treating obesity, regulating the growth of beneficial bacteria while reducing the number of harmful bacteria currently, *Bifidobacteria* and *Lactobacillus* are the most commonly used probiotics. Compared with non-obese people, obese people have different intestinal types and are more sensitive to intervention. The composition of bacteria in obese people is different from that in non-obese people, and regulation of these differences may be a therapeutic direction. Prebiotics improved the adverse symptoms of overweight and obesity. They not only reduce weight by regulating appetite but also reduce blood pressure, improve the negative mood caused by obesity, and improve cognitive performance. Further studies are needed on the synergistic effects of probiotics and probiotics, and strains, dosage forms, and formulations can be enhanced to optimize the combination of these dietary supplements.

Type 2 diabetes mellitus

Type 2 diabetes mellitus is relative insulin deficiency owing to β-cell dysfunction and insulin resistance (Eizirik et al., 2020). In the GDM population, the risk of subsequent development of T2DM is several times higher than those people with average blood glucose (Vounzoulaki et al., 2020). Pregnant women with type 1 and type 2 diabetes are more possibly to get adverse pregnancy outcomes (Murphy et al., 2021). The risk of such diseases also shows the importance of T2DM intervention and prevention (Vounzoulaki et al., 2020).

Type 2 diabetes mellitus is associated with gut microbiota. T2DM can be effectively managed through the regulation of gut microbiota by probiotics. Firouzi et al. randomized 136 T2DM patients to receive probiotics or placebo and found that glycosylated hemoglobin A1c (HbA1c) in the probiotics group decreased by 0.14%, while that in the placebo group increased by 0.02% ($p < 0.05$), fasting insulin decreased by 2.9 U/ml in the probiotic group and increased by 1.8 U/ml in the placebo group

($p < 0.05$), probiotics supplementation modestly improved fasting insulin and HbA1c (Firouzi et al., 2017). In addition, a study of 68 patients with T2DM confirmed that specific *Lactobacillus reuteri* strains reduced HbA1c and cholesterol by adjusting the abundance of *Bacteroides* and *Bifidobacteria*, and ADR-3 strain is superior to ADR-1 strain in terms of antihypertensive effect and reducing the number of *Firmicutes* (Hsieh et al., 2018).

Previous studies have shown that dietary fiber, lifestyle intervention, and hypoglycemic drugs metformin have been shown to reduce the incidence rate of T2DM (Wu et al., 2017; Zhao et al., 2018). The effectiveness of these interventions is enhanced by regulating the gut microbiota. A randomized controlled study by Palacios et al. studied 60 adults with BMI $\geq 25\ kg/m^2$ and who had diabetes prodromal stage or T2DM in the past 12 months. Participants received a multi-strain probiotic or placebo intervention. The primary and secondary outcome indicators were analyzed at baseline and 12 weeks after the intervention, and the fecal microbiota was analyzed by macrogenomic analysis. The results exhibited that for the subgroup taking metformin, FPG, HbA1c, and insulin resistance decreased, and plasma butyrate concentration increased; Compared with the placebo group, the microbial butyric acid production pathway was abundant in the probiotic group. Therefore, probiotics can be used as an adjuvant of metformin by increasing the production of butyrate, which enhance the management of T2DM (Palacios et al., 2020).

Prebiotics also improve T2DM by regulating gut microbiota disorder. Around 46 T2DM patients showed that 10 g of inulin rich in fructooligosaccharides per day for 2 months benefit for blood glucose, blood lipid levels, also including immune indicators (Dehghan et al., 2016). Fructooligosaccharides inulin also has beneficial effects on improving the level of metabolites of gut microbiota. Birkeland et al. studied 25 T2DM patients supplemented with fructooligosaccharides inulin for 6 weeks. Prebiotics had significant bifurcation and increase fecal SCFAs concentration but did not change the diversity of fecal flora (Birkeland et al., 2020). In addition, more and more attention has been paid to the intervention effect of synbiotic supplements on T2DM patients. Kanazawa et al. recruited 88 obese patients with T2DM and supplemented synbiotics for 24 weeks. The results revealed that *Bifidobacteria* and *Lactobacillus* increased after administration, the relative abundance of *Bifidobacteria* increased, as well as the concentration of acetic acid and butyric acid in feces. However, the inflammatory marker IL-6 did not change much. Thus, synbiotics administration at least partially improves the intestinal environment of obese T2DM patients (Kanazawa et al., 2021).

In short, probiotics regulate the gut microbiota to alleviate the adverse effects of T2DM, such as lowering the biochemical parameters related to glucose metabolism. Different strains have different intervention effects, and some strains show better effects in regulating the flora and improving the phenotype. Probiotics enhance the effectiveness of hypoglycemic drugs and exercise interventions, and the mechanism may be the increase of microbial butyric acid production after probiotics intervention to assist metformin treatment. Prebiotic supplements containing fructooligosaccharides inulin affect glucose and lipid status and immune regulation in T2DM patients. Synbiotics supplementation also improved the adverse intestinal environment and increases the content of beneficial bacteria. Therefore, probiotics, prebiotics, and their components alleviate or improve T2DM to some extent. Still, the specific efficacy of these interventions on diabetes needs to be further studied in clinical trials.

Conclusion and prospects

Gestational diabetes mellitus is nearly related to the disorder of gut microbiota. Although the role of probiotics, prebiotics, and synbiotics in the prevention of GDM is controversial, several studies suggest that dietary supplements have certain effect in reducing GDM (Wickens et al., 2017). In contrast, other studies prove no benefit (Callaway et al., 2019; Pellonperä et al., 2019; Shahriari et al., 2021). The differences are related to several factors, such as the timing of dietary supplements intervention, differences in probiotic strains, and the level of supplement dosage. Furthermore, the BMI of pregnant women affect whether pregnant women develop GDM and the role of targeting gut microbiota, specific populations benefit more from the current intervention, and the presence or absence of GDM status affects the sensitivity of intervention (Mokkala et al., 2021). For pregnant women with GDM, continued use of dietary supplements also delay the adverse progression of GDM and reduce blood glucose (Kijmanawat et al., 2019). However, dietary supplements also have some beneficial effects on GDM, including improving glucose metabolism, insulin metabolism, and lipid levels. Meanwhile, due to the limited number of trials and sample population, more studies on the pregnancy population are needed in the future.

At the same time, this paper also reviewed the intervention effects of dietary supplements on GDM related risk factors, such as for overweight, obesity, and T2DM. Prebiotics reduce weight by increasing satiation and reducing appetite (Bolognini et al., 2019), and partially improve glucose metabolism indicators of T2DM. It suggests that we should pay attention to not only the prevention and treatment of GDM but also the improvement of overweight and obesity before pregnancy, and intervention measures should be taken to prevent of T2DM in GDM population. In recent years, most studies have focused on probiotics and prebiotics effect of GDM, obesity, and T2DM. Some studies have also been carried out on neurological disorders such as Alzheimer's disease and major depressive disorder (Rudzki et al., 2019; Tamtaji et al., 2019), and digestive diseases such as nonalcoholic fatty liver disease (Bakhshimoghaddam et al., 2018). Dietary supplements have more or less improved the clinical symptoms of these diseases. In the future, researchers should invest more in researching

other conditions, such as digestive system metabolic diseases, allergic diseases, cardiovascular diseases, and so on. Studies on the targeting of probiotics, prebiotics, and other related gut microbiota are still in progress, and a large number of clinical trials are needed in the future.

Several clinical trials indicate regulating the composition of gut microbiota is the direct mechanism for improving a variety of diseases. The overall performance is to increase the abundance of *Bifidobacteria*, *Lactobacillus*, *Bacteroides*, *Prevotella*, and *Clostridium*, and regulate the number of *E. coli*, *Firmicum*, and TM_7 bacteria to decrease. In addition, probiotics and prebiotics also indirectly improve such diseases by regulating gut microbiota metabolites SCFAs, such as acetic acid, propionic acid, and butyric acid. Increasing intestinal permeability also plays a vital role in improving the overall metabolic system by improving intestinal barrier function. Previous studies have shown that dietary supplements work through various pathways, including improving intestinal barrier function by producing SCFAs and regulating immune function. Further cell and animal experiments are needed to explore the in-depth mechanism of gut microbiota and their regulation of signaling pathways to target diseases and play a beneficial role.

Dietary supplements intervention is a promising strategy with beneficial effects on the gut microbiome. Several different strains of probiotics, particularly *Lactobacillus* and *Bifidobacteria*, or prebiotics that modulate beneficial bacteria, have been shown to improve relevant indicators, demonstrating the importance of dietary supplements for disease prevention and symptom improvement. Due to the beneficial effect of dietary supplements intervention in improving a variety of diseases, the combined intervention effect of multiple strains is better. New processing methods such as the development of enzyme-modified prebiotics and probiotics to enhance beneficial effects, as well as the optimization of the formulation of biogenic preparations, are also worthy of further development to achieve optimal therapeutic effects.

Author contributions

JW conceived and drafted the manuscript and prepared tables and figures. JM reviewed and modified 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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Protective effect of *Limosilactobacillus fermentum* HFY06 on dextran sulfate sodium-induced colitis in mice

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Ulcerative colitis is one of the main gastrointestinal diseases that threaten human health. This study investigated the effect of *Limosilactobacillus fermentum* HFY06 (LF-HFY06) on dextran sulfate sodium (DSS)-induced murine colitis. The protective effect of LF-HFY06 was evaluated by examining the length and histopathological sections of colon, related biochemical indicators, and genes related to inflammation. Direct and microscopic observations showed that LF-HFY06 increased the length of the colon and ameliorated the pathological damage induced by DSS. The biochemical indicators showed that LF-HFY06 enhanced the activities of antioxidant enzymes total superoxide dismutase (T-SOD) and catalase (CAT) in serum, while reducing the level of malondialdehyde (MDA). It was also observed that the serum inflammatory cytokines levels of tumor necrosis factor-α (TNF-α), interferon (IFN)-γ, interleukin (IL)-1β, IL-6, and IL-12 were decreased, and the anti-inflammatory cytokine IL-10 level was increased. The qPCR experiment revealed that LF-HFY06 downregulated the mRNA expression levels of nuclear factor-κB-p65 (*Rela*), *Tnf*, *Il 1b*, *Il 6*, and prostaglandin-endoperoxide synthase 2 (*Ptgs2*) in colon tissues, and upregulated the mRNA expression of NF-κB inhibitor-α (*Nfkbia*) and *Il 10*. These data indicated that LF-HFY06 inhibited inflammation through the NF-κB signaling pathway to prevent the occurrence and development of colitis. This research demonstrates that probiotics LF-HFY06 have the potential to prevent and treat colitis.

KEYWORDS

***Lactobacillus fermentum*, dextran sulfate sodium (DSS), colitis, inflammation, probiotics**

Introduction

Ulcerative colitis is a non-specific inflammatory disease primarily involving the rectum, colonic mucosa, and submucosa (Feuerstein et al., 2019). The specific cause of ulcerative colitis is not completely clear at present, and the main factors affecting its pathogenesis are genetic and environmental factors, immunity, and gut microbes (Kaplan, 2015; Liu and Stappenbeck, 2016). Although the incidence of inflammatory bowel disease (IBD) in North America and Europe has stabilized in recent years, its incidence has been rising in the newly industrialized countries of Africa, Asia, and South America (Seyedian et al., 2019; Mak et al., 2020). The first-line drugs currently used in clinical are mainly 5-aminosalicylic acids, glucocorticoids, biological agents, and immunosuppression. However, the above-mentioned drugs have the problems of easy recurrence after drug withdrawal or toxic and side effects caused by long-term use (Damião et al., 2019; Cohen and Weisshof, 2020). Thus, it is very important to study new therapeutical alternatives for the prevention and treatment of IBD. In this context, probiotic microorganisms have been explored as an alternative therapeutic approach against intestinal inflammation (Jang et al., 2019; Hrdı et al., 2020; Barroso et al., 2022).

Probiotics as living microorganisms provide a health benefit to the host by regulating the intestinal microbiota (Juarez et al., 2013), inhibiting the colonization of pathogenic bacteria, regulating immunity, and secreting antibacterial substances (Kostic et al., 2014; Sonnenburg and Bäckhed, 2016; Bron et al., 2017; Shen et al., 2018). The most common probiotics are *Limosilactobacillus* and *Bifidobacterium* (Lee et al., 2018; Robertson et al., 2020; Yadav et al., 2022). A lot of research shows that the probiotics that can be used for the prevention and treatment of colitis include *Lactiplantibacillus plantarum* (Liu et al., 2011), *Lacticaseibacillus rhamnosus* (Zocco et al., 2006), *Lactobacillus bulgaricus* (Takamura et al., 2011), and *Bifidobacterium* (Nishida et al., 2018). They prevent or relieve colitis through antioxidant, immunomodulatory, and change the composition of gut microbiota. Therefore, the rational application of probiotics is an important strategy to prevent and treat colitis.

Limosilactobacillus fermentum HFY06 (LF-HFY06) is a potential probiotic strain isolated from yak yogurt, which was collected from the Aba Tibetan and Qiang Autonomous Prefecture of Sichuan province. In previous studies, LF-HFY06 has the effect of alleviating CCl_4 (carbon tetrachloride)-induced liver injury and d-galactose-induced oxidative stress and inflammation in mice (Li et al., 2020, 2021a,b). The mechanism of LF-HFY06 may be involved in the upregulation of antioxidant genes (*Nrf2*, *Gclc*, *Sod1*, *Sod2*, and *Cat*) expression and downregulation of inflammation-related genes (*Rela*, *Tnf*, and *Ptgs2*) expression. In a previous study, we reported the effect of the synbiotic composed of arabinoxylan and HFY06 on colitis. However, no comparative study with clinically positive drugs has been performed to further explain its effect. Based on the increased incidence of colitis in the above-mentioned regions, we selected the LF-HFY06 strain that has shown certain physiological activity to further explore its role in colitis.

Materials and methods

Source of strain

LF-HFY06 was isolated from Hongyuan yak yogurt, a unique product of Aba Tibetan and Qiang Autonomous Prefecture in Sichuan, China. NCBI's Basic Local Alignment Search Tool (BLAST) was used to identify the experimental strain LF-HFY06, which has been preserved in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China; CGMCC No. 16636).

Bacteria growth conditions

The LF-HFY06 (100 μL) was grown in 5 mL of MRS broth at 37°C for 24 h. To prepare the bacteria doses, the optical density of the bacterial suspension was measured at 595 nm, and its concentration was adjusted to 1×10^9 CFU/mL with normal saline.

Animals and experimental procedure

Animal experiments were approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (IACUC Number: 201906002B). All mice were housed at a room temperature of 20–22°C, 50 ± 10% humidity, under a 12 h diurnal light/dark cycle. During the experiment, the mice were allowed to eat standard rat chow and drink water freely. After a week of adjustable feeding, the experiment was performed as shown in **Figure 1**. Forty male Kunming mice (Chongqing Medical University, 6 weeks old) were randomly divided into four groups, namely, a normal group, model group, LF-HFY06 group (LF-HFY06), and salicylazosulfapyridine (SASP) group (Chen et al., 2017). The mice in the LF-HFY06 group received intragastric administration of 1.0×10^9 CFU/mL of LF-HFY06 (0.1 mL/10 g) every day for 28 days. From days 1 to 14, the mice in the LF-HFY06 group were treated as described above, and the other mice were treated with saline solution (0.1 mL/10 g) by intragastric administration. From days15 to 21, the mice in the normal group received sterile distilled water every day, and the other mice were given 5% DSS (molecular mass, 36–50 kDa; MP Biomedicals, Santa Ana, CA, United States) solution instead of sterile distilled water. At the same time, the mice in the SASP group received intragastric administration of 50 mg/mL (0.1 mL/10 g) SASP (Shandong

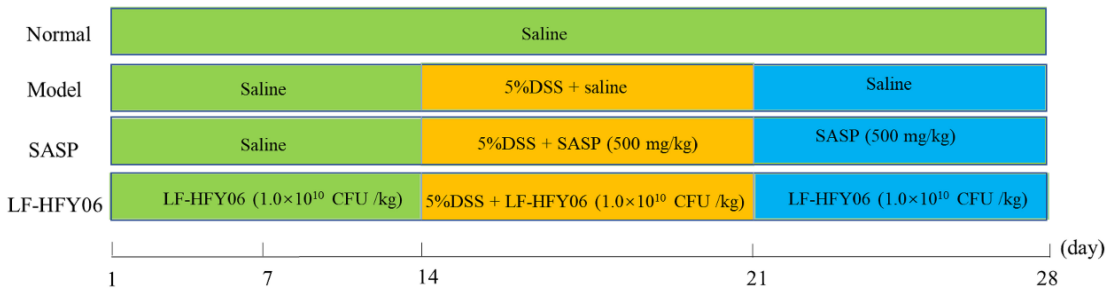

FIGURE 1
Animal experiment design of this study.

Huimeng Biotech Co., Ltd., Shandong, China). From days 22 to 28, the mice in the normal group and model group received sterile distilled water, and the mice in LF-HFY06 and SASP groups received intragastric administration as described above. On the day 29, the mice were anesthetized with ether for blood sampling. Afterward, the mice were sacrificed and the colon section was collected. The blood was centrifuged (4,000 rpm for 10 min at 4°C) to obtain the serum. The colon and serum were kept in an ultra-low temperature refrigerator (−80°C) for further testing.

Assessment of disease activity index score of colitis

The body weight loss, stool consistency, and rectal bleeding were observed during the modeling period (15–21 days). Each indicator is scored according to **Table 1** and the disease activity index (DAI) of mice was calculated (Yao et al., 2010). DAI = (combined score of weight loss, stool consistency, and bleeding)/3.

Histological analysis

The portion of the colon tissues was processed by the following procedure: 10% formalin solution fixation, dehydration, paraffin embedding, sectioning, hematoxylin, and eosin staining. Finally, the colon sections were fixed in neutral gum, and the pathological changes were examined by optical microscope (BX43 microscope, Olympus, Tokyo, Japan). Pathological damage of colon tissue was scored based on specific criteria shown in **Table 2** (Takagi et al., 2011). The total histological score was the sum of the epithelium and infiltration scores (total score = E + I), and thus ranged from 0 to 8.

TABLE 1 Evaluation of disease activity index (DAI) scores.

Score	Weight loss	Stool consistency	Occult/gross bleeding
0	(-)	Normal	Normal
1	1–5%		
2	6–10%	Loose	Occult bleeding
3	11–15%		
4	>15%	Diarrhea	Gross bleeding

Total-superoxide dismutase, catalase, and malondialdehyde levels in the serum of mice

The level of antioxidants markers T-SOD, CAT, and MDA in serum was performed using conventional biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the recommended instructions.

Cytokines levels

The determination of the serum cytokine levels TNF-α, IFN-γ, IL-1β, IL-6, IL-12, and IL-10 was processed under the instructions of the ELISA kit (Beijing Chenglin Biotechnology Co., Ltd., Beijing, China).

TABLE 2 Evaluation of histological scores.

Epithelium (E)	Score	Infiltration (I)	Score
Normal morphology	0	No infiltration	0
Loss of goblet cells	1	Infiltration around crypt bases	1
Loss of goblet cells in large areas	2	Infiltration reaching the muscularis mucosa	2
Loss of crypt	3	Extensive infiltration reaching the muscularis mucosa and thickening of the mucosa with abundant edema	3
Loss of crypts in large areas	4	Infiltration of the submucosa	4

Colonic gene expression analysis

The distal colonic (100 mg) was grounded in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, United States) to afford tissue homogenate, and then it was added with 200 μL chloroform. After the mixed solution was centrifuged at 14,000 rpm/min at 4°C for 15 min, the residue was discarded and isopropyl alcohol (500 μL) was subsequently added to the supernatant. The solution was fully mixed and placed at 4°C for 15 min, followed by centrifuging at 14,000 rpm/min and 4°C for 20 min. After discarding the supernatant, the precipitate was washed with 75% ethanol solution. After centrifugation once more, the upper water phase was removed, and the RNA precipitate was dissolved in 20 μL of enzyme-free water. The concentration and purity of the total RNA were tested *via* ultra-microspectrophotometry (Nano-100, All for Life Science, Hangzhou, Zhejiang, China).

To prepare a cDNA template, 1 μg/μL of RNA solution and a reverse transcription kit (Tiangen Biotech Co., Ltd., Beijing, China) were employed according to the instructions. A solution of cDNA (1 μg/μL, 1 μL) and SYBR Green PCR Master Mix (10 μL) was added with upstream and downstream primers (1 μL, **Table 3**) to afford a qPCR reaction solution (Zhou et al., 2019). Then, amplification was performed using the Applied Biosystems StepOnePlus Real-Time PCR Instrument (Thermo Fisher Scientific Co., Ltd., MA, United States). The cycling parameters were: 95°C for 90 s, 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, then, 95°C for 30 s, and 55°C for 35 s. With β-actin as a housekeeping gene, the $2^{-\Delta\Delta Ct}$ method was selected to calculate the relative expression of the related gene (Livak and Schmittgen, 2001).

TABLE 3 Sequences of primers used in this study.

name	Sequence	bp	Genbank accession no.
Rela	Forward: 5′-ATGGCAGACGATGATCCCTAC-3′	167	XM_006501107
	Reverse: 5′-CGGAATCGAAATCCCCTCTGTT-3′		
Nfkbia	Forward: 5′-TGAAGGACGAGGAGTACGAGC-3′	127	XM_003987542
	Reverse: 5′-TGCAGGAACGAGTCTCCGT-3′		
Tnf	Forward: 5′-CTGAACTTCGGGGTGATCGG -3′	122	XM_021149735
	Reverse: 5′-GGCTTGTCACTCGAATTTTGAGA-3′		
Il 1b	Forward: 5′-GAAATGCCACCTTTTGACAGTG -3′	116	NM_008361
	Reverse: 5′-TGGATGCTCTCATCAGGACAG-3′		
Il 6	Forward: 5′-CTGCAAGAGACTTCCATCCAG-3′	131	XM_021163844
	Reverse: 5′-AGTGGTATAGACAGGTCTGTTGG-3′		
Il 10	Forward: 5′-CTTACTGACTGGCATGAGGATCA-3′	101	XM_021175612
	Reverse: 5′-GCAGCTCTAGGAGCATGTGG-3′		
Ptgs2	Forward: 5′-GGTGCCTGGTCTGATGATG-3′	116	MW395257
	Reverse: 5′-TGCTGGTTTGGAATAGTTGCT-3′		
Actb	Forward: 5′-AACTCCATCATGAAGTGTGA-3′	247	XM_049111166
	Reverse: 5′-ACTCCTGCTTGCTGATCGAC-3′		

Rela, nuclear factor-κB p65; Nfkbia, NF-κB inhibitor α; Tnf, tumor necrosis factor-α; Il 1b, interleukin-1β; Il 6, interleukin-6; Il 10, interleukin-10; Ptgs2, prostaglandin-endoperoxide synthase 2; Actb, beta-actin.

Statistical analysis

The experimental data analysis was accomplished in SPSS 17.0 (SPSS Inc., Chicago, IL, United States) and GraphPad Prism 7 statistical software (Graph Pad Software Inc., La Jolla, CA, United States). The mean ± standard deviation was the form to express the results. Comparisons among groups were obtained by one-way analysis of variance (ANOVA) followed by Tukey's test, in which $P < 0.05$ indicated a significant difference.

Results

Colon length

Figure 2A shows the general appearance of colon tissue after the LF-HFY06 treatment. The colon length of mice in the model group was 7.86 ± 0.64 cm, which was significantly shorter compared with the normal group (9.82 ± 0.52 cm) ($P < 0.05$). Treatment groups significantly increased the colon length (LF-HFY06: 9.3 ± 0.64 cm; SASP: 9.28 ± 0.71 cm) when compared to the model mice group ($P < 0.05$).

Assessment of disease activity index

During the experimental period, a gradual weight increase was observed for the animals in the normal group, while a significant weight loss was observed for the model group. Treatment with LF-HFY06 and SASP was able to attenuate this weight loss (**Figure 2B**). DAI scores of mice in each group are shown in **Figure 2C**. There was no significant difference between the treatment groups and model group on days 15–16. On day 17, DAI increased significantly in the model group

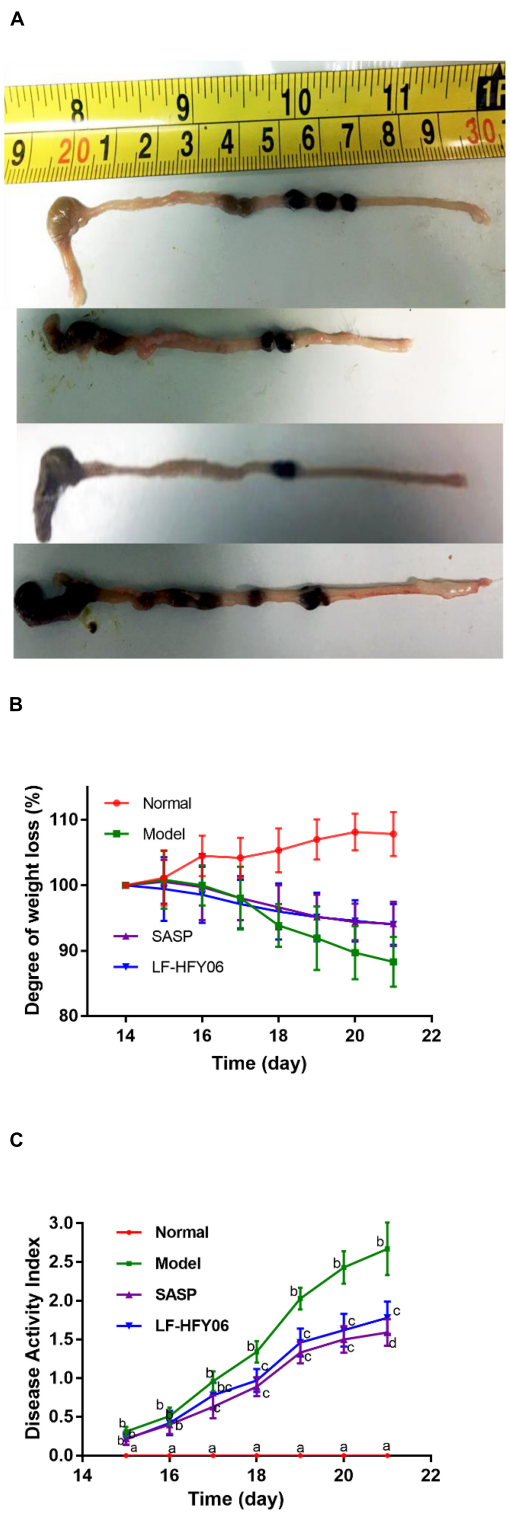

FIGURE 2
(A) The general appearance of colon tissue. **(B)** The degree of weight loss, $^{a-c}$Mean values with different letters on the same day indicate a significant difference after Tukey's test ($P < 0.05$). **(C)** Disease activity index of mice in different groups. $^{a-d}$Mean values with different letters on the same day indicate a significant difference ($P < 0.05$) after two-way ANOVA.

(Figure 2C, $P < 0.05$), and the increasing trend was faster than that in the treatment groups. However, no significant difference was observed in DAI between the treatment groups during the establishment of the colitis model (days17–20; $P > 0.05$).

Histological analysis

As shown in Figure 3, the colonic mucosal epithelial cells in the healthy mice were intact, and the glands were arranged in an orderly manner. Also, it was obvious that the crypts were normal with no ulcers. However, the mice in the model group exhibited severe colonic mucosal erosion, and the glands were disorderly arranged. Many crypts were dramatically destroyed and goblet cells were drastically reduced, accompanied by inflammatory cell infiltration (Figure 3A, black arrow). After the administration of LF-HFY06 and SASP, only a few ulcers appeared, and the crypts and the goblet cells were relatively complete with neatly arranged glands (Figure 3A, black arrow). The injury in the treatment group was less severe than that of the model group. Compared to the model group, the histological score of the treatment group was significantly lower ($P < 0.05$). The SASP group had a strikingly lower histological score than the LF-HFY06 group ($P < 0.05$; Figure 3B).

Total-superoxide dismutase, catalase, and malondialdehyde levels in the serum of mice

The activities of T-SOD and CAT antioxidant enzymes in the model mice receiving DSS were significantly decreased, while the MDA production was significantly increased compared to those in the healthy mice ($P < 0.05$; Table 4). In contrast to the model group, LF-HFY06 and SASP significantly increased the activities of SOD and CAT, and significantly reduced the amount of MDA ($P < 0.05$; Table 4).

Serum TNF-α, IL-1β, IL-6, IL-12, IFN-γ, and IL-10 levels in mice

A healthy intestinal barrier is critical for intestinal health, of which mucosal epithelial cells are an important part. When intestinal inflammation occurs, intestinal mucosal epithelial cells exert innate immune function, and thus, IL-4, IL-6, IL-10, and other cytokines in the tissue participate in the immune regulation response (Stevens et al., 1992). The pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, and IFN-γ levels in mice with DSS-induced colitis were apparently higher than those of mice in the normal group,

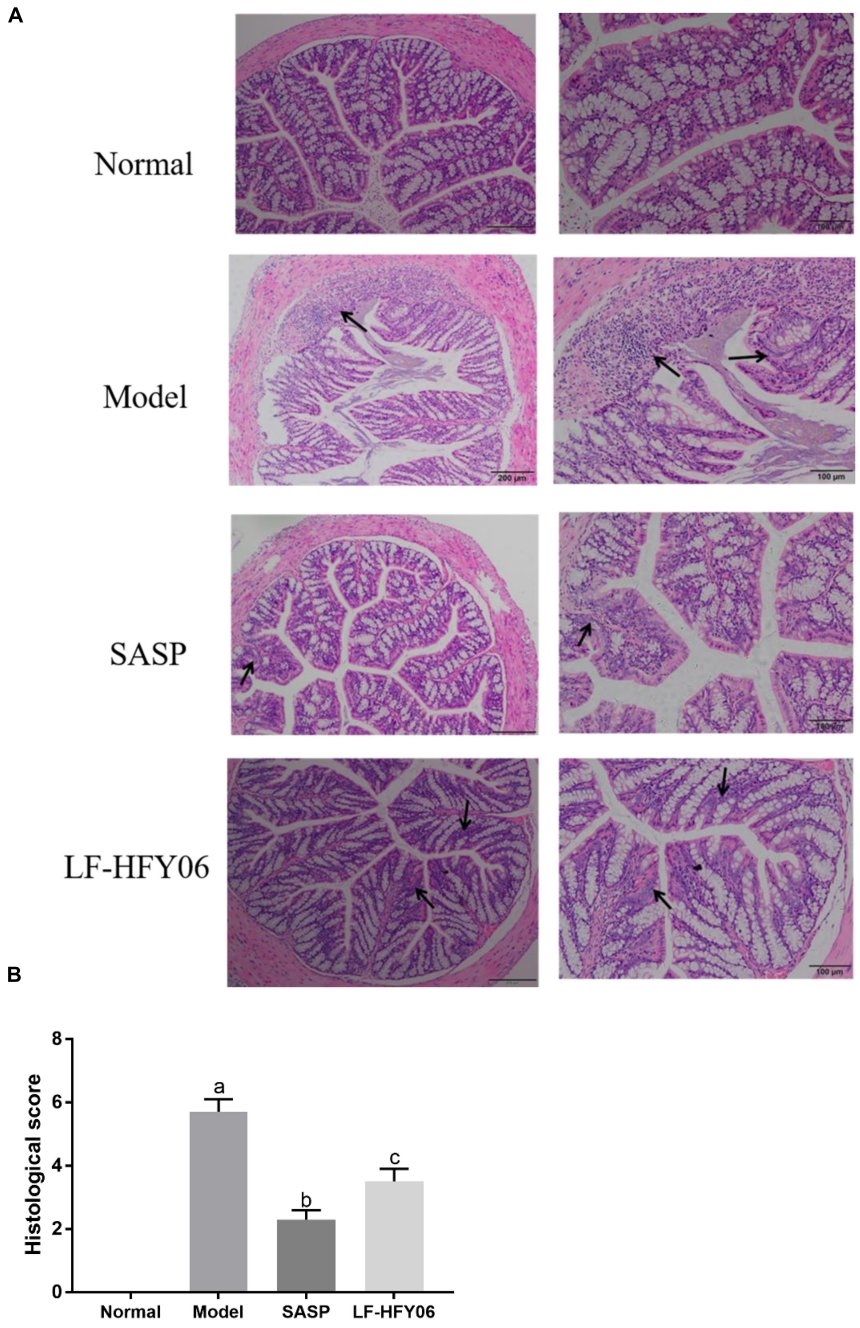

FIGURE 3
(A) Histopathological examination of colon tissue sections. **(B)** Histological disease score of colon tissue sections in mice of the different groups.

but anti-inflammatory cytokine IL-10 was lower (Table 5, $P < 0.05$). In comparison with the DSS model group, the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, and IFN-γ in the treatment groups showed a decreasing trend, and the anti-inflammatory factor IL-10 showed an increasing trend (Table 5, $P < 0.05$). The results showed that the inhibition of inflammation of LF-HFY06 was comparable to that obtained with SASP.

Rela, *Nfkbia*, *Tnf*, *Il 1b*, *Il 6*, *Il 10*, and *Ptgs2* mRNA expression levels in colonic tissue

The NF-κB signaling pathway is closely associated with the occurrence of inflammation. The mRNA expression levels of *Rela*, *Tnf*, *Il 1b*, *Il 6*, and *Ptgs2* were all upregulated in the model group, and the *Nfkbia* and *Il 10* mRNA expression

TABLE 4 Serum levels of T-SOD, CAT, and MDA in mice of each group.

Group	Normal	Model	SASP	LF-HFY06
T-SOD (U/mL)	218 ± 11^{a}	127 ± 11^{b}	184 ± 19^{c}	198 ± 14^{c}
CAT (U/mL)	57.6 ± 9.4^{a}	17.9 ± 3.6^{b}	43.2 ± 6.2^{c}	35.2 ± 3.9^{c}
MDA (nmol/mL)	3.8 ± 1.0^{a}	13.1 ± 1.9^{b}	6.9 ± 0.5^{c}	6.8 ± 1.1^{c}

Values are presented as the mean ± standard deviation (n = 8/group). Normal, mice were treated with saline; Model, mice were treated with 5% DSS; SASP, mice were treated with 50 mg/mL of salicylazosulfapyridine + 5% DSS; LF-HFY06, mice were treated with 1.0×10^{9} CFU/mL of LF-HFY06 + 5% DSS; $^{a-c}$significant difference ($P < 0.05$) was indicated by different letters in the same row after Tukey's test.

TABLE 5 Serum levels of TNF-α.

Group	Normal	Model	SASP	LF-HFY06
TNF-α (ng/L)	993 ± 82^{a}	$1{,}415 \pm 45^{b}$	$1{,}168 \pm 82^{c}$	$1{,}229 \pm 59^{c}$
IL-1β (ng/L)	76.9 ± 4.9^{a}	96.6 ± 6.4^{b}	85.8 ± 5.8^{c}	84.1 ± 4.7^{c}
IL-6 (pg/L)	275 ± 16^{a}	332 ± 15^{b}	278 ± 19^{a}	280 ± 15^{a}
IL-12 (ng/L)	138 ± 7^{a}	167 ± 8^{b}	159 ± 6^{bc}	150 ± 9^{c}
IFN-γ (ng/L)	841 ± 76^{a}	1485 ± 122^{b}	$1{,}206 \pm 67^{c}$	$1{,}103 \pm 65^{c}$
IL-10 (pg/L)	$1{,}437 \pm 84^{a}$	$1{,}014 \pm 113^{b}$	$1{,}257 \pm 60^{c}$	$1{,}366 \pm 68^{ac}$

Values are presented as the mean ± standard deviation (n = 8/group). Normal, mice were treated with saline; Model, mice were treated with 5% DSS; SASP, mice were treated with 50 mg/mL of salicylazosulfapyridine + 5% DSS; LF-HFY06, mice were treated with 1.0×10^{9} CFU/mL of LF-HFY06 + 5% DSS; $^{a-c}$significant difference ($P < 0.05$) was indicated by different letters in the same row after Tukey's test.

were downregulated in contrast to the normal group (**Figure 4**, $P < 0.05$). After using LF-HFY06 and SASP to intervene, the *Rela*, *Tnf*, *Il 1b*, *Il 6*, and *Ptgs2* mRNA expression was suppressed, and the mRNA expression of *Nfkbia* and *Il 10* was enhanced as compared to that in the DSS model group ($P < 0.05$). The variation trend revealed that LF-HFY06 may regulate the expression of inflammatory cytokines *via* inhibiting the NF-κB signaling pathway.

Discussion

DSS is a commonly used reagent for colitis modeling (Strober et al., 2002; Wirtz et al., 2007). DSS induces colonic inflammation in mice, and the resulting colonic congestion, edema, thickening of the colon wall, and the formation of an ulcerated surface will shorten the length of the colon. Therefore, the length of the colon can directly reflect the degree of colitis, which is considered to be negatively correlated with the severity of enteritis (Hu et al., 2021). In the current study, it was found that the length of the colon was shortened by DSS. The histopathological observation indicated that DSS caused the destruction of colonic crypts, as well as injury and apoptosis of colon cells (Hu et al., 2021). From the colon length and pathological examination, the intervention of LF-HFY06 alleviated the shortening of the colon length induced by DSS and assisted in maintaining the structural integrity of the colon. The results are the same as those of studies that have reported that lactic acid bacteria alleviated IBD (Vemuri et al., 2017; Amer et al., 2018).

Excessive activation of oxidative stress is another important mechanism of ulcerative colitis (Piechota-Polanczyk and Fichna, 2014). The enhancement of oxidative stress causes the increase of protein and DNA damage, cell degeneration, necrosis, and then promotes the occurrence and exacerbation of colitis (Zhu and Li, 2012). The TNF, NF-κB, and JNK signaling pathways could be activated by reactive oxygen species (ROS) to induce apoptosis and aggravate intestinal inflammation (Blaser et al., 2016). The body's antioxidant system consists of enzymatic and non-enzymatic systems (Birben et al., 2012). The enzymatic antioxidant system includes SOD, CAT, and glutathione peroxide. SOD is an antioxidant enzyme that can specifically clear superoxide anions by decomposing it into harmless H_2O and oxygen molecules (Birben et al., 2012). CAT is an enzyme that catalyzes the decomposition of hydrogen peroxide into oxygen and H_2O (Birben et al., 2012). MDA is a product of lipid peroxidation, by which nucleic acids and proteins are further damaged. Under the oxidative stress associated with colitis, LF-HFY06 could increase the activities of T-SOD, CAT, and reduce the level of MDA to suppress oxidative stress.

A complex regulatory network of cytokines is involved in colitis (Izcue et al., 2009; Chen and Sundrud, 2016). As a pro-inflammatory cytokine, TNF-α and IL-6 play important roles in the pathogenesis of ulcerative colitis. They induce the release of other cytokines, which aggravate the inflammatory response (Drastich et al., 2011; Billiet et al., 2014; Xiao et al., 2016). IL-1β can promote chemotactic neutrophils and other inflammatory cells to enter the related lesions of the intestine (Mulay et al., 2013). IL-12 is also one of the strongest NK cell-activating factors, and it stimulates NK cells to produce a variety of cytokines, such as IFN-γ, IL-8, and TNF-α (Zelante et al., 2007). Many studies have shown that the IFN-γ level was increased in intestinal inflammation, which exerts immunomodulatory effects (Ferrier et al., 2003). However, IL-10 inhibits inflammation in the microenvironment of the intestinal tissue. It modulates the occurrence and development of intestinal inflammation and maintains the intestinal mucus barrier (Huber et al., 2011; Shouval et al., 2014). DSS stimulates the production of inflammatory factors TNF-α, IL-6, IFN-γ, IL-12, IL-1β, and decreases the levels of anti-inflammatory factor IL-10. LF-HFY06 evidently suppressed the levels of inflammatory factors TNF-α, IL-6, IFN-γ, IL-12, and IL-1β. Meanwhile, it significantly elevated the IL-10 level to inhibit inflammation in DSS-induced colitis.

As a vital transcription factor during the process of the body's immune response, NF-κB binds to its inhibitory protein IκB in an inactive manner under normal conditions. It is reported that DSS activates NF-κB by accelerating the phosphorylation of IκB protein (Onizawa et al., 2009;

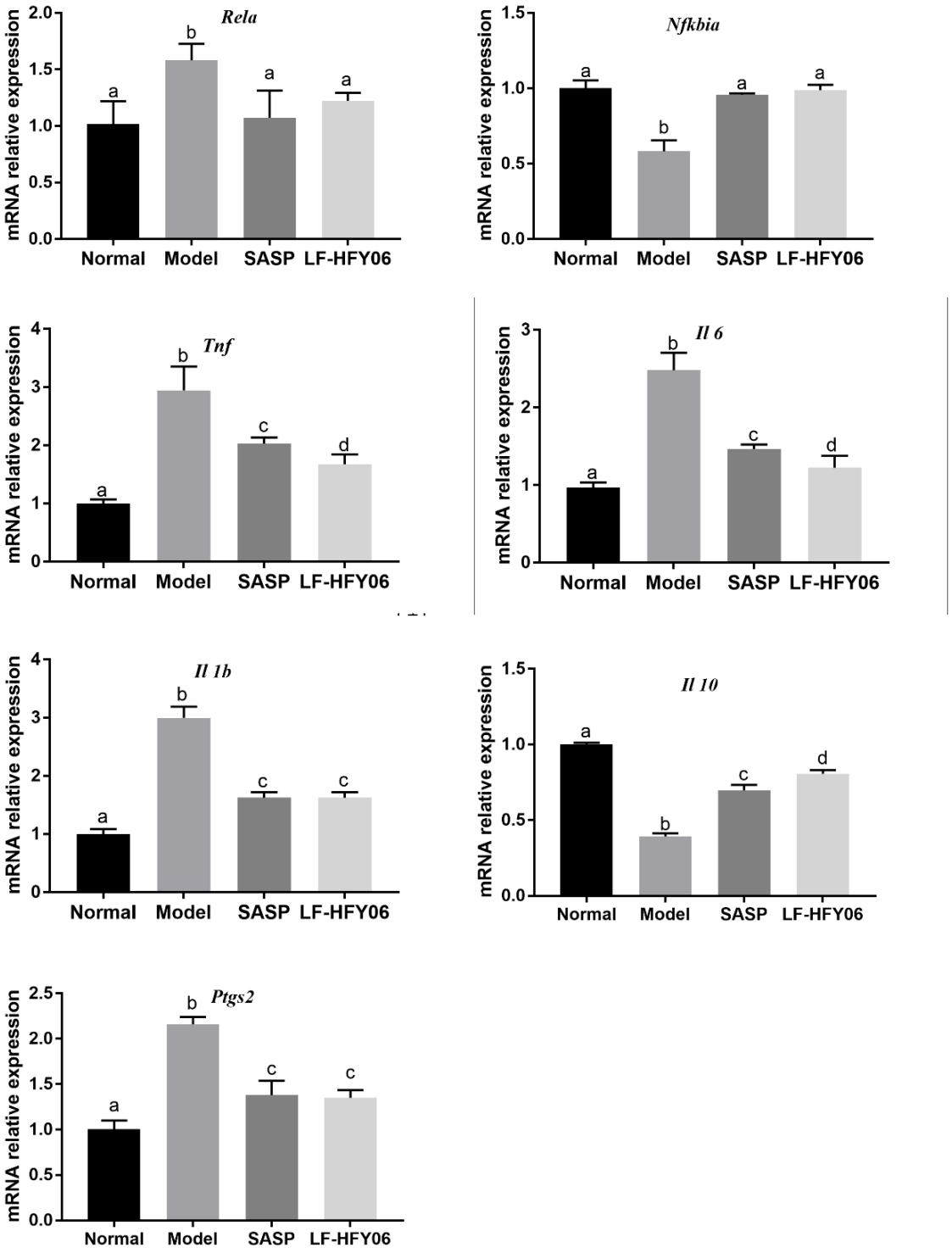

FIGURE 4
The *Rela, Nfkbia, Tnf, Il 1b, Il 6, Il 10,* and *Ptgs2* mRNA expression levels in colon tissues of the different groups. The data are shown as mean ± standard deviation (n = 8). $^{a-d}$Significant difference ($P < 0.05$) was indicated by different letters after Tukey's test.

Liu et al., 2016). The activated NF-κB further facilitates the release of pro-inflammatory factors, causing severe inflammatory damage to the body (Onizawa et al., 2009; Liu et al., 2016). At the same time, the induced inflammatory cytokines, such as IL-6 and TNF-α, promote the expression of the NF-κB gene to form a positive feedback loop. This triggers

an excessive immune response in colon tissues and damages colon mucosa, leading to the occurrence of colitis (Wang et al., 2003; Hu et al., 2014).

The core role of the NF-κB signaling pathway is to regulate the balance between inflammatory factors and anti-inflammatory factors. Studies have reported that *Lactobacillus* sp. relieved colitis symptoms *via* the NF-κB signaling pathway (Chen et al., 2017; Zhou et al., 2019). COX-2 is an inducible enzyme and could catalyze arachidonic acid to produce endogenous prostaglandins, which is coded by *Ptgs2* (Lee et al., 2004). In the current study, the administration of LF-HFY06 suppressed the mRNA expression of *Rela* and elevated the mRNA expression of *Nfkbia* to inhibit the activation of the NF-κB signaling pathway. Then, the mRNA expression of pro-inflammatory cytokines, *Tnf*, *Il 1b*, *Il 6*, and *Ptgs2* was suppressed, and that of anti-inflammatory cytokines *Il 10* was elevated in colon tissue. This indicates that LF-HFY06 may relieve colitis *via* the inhibition of the NF-κB signaling pathway.

According to literature reports, SASP could be decomposed into sulfadiazine and 5-aminosalicylic acid in the effect of intestinal microorganisms. 5-Aminosalicylic acid has antibacterial, anti-inflammatory, and immunosuppressive effects (Zheng, 2006). In this study, LF-HFY06 exerts antioxidant and anti-inflammatory activities by inhibiting the NF-kB signaling pathway. **Tables 4**, **5** show that insignificant differences were emerged between SASP and LF-HFY06 in regulating the levels of related biochemical indicators. The qPCR analysis showed that higher mRNA expression of the anti-inflammatory IL-10 in the SASP group was observed compared with that of the LF-HFY06 group. However, the expression of the inflammatory gene *Tnf* and *Il 6* in SASP group was higher than those of LF-HFY06. There were no significant differences in other genes. Overall, the ability to regulate gene expression is approximately equal between SASP and LF-HFY06.

In recent years, *Lactobacillus* sp. is used to prevent or alleviate colitis in mice mainly through immunomodulatory effects and regulation of the gut microbiome. *Limosilactobacillus fermentum* CQPC04 could inhibit oxidative stress and inflammation in mice with colitis, and its mechanism of it is to regulate the NF-κB signaling pathway (Zhou et al., 2019). *Limosilactobacillus fermentum* HY01 and *Limosilactobacillus fermentum* IM12 also inhibited the inflammatory symptoms of colitis through this pathway (Chen et al., 2017; Lim et al., 2017). Moreover, it is reported that *Limosilactobacillus fermentum* KBL375 could regulate the immune response and increase the abundance of beneficial microorganisms to change the composition of gut microbiota, thus achieving the purpose of alleviating intestinal inflammatory diseases (Jang et al., 2019). Similarly, Rodríguez-Nogales et al. (2017) reported that *Limosilactobacillus fermentum* CECT5716 could counteract an enrichment in *Bacillus* and *Paenibacillus*, together with a reduction in *Cytophaga*, achieving an anti-inflammatory effect in colitis mice. The regulation of antioxidant levels is also one of the important mechanisms. Chauhan et al. reported that the antioxidant levels were increased by *Limosilactobacillus fermentum* Lf1 to ameliorate colitis (Chauhan et al., 2014). The mechanism of LF-HFY06 in relieving colitis is similar to previous studies. LF-HFY06 ameliorates ulcerative colitis in mice by modulating the NF-κB signaling pathway to downregulate inflammatory cytokines and upregulate anti-inflammatory factors.

Also, short fatty acids (mainly butyric acid) produced by probiotics promote the development of regulatory T cells, which in turn continuously strengthen the mucosal barrier (Schirmer et al., 2019). Second, probiotics can also secrete antibacterial substances, which could inhibit the growth of intestinal pathogens (Schirmer et al., 2019). Those are the directions for further in-depth research on LF-HFY06.

Conclusion

In our research, 5% DSS was used to establish a colitis model in mice. After intervention with LF-HFY06, the colon tissue damage was significantly alleviated, the antioxidant capacity was enhanced, and the inflammatory response was inhibited. The result of qPCR detection showed that LF-HFY06 may protect against colitis through the NF-κB signaling pathway. The mechanism of LF-HFY06 was involved in the upregulation of *Nfkbia* and *Il 10* mRNA expression, and downregulation of *Rela*, *Tnf*, *Il 1b*, *Il 6*, and *Ptgs2* mRNA expression. However, its experiments on humans have not been carried out yet. In future, the mechanism of LF-HFY06 and relevant basic scientific research will be further analyzed to provide more references for carrying out clinical trials.

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/s.

Ethics statement

The Animal experiments were approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (IACUC Number: 201906002B).

Author contributions

XZ conceived the study. BL and LY completed the writing of the manuscript, completed the inspection, and modification of

the manuscript. BL and YW completed the main experimental work. YW mainly completed data sorting and statistical analysis. All authors discussed the data and reviewed 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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Effect of *Lactobacillus fermentum* ZS40 on the NF-κB signaling pathway in an azomethane-dextran sulfate sodium-induced colon cancer mouse model

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The occurrence of intestinal diseases such as colon cancer is closely related to the intestinal flora. *Lactobacillus fermentum* is a gut probiotic that plays an important role in chronic intestinal inflammation and colon cancer. In the current study, we investigated the effect of *Lactobacillus fermentum* ZS40 on NF-κB signaling pathway of azomethane-dextran sulfate sodium (AOM-DSS) -induced colon cancer in mice. Animals were divided into control group (NC), AOM-DSS-induced model group (CRC), AOM-DSS plus high-dose *Lactobacillus fermentum* ZS40 (ZS40-H), AOM-DSS plus low-dose *Lactobacillus fermentum* ZS40 (ZS40-L), AOM-DSS plus *Lactobacillus bulgaricus* (BLA), and AOM-DSS plus sulfasalazine (SD)-treated group. Observation of animal physiological activity (body weight and defecation), biochemical measurements, histopathological examination of colon tissue, qPCR to evaluate the expression of inflammation-related genes, immunohistochemical analysis of CD34 and CD117, and Western blot analysis of NF-κB signaling pathway were performed. Compared with the CRC group, the ZS40-H, ZS40-L, BLA, and SD groups had decreased levels of colon cancer marker proteins CD34 and CD117, and the number of abnormal colonic lesions observed by colon histology decreased, while the ZS40-H group showed excellent results. In addition, all probiotic interventions showed weight loss effects. The expression of inflammatory stimulators TNF-α and IL-1β in the probiotic treatment group decreased; the expression of key proteins IκBα and p65 in the NF-κB signaling pathway also decreased, resulting in a decrease in the expression of the target protein Cox-2. Therefore, administration of *Lactobacillus fermentum* ZS40 as a probiotic can alleviate intestinal inflammation and prevent colon cancer in mice.

KEYWORDS

NF-κB signaling pathway, AOM-DSS, colon cancer, *Lactobacillus fermentum*, inflammation-related genes

Introduction

With high morbidity and mortality, colon cancer is a malignant disease that threatens both human health and life (Arnold, 2018). Colon cancer ranks third in the incidence of cancer diseases among both men and women, globally (Tang and Evans, 2021). In China, the number of new cases of colon cancer each year is as high as 400,000, and there are about 10 million new cases worldwide annually (Jia et al., 2022). There may be no symptoms in the early stages of colon cancer. In the middle and late stages, the disease may manifest as abdominal distension and indigestion, followed by changes in bowel habits, abdominal pain, or blood in the stool (Pacal et al., 2020). The methods for early detection of colon cancer are imprecise because the symptoms in the early stages of disease are not obvious, which leads to the late detection of colon cancer (Birgisson et al., 2021). At present, some patients with colon cancer can be treated with surgery, but the risks involved in surgery are high and the adverse postoperative effects can be considerable (Wahab et al., 2021).

Inflammation is the body's defense response to injury, but uncontrolled inflammation is often closely associated with cancer development and metastasis (Schmitt and Greten, 2021). Sometimes, the chronic inflammation that causes cancer stems from a disease characterized by inflammation. For example, the inflammatory diseases colitis, pancreatitis, and hepatitis are associated with an increased risk of colon cancer, pancreatic cancer, and liver cancer, respectively. Clinical investigations have shown that many cancer patients have a history of chronic inflammatory diseases (Beckmann et al., 2019). Numerous studies have confirmed that inflammation may also disrupt the balance of intestinal flora and induce intestinal diseases (Jackson and Theiss, 2020). In chronic inflammation, cytokines and chemokines produced by inflammatory cells can spread the focal inflammatory response to surrounding tissues through the NF-κB signaling pathway (Zhao et al., 2021). After induction by pro-inflammatory factors and tumor necrosis factors, the NF-κB signaling pathway regulates the expression of various genes such as interleukin-related genes and apoptotic factors downstream. This process also increases the immune evasion ability of precancerous cells. The inflammatory process produces molecules called cytokines that stimulate the growth of blood vessels that bring oxygen and nutrients to the tumor, and the process can also produce molecules called free radicals that further damage DNA. These inflammatory side effects may help maintain and promote cancer growth. Therefore, chronic inflammation is the initiating factor of tumorigenesis, and NF-κB plays an important role in the occurrence and development of inflammatory tumors.

The human colon is an important metabolic organ with a complex intestinal flora structure (Zhao Y. L. et al., 2018). The number of viable bacterial cells per gram of intestinal content is far greater than 10^{11}—and can even reach 10^{14} (Li C. et al., 2019). In general, the intestinal flora can protect the colon, but when the number of florae is reduced by more than 50%, the ability to protect the colon from carcinogens is lost (Zhou et al., 2019). Gut microbes regulate gut health by releasing metabolites, and dysbiosis is a hallmark of colorectal cancer (CRC), leading to inflammation, tumor growth, and response to therapy (Bell et al., 2022). Studies conducted at the beginning of the twentieth century found that the longevity of people from Bulgaria was related to their long-term use of fermented dairy products (Salvetti and O'Toole, 2017). Since then, a large amount of research data has shown that the intake of fermented dairy products is beneficial to human health (Warren et al., 2018).

Lactobacillus fermentum ZS40 (China General Microbiological Culture Collection Center, CGMCC No.: 18226) is an active strain isolated from the traditionally fermented yogurt in Zhaosu County, Xinjiang, by the Chongqing Collaborative Innovation Center for Functional Food. The preliminary activity test results have shown that the ability of *Lactobacillus fermentum* ZS40 to tolerate artificial gastric juice reached 79.3%. In this study, a mouse colon cancer model induced by azoxymethane-dextran sulfate sodium (AOM-DSS) was used to explore the influence of the NF-κB signaling pathway in the occurrence and development of colon cancer and the ameliorative effect of *Lactobacillus fermentum* ZS40.

Materials and methods

Strain culture

Lactobacillus fermentum ZS40 and *Lactobacillus bulgaricus* strains were inoculated into Man, Rogosa (MRS) liquid medium at an inoculum of 2%. and was incubated in a constant temperature water shaker at 37°C at 100 rpm for 24 h. The solution was then centrifuged at 12,000 rpm for 10 min and the supernatant discarded. The bacterial pellet was then resuspended in 0.9% saline. The purity of the bacterial solution was checked with Gram stain microscopy. Using the gradient dilution method, the *Lactobacillus fermentum* ZS40 bacterial solution was diluted to 10^{11} and 10^{9} colony-forming units (CFU), and *Lactobacillus bulgaricus* was diluted to 10^{11} CFU for use.

In vitro resistance of probiotic

The MRS-THIO medium with porcine bile salt concentrations of 0, 1, 2, and 3 g/L was prepared, the activated probiotic were inoculated into the MRS-THIO medium at a volume of 2%, and

Abbreviations: AOM-DSS, azoxymethane-dextran sodium sulfate; MRS, Man Rogosa; IL-1β, interleukin 1β; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; MIP-1β, macrophage inflammatory protein 1β; VCAM-1, vascular endothelial cell adhesion molecule 1; TRAF, TNF receptor-associated factor; IκBα, nuclear factor inhibitor protein; IKKα/β, IκB kinase; Bcl-XL/2, B-cell lymphoma/Leukemia; Cox-2, Cyclooxygenase-2.

the blank medium with a bile salt concentration of 0 was used as a control. After culturing at 37°C and 100 rpm for 24 h, the absorbance A at OD600nm was measured, and the tolerance of probiotic to bile salts was calculated according to formula 1.

$$\text{Bile salt tolerance} = A1 / A0 \times 100\% \quad (1)$$

A1: OD600 nm of bile salt-containing medium; A0: OD600 nm of blank medium.

Artificial gastric juice prepared with 0.2% NaCl and 0.35% pepsin was used, pH3.0 was adjusted to use 1 mol/L HCl, and the bacteria were filtered through a 0.22 μm filter. The activated probiotic and artificial gastric juice were mixed in a volume ratio of 1:9. They were incubated at 37°C and 100 rpm. The culture medium after 0 h and 3 h was taken and diluted 10 times with 0.9% NaCl. The diluted solution was spread on MRS solid medium plate, and cultured at 37°C for 48h. The survival rate of probiotic was calculated according to formula 2.

$$\text{Survival rate} = 3\text{h number of viable probiotic} / 0\text{h number of viable probiotic} \times 100\% \quad (2)$$

Experimental animals

Male C57BL/6J mice (4 weeks old, 16–18 g) were purchased from Chongqing Medical University. Mice were housed at 25°C under a 12-h light/dark cycle and provided with standard rodent chow (ENSIWEIER Biotechnology Co., Ltd., Chongqing, China) and purified water. The entire trial period was 12 weeks. The 1st week was the adaptive culture cycle. All experimental animals were provided with standard feed and purified water. After the adaptation period, the experimental animals were randomly divided into Normal group (NC); Colorectal cancer control group (CRC); Probiotic intervention group: high-dose ZS40 bacterial solution (ZS40-H), low-dose ZS40 bacterial solution (ZS40-L) and Bulgarian bacteria bacterial solution (BLA), and Drug control group: sulfasalazine solution (SD). The 2nd to the 11th week were model induction cycle, NC and CRC were given 0.2 ml of 0.9% Nacl/Day; ZS40-H, ZS40-L, and BLA group were administered by gavage with 0.2 ml of 10^{11} CFU, 10^{9} CFU, and 10^{11} CFU bacterial solution, respectively, as the number of viable bacteria ingested per day; SD was given 0.2 ml of 2.5% sulfasalazine solution by intragastric administration per day. In addition to the above continuous treatment operations, the following treatments are also required. On the first day of the 2nd week, all groups except NC were intraperitoneally injected with AOM reagent at a dose of 10 mg/kg, and purified water was provided during the week. From 3th to 5th week, drinking conditions of 2.5% DSS solution for 7 days and purified water for 14 days were provided. These drinking conditions were repeated 3 times (3th–11th week). The 12th week was the recovery week, and all treatments of all experimental animals were stopped and were provided with standard feed and purified water (Figure 1). After all experiments (13th week), all animals blood was collected by orbital draw method and centrifuged at 9,500*g* at 4°C in a refrigerated high-speed centrifuge (iCEN-24R, Hangzhou Aosheng Instrument Co., Ltd., Zhejiang, China) to obtain serum, and dissected and collected tissues. The colon were quickly removed, and photographed. The intercepted part of the sample was immersed in the fixative at 4°C and sent to the biological company for staining. The remaining colon samples were immediately frozen in liquid nitrogen and stored at −80°C for further analysis. Animal experiments were approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (IACUC Number: 202106009B).

Colon morphology observation

The sacrificed mice were fixed on the dissecting board, the intestinal tissue of the mice was removed, the connection between the cecum and the small intestine was carefully cut, the connection between the colon and the anus was cut off, and the colon tissue was obtained and weighed. The colon was carefully unfolded. Been careful not to artificially lengthen the colon. Be careful not to artificially increase the length of the colon. The 0-scale position was aligned with the junction of the colon and the cecum.

Colon pathological observation

Colon tissue samples from each animal were pathologically examined. These tissue samples were fixed in a tissue fixative (Sevier Biotechnology Co., Ltd., Chongqing, China) at 4°C (Collected in 2.1). Within 24 h, the fixed tissue samples were sent to Chongqing Sevier Biological Company to prepare paraffin sections and undergo hematoxylin and eosin (H&E) staining. Additionally, the obtained tissue sections were sent to Chongqing Servicebio Biological Company again for CD34 (GB121693, Servicebio) and CD117 (GB11073-2, Servicebio) immunohistochemical staining. The stained samples were viewed with an upright microscope (BX43F; Olympus, Tokyo, Japan). And the severity of intestinal pathology was double-blindly scored, pathology score ≥ 2, regarded as intestinal injury.

Serum enzyme-linked immunosorbent assay (Elisa)

To prepare serum samples, the collected blood *via* retro-orbital sampling was allowed to stand at 4°C for 1 h and centrifuged at 9,500*g* for 15 min at 4°C (Collected in 2.1). A commercial ELISA kit (mlibio, Shanghai, China) was then used to measure levels of interleukin 1β (IL-1β), interleukin 8 (IL-8), tumor necrosis factor-α (TNF-α), macrophage inflammatory

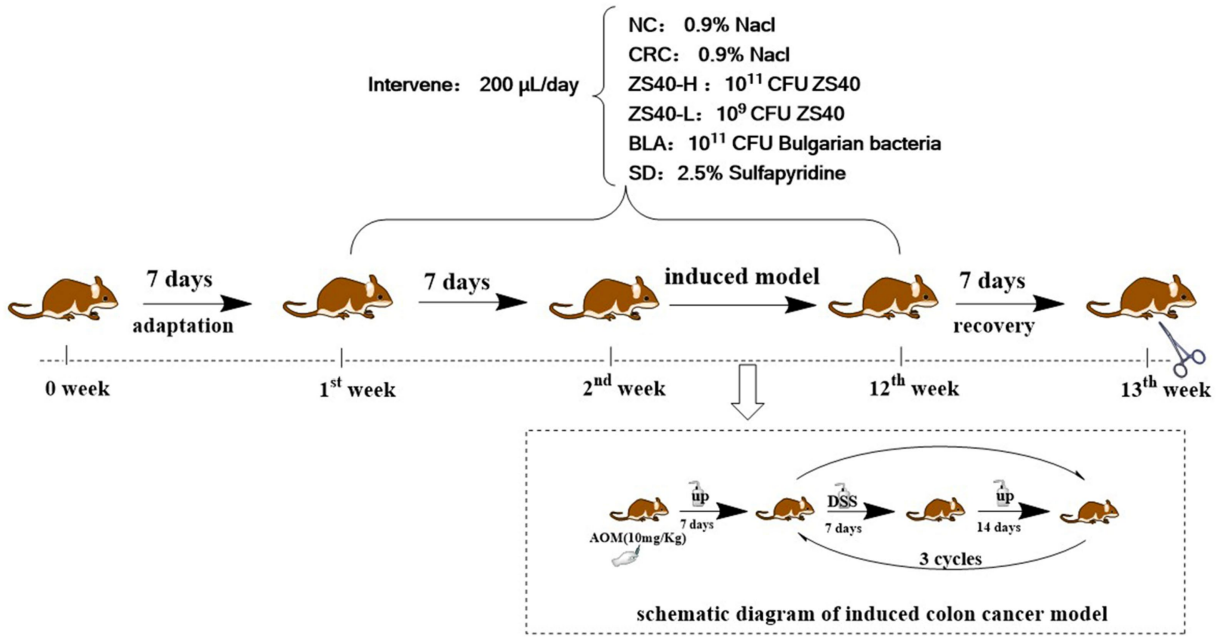

FIGURE 1
Schematic diagram of experiment cycle.

protein 1β (MIP-1β), and vascular endothelial cell adhesion molecule 1 (VCAM-1).

Real-time fluorescence quantitative PCR

The mRNA expressions of IL-1β, TNF-α, nuclear factor kappa-B (NF-κB, p65), TNF receptor-associated factor 1/2/6 (TRAF1/2/6), nuclear factor inhibitor protein (IκBα), IκB kinase (IKKα/β), B-cell lymphoma/leukemia (Bcl-XL, Bcl-2), and Cyclooxygenase (Cox-2) were measured using real-time fluorescence quantitative PCR (RT-qPCR). The total RNA was extracted and cDNA reverse transcription was performed, according to instructions of the RNA extraction kit (BaiMaiKe Technologies, Beijing, China) and cDNA reverse transcription kit (Yeasen Technologies, Shanghai, China), respectively. A microspectrophotometer (Nano-300; ALLSHENG, Zhejiang, China) was used for quantification by measuring the absorbance at 260 nm. Then, RT-qPCR was performed using an RT-qPCR instrument (StepOne Plus, ABI, United States), normalized with actin. The final RT-qPCR product expression was calculated using the $2^{-\triangle\triangle Ct}$ method. Each sample was replicated 4 times and data were statistically analyzed using Prism 7.0 software.

Western blot analysis

Colon samples were homogenized using a protein extraction kit, in which the phenylmethanesulfonyl fluoride, protease inhibitors, and phosphatase inhibitors were mixed (Solarbio Life Sciences, Beijing, China), and then centrifuged at 12,000*g* for 20 min at 4°C. The protein concentration was determined using a bicinchoninic acid protein determination kit (Yeasen Technologies, Shanghai, China). For Western blot analysis, 50 μg of protein extract was separated with 10% NuPAGE (NP0302BOX, Invitrogen) and then transferred to a polyvinylidene fluoride (PVDF) membrane. This was sealed with 5% skim milk for 1 h at 28°C, using anti-p65 (51-0500, Invitrogen), anti-IL-1β (MM425B, Invitrogen), anti-TNF-α (AMC3012, Invitrogen), anti-IκBα (MA5-16152, Invitrogen), anti-Cox-2 (PA5-17614, Invitrogen), and anti-β-actin (MA1-140, Invitrogen); incubated in the PVDF membrane; washed five times with 1× TBST; and then combined with Horseradish peroxidase (HRP) secondary antibodies (A32723, Invitrogen). Next, the mixture was incubated for 1 h at room temperature and the PVDF membrane was washed five times with 1× TBST. Antibody binding was observed using enhanced chemiluminescence (Solarbio Life Sciences, Beijing, China). Finally, ImageJ software (U.S. National Institutes of Health, Bethesda, MD, United States) was used to quantify protein expression.

Data analysis

The data were expressed as mean ± standard deviation (SD). GraphPad 7.0 (GraphPad Software, San Diego, CA, United States) and IBM SPSS 21.0 (IBM Corp., Armonk, NY, United States) statistical software packages were used for analysis. We used one-way analysis of variance and Duncan's multiple range test to evaluate the difference between the mean values in each group. A value $p < 0.05$ was considered statistically significant.

Results

Viability of probiotics

Both *Lactobacillus fermentum* ZS40 and *Lactobacillus bulgaricus* could grow in an MRS medium containing different concentrations of bile salts, but the growth efficiency varies greatly under different concentrations. In 1 g/L MRS-THIO medium, the growth efficiency of *Lactobacillus fermentum* ZS40 and *Lactobacillus bulgaricus* was over 50%, and the growth efficiency of *Lactobacillus fermentum* ZS40 reached 79%; with the increase of bile salt concentration, the growth efficiency of *Lactobacillus fermentum* ZS40 and *Lactobacillus bulgaricus* was inhibited. Under the condition of concentration of 2 g/L, the growth efficiency of *Lactobacillus fermentum ZS40* reached 43%; under the condition of concentration of 3 g/L, the growth efficiency of *Lactobacillus fermentum ZS40* was 28%.

There was a difference in the survival rates of *Lactobacillus fermentum* ZS40 and *Lactobacillus bulgaricus* in artificial gastric juice at pH3.0. The viable counts of *Lactobacillus fermentum* ZS40 and *Lactobacillus bulgaricus* in artificial gastric juice were 6.3×10^7 CFU/ml and 4.8×10^7 CFU/ml after 0 h; the viable counts after 3 h were 4.27×10^6 CFU/ml and 2.88×10^6 CFU/ml; the survival rates of *Lactobacillus fermentum* ZS40 and *Lactobacillus bulgaricus* in the artificial gastric juice of pH3.0 within 3 h were, respectively, 6.7 and 6%.

Effect of probiotic samples on body weight

During the experiment, the body weight changes of the experimental mice were recorded. As shown in Figure 2, from the 2th experimental week after the injection of AOM, compared with the normal group, the body weight of the mice in the CRC group showed a significant decline. In the later stage of the experiment, the body weight of the mice in the CRC group kept decreasing under the action of DSS. Although the body weight of the mice in the intervention group of *Lactobacillus fermentum* ZS40-H was affected by AOM-DSS, the body weight was upregulated, showing differences from CRC mice. The same results were also shown in the ZS40-L, BLA, and SD groups. The body weight of mice in these intervention groups all showed a certain degree of upregulation.

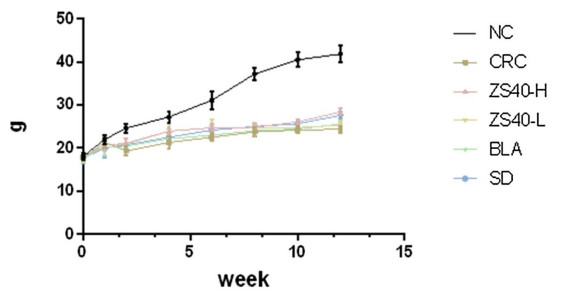

FIGURE 2
Body weight of mice. NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose *Lactobacillus fermentum* ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose *Lactobacillus fermentum* ZS40 (10^9 CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD, mice treated with sulfasalazine (25%).

Effect of probiotic samples on colon morphology

As shown in Figure 3A, the length and morphology of the mice colon were compared after dissection at the end of the experiment, and the colon weight was recorded (as shown in Figure 3B). In the anatomical image, we can see that compared with NC, owing to the large amount of inflammation produced during the process of inducing colon cancer, the length of the colon in CRC mice was significantly shortened (NC:8.3 cm; CRC:4.9 cm), the intestine was filled with pus-like material, colon weight increased significantly(NC:0.5370 ± 0.08 g; CRC:0.7245 ± 0.06 g), and colon lumps were found at multiple locations (Where the arrow points). On the contrary, in ZS40-H and SD mice, there was less shortening of the colon (ZS40-H:7.0 cm; SD:6.9 cm), less pus-like material, fewer colon lumps, and the volume of the lumps was also reduced (ZS40-H:0.603 ± 0.05 g; SD:0.6555 ± 0.10 g). Even, no lumps were found in some tissues. Similar situations were found in the ZS40-L and BLA groups (ZS40-L:6.7 cm, 0.6729 ± 0.05 g; BLA:6.0 cm, 0.6926 ± 0.09 g), but the improvement was not more obvious than that in ZS40-H and SD mice. This shows that after the interference of probiotics and drugs, inflammation in the mouse colon was alleviated, and the effects of high-dose ZS40 and drugs were the most significant.

Effect of probiotics samples on serum indexes

As shown in Table 1, compared with NC mice, the serum levels of IL-1β, IL-8, TNF-α, MIP-1β, and VCAM-1 in the CRC group were significantly increased. Compared with CRC, ZS40-H treatment reduced the serum levels of IL-1β, IL-8, TNF-α, MIP-1β, and VCAM-1 in CRC mice. Moreover, compared with the CRC group, mice in the ZS40-L and BLA treatment groups and the SD control group had effectively reduced levels of IL-1β, IL-8, TNF-α, MIP-1β, and VCAM-1; SD showed a better effect in reducing the levels of inflammatory factors ($p < 0.05$). This indicates that *Lactobacillus fermentum* ZS40 could reduce the levels of inflammatory factors and vascular cell adhesion factors in the serum of C57 mice induced by AOM-DSS.

Effect of probiotic samples on colon tissue

The morphology of colon tissue was observed by analyzing the H and E-stained sections of colon tissue (Figure 4A), and the

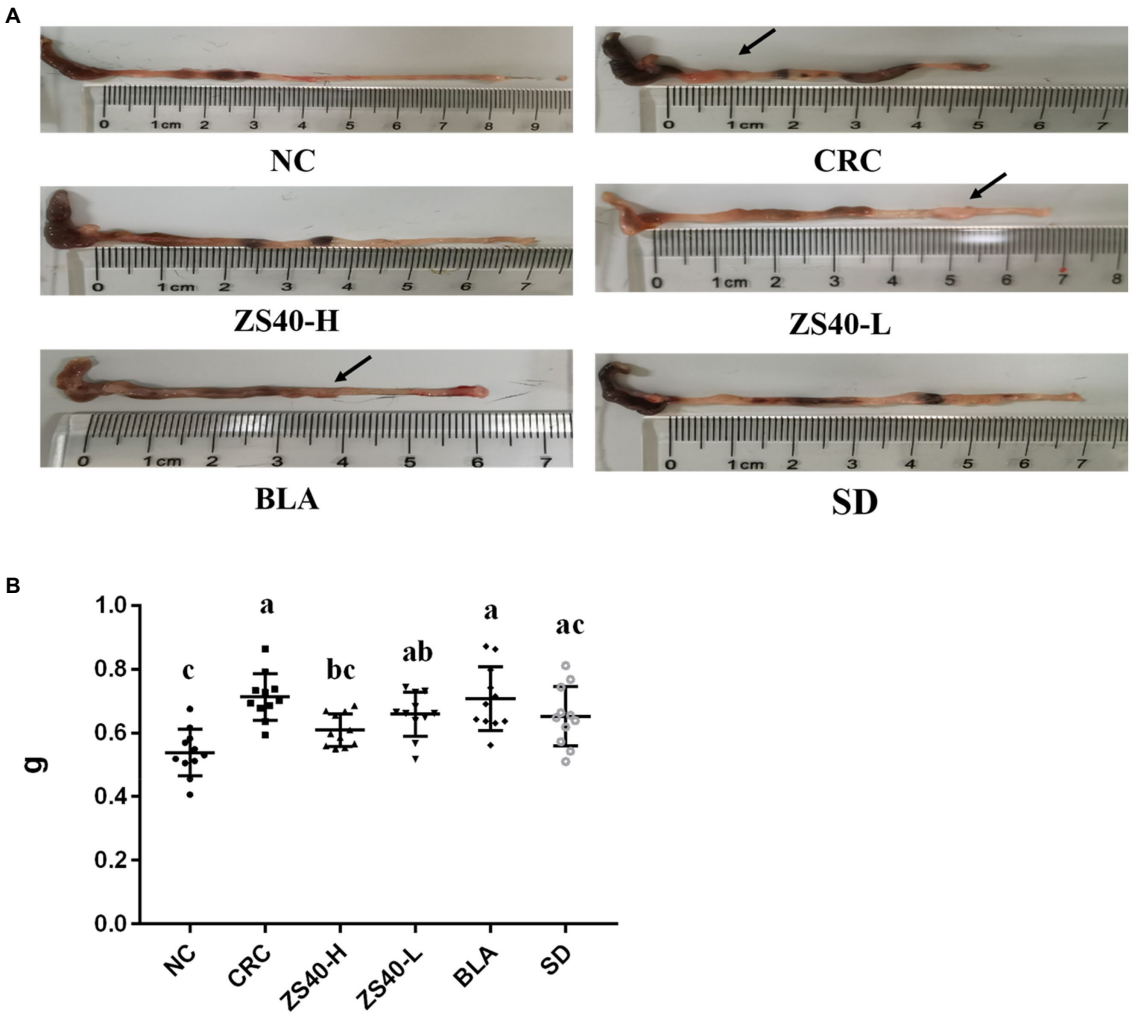

FIGURE 3
Anatomy of mice colon **(A)**: Colon length; **(B)** Colon weight. [a–c]Mean values with different letters over the same column are significantly different (p<0.05) according to Duncan's multiple range test. NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose *Lactobacillus fermentum* ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose *Lactobacillus fermentum* ZS40 (10^{9} CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD, mice treated with sulfasalazine (25%).

TABLE 1 Levels of inflammatory indexes in mouse serum samples.

	IL-1β (pg/ml)	IL-8 (pg/ml)	TNF-α (pg/ml)	MIP-1 (pg/ml)	VCAM-1 (pg/ml)
NC	22.675 ± 1.32^{bc}	24.274 ± 1.46^{c}	162.356 ± 15.04^{c}	5.209 ± 0.37^{c}	92.830 ± 6.75^{c}
CRC	30.697 ± 2.95^{a}	34.750 ± 2.78^{a}	257.238 ± 19.32^{a}	8.113 ± 0.50^{a}	143.874 ± 15.11^{a}
ZS40-H	24.432 ± 1.08^{c}	25.436 ± 1.22^{c}	202.718 ± 15.76^{b}	5.665 ± 0.69^{bc}	94.332 ± 7.11^{c}
ZS40-L	26.069 ± 1.22^{b}	26.661 ± 1.71^{bc}	212.658 ± 13.28^{b}	7.618 ± 0.38^{a}	129.738 ± 14.81^{b}
BLA	26.639 ± 1.46^{b}	27.681 ± 1.36^{b}	209.094 ± 17.04^{b}	7.678 ± 0.62^{a}	119.048 ± 11.73^{b}
SD	26.058 ± 1.85^{b}	24.650 ± 1.39^{c}	193.632 ± 21.78^{b}	6.106 ± 0.42^{b}	100.738 ± 6.36^{c}

Values are mean ± standard deviation (N = 10/group).
[a–c]Mean values with different letters over the same column are significantly different (p < 0.05) according to Duncan's multiple range test. NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose Lactobacillus fermentum ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose Lactobacillus fermentum ZS40 (10^{9} CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD, mice treated with sulfasalazine (25%); IL-1β, interleukin 1β; IL-8, interleukin 8; TNF-α, tumor necrosis factor-α; MIP-1β, macrophage inflammatory protein 1β; VCAM-1, vascular endothelial cell adhesion molecule 1.

pathological score of the severity of the colon of the mice (Figure 4B). In the CRC group, epithelial cells fall off in colon tissue, and lymphocytes and plasma cells infiltrate in lamina propria. We observed that the ZS40-H intervention treatment could reduce the accumulation of inflammatory factors in the colon tissue of CRC mice. Also, compared with the CRC group, the ZS40-L and

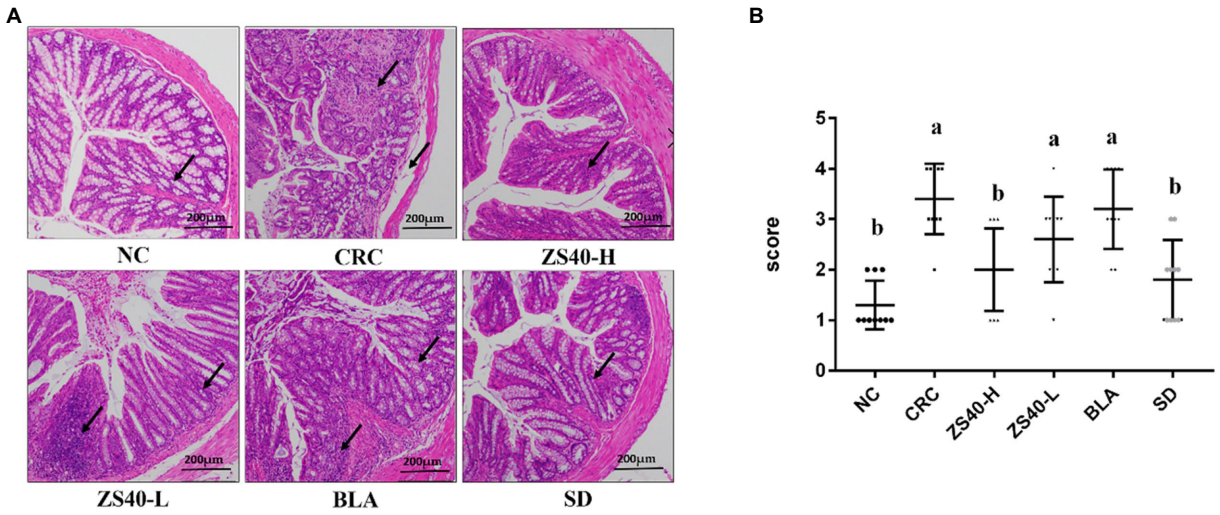

FIGURE 4
(A) Pathological section of mice colon (H&E) Magnification 10×. **(B)** Colon severity pathological scores 0 marks: intact villi and epithelium; 1 marks: slight submucosal or lamina propria swelling and separation; 2 marks: moderate submucosa or lamina propria swelling and separation and plasma cell infiltration; 3 marks: severe submucosal or lamina propria swelling and plasma cell infiltration, local villi atrophy and shedding; 4 marks: intestinal villi disappeared with intestinal wall necrosis Pathology score ≥2, regarded as intestinal injury. [a,b]Mean values with different letters over the same column are significantly different ($p<0.05$) according to Duncan's multiple range test. NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose *Lactobacillus fermentum* ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose *Lactobacillus fermentum* ZS40 (10^{9} CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD, mice treated with sulfasalazine (25%) The arrows point to the colonic mucosa and goblet cell structure.

BLA treatment groups and the BD control group had significantly reduced inflammatory infiltration of mouse colon tissues.

Effect of probiotic samples on expression of tumor markers

The staining intensities of CD34 and CD117 expression in colon tissue were observed with immunohistochemistry. As shown in Figure 5, compared with the NC group (AOD: CD34: 0.334 ± 0.01; CD117: 0.316 ± 0.01), the expression intensities of CD34 and CD117 were higher in colon tissue samples of the CRC group. In colon lesions, the expression levels of target cells (CD34 and CD117) were increased (AOD: CD34: 0.403 ± 0.01; CD117: 0.458 ± 0.02), the expression aggregation area of the target protein could be clearly observed, and the expression rates of positive results were higher. Compared with CRC, after intervention with ZS40-H, ZS40-L, BLA, and SD, the positive expression rate decreased (AOD: CD34 ZS40-H: 0.335 ± 0.05, ZS40-L: 0.398 ± 0.01, BLA: 0.349 ± 0.01, SD: 0.376 ± 0.01; CD117 ZS40-H: 0.367 ± 0.01, ZS40-L: 0.392 ± 0.04, BLA: 0.389 ± 0.01, SD: 0.369 ± 0.01). Among these, the ZS40-H and SD groups were more obvious.

Effect of probiotic samples on mRNA

RT-qPCR analysis confirmed that the accumulation of inflammation caused an increase of IL-1β, TNF-α, p65, IKKβ, TRAF-6, and Cox-2 expression levels in the colon tissue of the CRC mice. At the same time, mRNA expression of TRAF-1/2, IκBα, IKKα, Bcl-2, and Bcl-xL decreased (Figure 6). An intervention with probiotic *Lactobacillus fermentum* ZS40 and anti-inflammatory drugs could reduce the expression levels of IL-1β, TNF-α, p65, IKKβ, TRAF-6, and Cox-2 in the colon tissue of CRC mice and increase those of TRAF-1/2, IκBα, IKKα, and Bcl-2. As for the mRNA expression level of Bcl-xL, SD had a better intervention effect, and high-dose *Lactobacillus fermentum* ZS40 showed better effects in relieving inflammation.

Expression of key proteins in the NF-κB signaling pathway

We analyzed the protein expression in mouse colon tissues using Western blot analysis (Figure 7). Compared with the NC group, the expression levels of IL-1β and TNF-α in colon tissue of the CRC group were increased. The ZS40-H, ZS40-L, BLA, and SD groups had reduced expression of inflammatory factors in colon tissues, among which the ZS40-H and SD groups showed the most obvious reduction effect. The expression levels of p65, IκBα, and Cox-2 in colon tissue also showed a decreasing trend in the ZS40-H, ZS40-L, BLA, and SD groups, as compared with the CRC group. However, the difference between the ZS40-H and SD groups was not obvious.

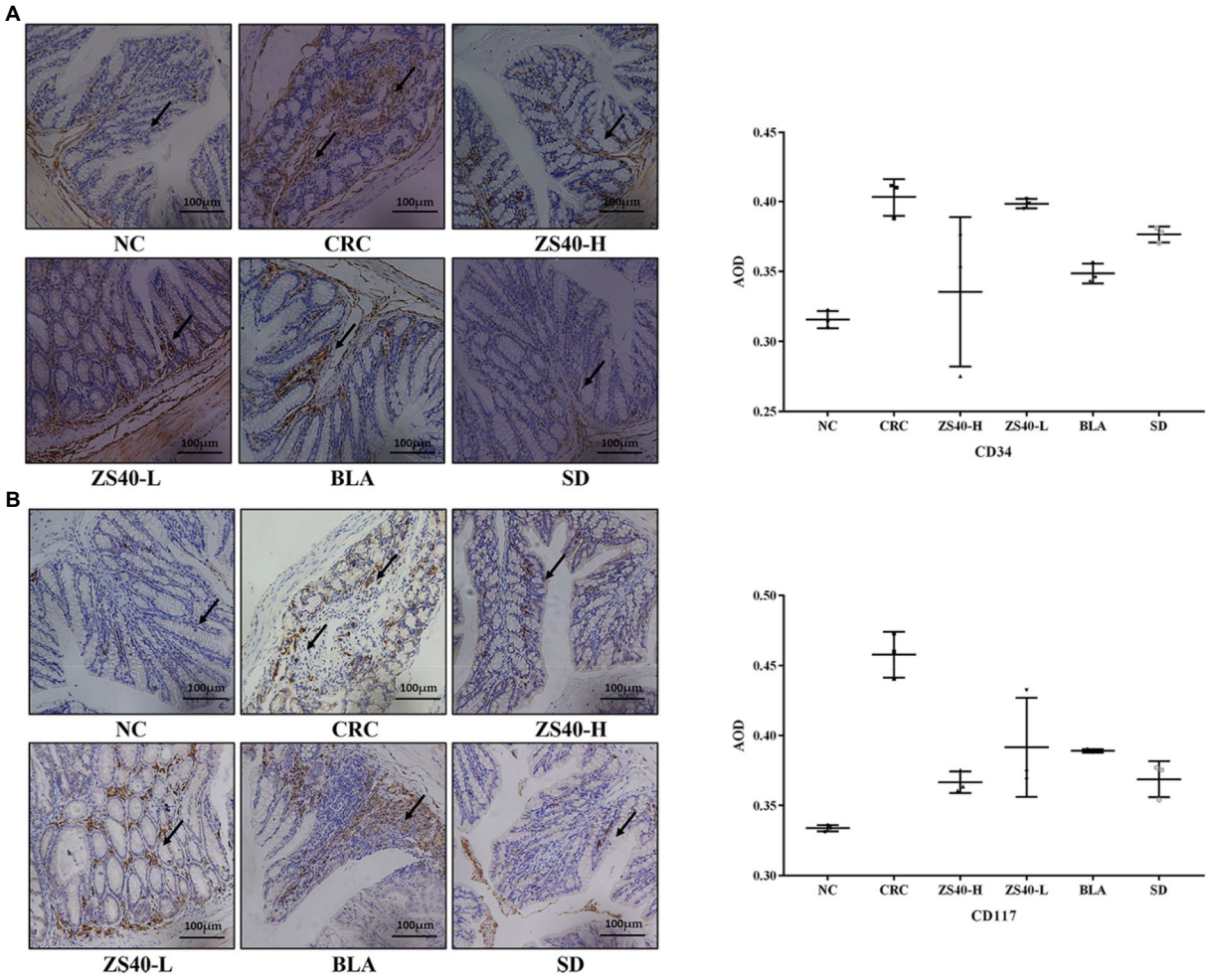

FIGURE 5
Pathological section of mice colon (immunohistochemical staining) Magnification 20× **(A)**: Indicator CD34; **(B)**: Indicator CD117; NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose *Lactobacillus fermentum* ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose *Lactobacillus fermentum* ZS40 (10^{9} CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD, mice treated with sulfasalazine (25%) The arrows point to the expression of the target protein.

Discussion

Epidemiological studies have confirmed that many tumors are caused by the repeated stimulation of inflammation (Li K. L. et al., 2019). Moreover, studies have shown that anti-inflammatory drugs can reshape the tumor immune environment and enhance the immune blocking effect (Pelly et al., 2021). Clinical studies have found that there may be a correlation between ulcerative colitis and colon cancer and that inflammatory cells are present in tumor biopsy samples (Wang et al., 2021). The clinical utility of inflammation-related biomarkers in routine blood tests has been reported in a variety of cancers, and Yamamoto, T. summarizes the prognostic impact of each inflammation-related marker on CRC (Yamamoto et al., 2021). Many previous studies have shown that long-term consumption of DSS solution can cause colitis, and the consumption of lactic acid bacteria has an intervening effect in preventing colon cancer caused by DSS (Wang C. Z. et al., 2018). The combined stimulation of AOM and DSS may induce colon cancer (Jeon et al., 2018). Research on the use of lactic acid bacteria to induce apoptosis in colon cancer cells is a popular research topic. In our study, the reason for successful interference of combined stimulation with AOM and DSS to induce colon cancer in mice can be attributed to the effect of long-term consumption of lactic acid bacteria.

Our results showed under the combined effect of AOM and DSS, obvious lump-like foreign bodies were found in the colon tissue of CRC mice, and the number and volume were larger. The intestinal tract is filled with a large amount of tissue mucus, the intestinal wall is thinned, and the blood vessels are obvious. It shows that the combined effect of AOM and DSS can form more obvious colon lesions in mice, and the intestinal inflammation is obvious. At the same time, the variation of lesions in the intestinal tissue of mice treated with *Lactobacillus fermentum* CQZS40 was improved. It is manifested as a smooth and elastic intestinal

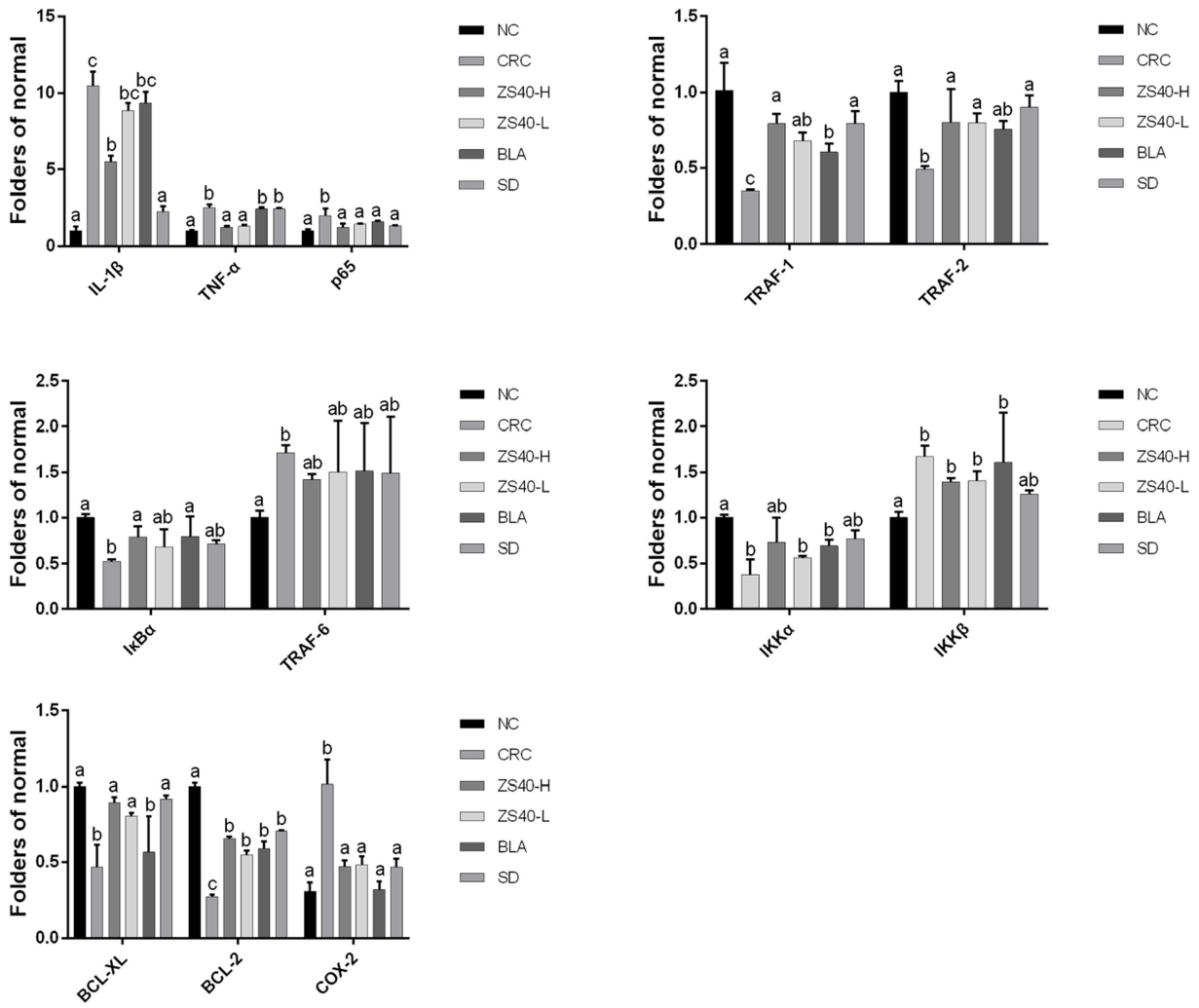

FIGURE 6
mRNA expression levels of inflammatory factors in colon tissue the difference in variance between the two groups was significant ($p<0.05$), [a–c]Mean values with different letters in the same column are significantly different ($p<0.05$) according to Duncan's honestly significantly different tests. NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose *Lactobacillus fermentum* ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose *Lactobacillus fermentum* ZS40 (10^{9} CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD: mice treated with sulfasalazine (25%).

surface, and a decrease in the number and volume of foreign body masses in the intestinal tract. The results showed that ingestion of active *Lactobacillus fermentum* was effective in preventing colon cancer induced by the combination of AOM and DSS.

The active cytokine TNF-α is secreted by macrophages and lymphocytes activated by endotoxins. It has been reported that the main role of TNF-α in the body is not to kill tumors but rather to promote tumor development as an inflammatory factor (Lv et al., 2020). Moreover, TNF-α is highly correlated with colorectal cancer (Hu et al., 2019). TNF-α can induce tumor immunosuppressive microenvironment formation by activating inflammation-related signaling pathways, as well as promoting tumor angiogenesis and tumor spread (Shen et al., 2019). Interleukins transmit information; activate and regulate immune cells; mediate the activation, proliferation, and differentiation of T and B cells; and play an important role in inflammation (Lerner, 2020). Among them, IL-1β is an inflammatory cytokine, which is widely involved in various pathological damage processes such as human tissue destruction and edema formation. IL-8 is an important inflammatory mediator (Xiao et al., 2018). Moreover, when the body is affected by infection and certain autoimmune diseases, IL-8 significantly increases local inflammation and is elevated in serum. In the detection of cytokine levels, the mouse serum levels of TNF-α, IL-1β, and IL-8 were in line with expectations. The increase in the levels of these factors is the basic condition for promoting the occurrence of inflammation.

Together with the action of various factors that promote inflammation, the body triggers a series of acute reaction and fever reactions and can also activate endothelial cells to increase vascular permeability. MIP belongs to a class of chemokine

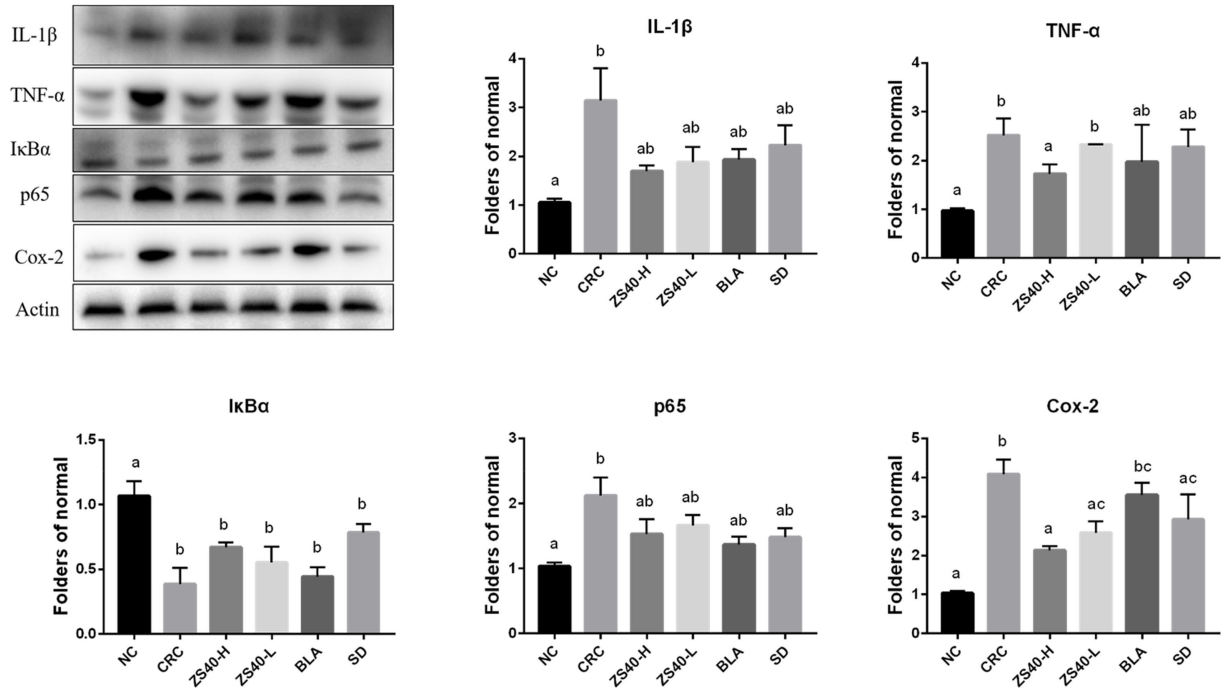

FIGURE 7
Expression of key proteins in the NFκB signaling pathway in colon tissue The difference in variance between the normal group and intervention was significant (p<005), $^{a-c}$Mean values with different letters in the same column are significantly different (p<005) according to Duncan's honestly significantly different tests. NC, normal untreated mice; CRC, colon cancer model mice induced with AOM-DSS; ZS40-H, mice treated with high-dose *Lactobacillus fermentum* ZS40 (10^{11} CFU); ZS40-L, mice treated with low-dose *Lactobacillus fermentum* ZS40 (10^{9} CFU); BLA, mice treated with Bulgarian strain (10^{11} CFU); SD, mice treated with sulfasalazine (25%).

(Chahar et al., 2018). MIP can activate granulocytes, regulate the adhesion of CD8+ T cells and vascular endothelial cells, participate in hematopoiesis regulation, and induce natural killer cell proliferation and activation. As an important factor that mediates the adhesion of leukocytes to vascular endothelial cells, vascular cell adhesion molecules play an important role in vascular injury. Studies have shown that the increase in MIP expression has an important role in the occurrence of vascular-related diseases (Yang et al., 2018). MIP-2 released during the early stage of inflammation chemotactically activates neutrophils to the site of inflammation, which then release a large number of proteolytic enzymes to cause inflammation (Subramanya et al., 2018). Vascular cell adhesion molecule (VCAM)-1 mediates cell-to-cell interactions and can induce the expression of VCAM-1 in the inflammatory tumor microenvironment by high expression of IL-1β (Shen et al., 2021). The results of the present research are similar to the abovementioned results of previous studies. The expression levels of MIP-1β and VCAM-1 were increased in the model group, but decreased after the *Lactobacillus fermentum* ZS40 intervention. This may be related to the reduction in inflammation factors TNF-α, IL-1β, and IL-8.

The expression level of protein indicators can express the condition of the body. The practical value of immunohistochemistry in tumor diagnosis and differential diagnosis has been generally recognized, and its accuracy rate can reach 50%–75% in the differential diagnosis of poorly differentiated or undifferentiated tumors. CD117 is a specific marker for gastrointestinal stromal tumors and is generally diagnosed in combination with CD34. At present, CD117 is mainly used in combination with CD34 in research of gastrointestinal stromal tumors. The adhesion of leukocytes and displacement of vascular endothelial cells to the inflammatory area are important processes in inflammation because of the interaction of adhesion molecules (Takayuki et al., 2019). Increasingly more research results show that CD34 molecules have an important role in mediating cell adhesion. In this process, CD34 can mediate the accumulation of leukocytes, initiate an inflammatory response, and simultaneously cooperate with chemokines to enhance the inflammatory response (Zhao D. W. et al., 2018). The CD117 protein is a type III tyrosine kinase growth factor, which results in strong membrane and cytoplasmic expressions in gastrointestinal inflammatory cells (Jelena et al., 2020). Our results support previous studies and provide evidence that high doses of *Lactobacillus fermentum* and sulfasalazine have better protection against colon cancer compared to *Lactobacillus bulgaricus* administration. This observation can be clearly observed in the significantly lower AOD levels, colon cancer morphology, and histological malignant changes in the probiotic-treated group compared with the AOM-DSS-treated group.

The NF-κB signaling pathway regulates key processes in the occurrence and development of various types of cancer (Zhu et al., 2018). The transcription factor NF-κB is a key mediator of

inflammatory responses (Barnabei et al., 2021). NF-κB may be one of the most common regulators of cancer because of the extensive involvement of target genes and tissues. NF-κB protein has been detected in epithelial cells and macrophages of patients with ulcerative colitis, which provides evidence for constitutive NF-κB activation (Tong et al., 2019). Under the stimulation of pro-inflammatory cytokines TNF-α, interleukin IL-8, and other extracellular factors, IKKβ is activated after phosphorylation of TAK1 protein, resulting in the degradation of the IκB protein and the release of NF-κB dimers. Pro-inflammatory cytokines also induce p65 and activate the NF-κB pathway (Yang et al., 2019). Our detection of serum inflammatory cytokine levels and the detection of intestinal tissue gene and protein expression levels are in line with previous studies, and provide evidence that under the combined induction of AOM-DSS, the NF-κB signaling pathway in mice is activated, which is manifested in the body increased levels of inflammation. Compared with the CRC group, the *Lactobacillus fermentum*-treated group had lower detection levels of inflammatory signaling pathway stimulators IL-1β and TNF-α, and increased expression of the activating protein IKKβ, resulting in the degradation of IκBα and the increase in the expression of aggregate NF-κB. The above results can indicate that the role of *Lactobacillus fermentum* can protect mice from reducing inflammatory stimulation.

At the same time, studies have found that when the NF-kB signaling pathway is activated, a series of changes in the expression of oncogenes and proteins will occur. The expression of Cox-2 is closely related to NF-kB. It has been reported that in the research on Cox-2 selective inhibitors, it has a clear efficacy similar to NSAIDs in terms of analgesia and anti-inflammatory, and can protect gastrointestinal cells and prevent ulcer formation (El-Malah et al., 2022). Kitagawa et al. compared the expression of Cox-2 in colon cancer and normal colon tissue, and the results showed that Cox-2 was overexpressed in colon cancer tissue, while the expression of Cox-2 in normal colon mucosa was negative (Kitagawa et al., 2022). Bcl-2 is a major inhibitor of apoptosis gene. Theoretically, when it shows a high-intensity level, it causes a stronger result of inhibiting apoptosis. Corresponding results showed that the greater the probability of tumor cells evading apoptosis, the higher the malignancy of the tumor. The expression of Bcl-2 in various tumors has been confirmed. For example, Abdel-Wahab et al. showed that Bcl-2 protein expression is positively correlated with the differentiation of colorectal cancer and is a useful indicator for judging the malignancy of colorectal cancer (Abdel-Wahab et al., 2021). Bcl-xL is an anti-apoptotic protein belonging to the Bcl-2 family, which is involved in the regulation of cell apoptosis. Play an important role, a large number of studies have reported that Bcl-xL is highly expressed in colorectal cancer (Ramesh et al., 2021). The changes of Cox-2, Bcl-2, Bcl-XL are also reflected in our detection results, Cox-2, Bcl-2. The higher level of expression of Bcl-XL was found after the activation of the signaling pathway. This trend was suppressed in the *Lactobacillus fermentum* treatment group. This result is also in line with the experimental expectation.

Activation of the NF-κB canonical pathway ultimately leads to increased expression of NF-κB target genes such as IL-8, Bcl-2, and Cox-2. High expression of these pro-inflammatory factors, in turn, further exacerbates the stimulatory pathways that continue to affect cell proliferation or renewal. Therefore, NF-κB may contribute to the occurrence of colitis-associated colon cancer by maintaining a continuous inflammatory process in the intestinal mucosa. The direct and indirect effects of this transcription factor complex on tumorigenesis and progression have been validated in various animal models such as hepatocellular carcinoma, gastric cancer, and lung cancer (Ji et al., 2018; Wang J. J. et al., 2018, 2020). In our study, the *Lactobacillus fermentum* treatment group blocked the continuous development of the signaling pathway by affecting the important turning points of the NF-κB classical pathway, such as IκBα, p65, and IKKβ, as well as the release of pro-inflammatory factors.

Conclusion

Our present study showed that the consumption of *Lactobacillus fermentum* CQZS40 can reduce the occurrence of colon cancer tumors induced by AOM and DSS by inhibiting the NF-κB classical signaling pathway. The present results confirmed that experimental animals in the ZS40-H intervention group showed a decrease in the number of intestinal tissue cysts and a decrease in the level of intestinal inflammation compared with the CRC group, by affecting the release of inflammatory cytokines, gene and protein expression in colon tissue. Moreover, both high and low doses of Lactobacillus fermentum ZS40 could effectively inhibit the NF-κB signaling pathway. Among them, high doses of *Lactobacillus fermentum* ZS40 were more effective in inhibiting pro-inflammatory factors and regulating key proteins in the signaling pathway. These results may be related to the regulation of intestinal flora in the body after the ingestion of *Lactobacillus fermentum* ZS40. The present research finding is of great value for subsequent research on related areas and has inspired the authors' interest in researching the mechanism of *Lactobacillus fermentum* ZS40 in the intestine.

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

This study was approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food (20190902B, Chongqing, China) and followed the national standard of the People's Republic of China (GB/T 35892-2018) laboratory animal guidelines for ethical review of animal welfare.

Author contributions

JL and SW performed the majority of the experiments and wrote the manuscript. RY and XL contributed to the data analysis. XZ designed and supervised the study, and checked the final 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/fmicb.2022.953905/full#supplementary-material

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Potential application of the probiotic *Bacillus licheniformis* as an adjuvant in the treatment of diseases in humans and animals: A systematic review

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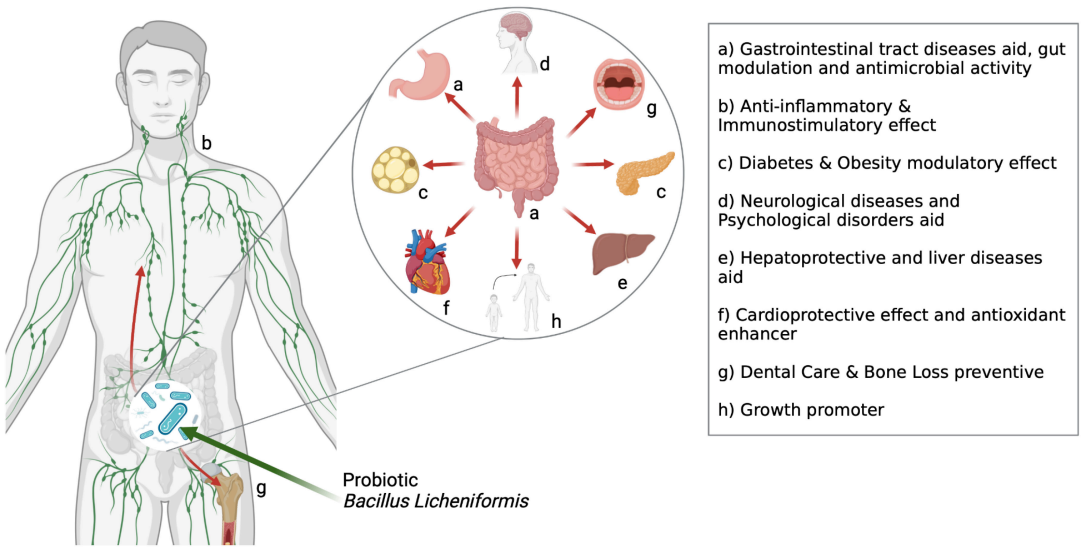
The use of *Bacillus licheniformis* as a probiotic has increased significantly in recent years. Published reports demonstrate that it provides multiple benefits for health. Although there are already studies in humans and is marketed, it is mostly used in the veterinary industry still. However, its benefits could be extrapolated to humans in future. This review addresses the application of *B. licheniformis*, its sporulation, mechanisms of action, and its role in the resolution, treatment, and prevention of different conditions and diseases. It focuses on scientific advances from 2016 to mid-2022 and emphasizes the most common diseases in the general population. Most of the 70% of published studies about the health benefits of *B. licheniformis* have been published from 2016 until now. The intake of *B. licheniformis* has been related to the effects of modulation of the intestinal microbiota, antimicrobial activity, growth promotion, anti-inflammatory and immunostimulatory effects, promotion of the regulation of the lipid profile, increase of neurotransmitters, and stress reduction, among others. These results provide novel possible applications of this and other probiotics in general. Although many benefits can be reported on a microorganism, the combination with others could provide a better effect. Further studies like this need to be done to understand the specific advantages of each probiotic and its strains and therefore achieve a better selection of them for a specific disease or disorder.

KEYWORDS

***Bacillus licheniformis*, functional ingredients, adjuvant in treatment, human disease, animal disease**



GRAPHICAL ABSTRACT

Mechanisms of action reported for *B. licheniformis*. More than 70% of studies about the effect of *B. licheniformis* on health have been published from 2016 to 2022. The most published health effects are supported in gastric and liver diseases, diabetes, and obesity. It has also been reported for its immunological, antimicrobial, anti-inflammatory effects.

Introduction

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" according to the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) (Bielecka, 2006). Probiotics are also employed frequently to maintain the balance of the internal microbiota and therefore human health (Sanders et al., 2011). Recently, probiotic application is focused on reducing the risk of developing a range of illnesses since gut microbial populations are not permanent and can be altered by numerous factors such as lifestyle, diet, and antibiotics. A variety of research has established the positive effects of probiotics and their link with intestinal disorders. However, there is still information that could delve into diseases apart from gastrointestinal disorders, which can be concentrated directly or indirectly.

The gut microbiota is regulated by several factors that have also been related to disease prevention and treatment, for example, age, since it modifies proportions of microbial phylum during the different stages of life (Nagpal et al., 2018). Furthermore, dysbiosis of the intestinal microbiota has been linked to the development of disorders. A homeostatic gut microbiota population is required for the host and the microbiome to coexist in symbiotic association (Kim et al., 2019).

Abbreviations: BL, *Bacillus licheniformis*.

Bacillus Licheniformis is a gram-positive, endospore-forming, mesophilic bacterium belonging to the species of firmicute in the family *Bacillaceae* (Makowski et al., 2021). It can be found in raw milk as a contaminant, it is ubiquitous in soil and food on farms (Banykó and Vyletělová, 2009). It has also been isolated from buttermilk powder, pea, or mushroom soups, and in general in food as spoiling bacteria (Krawczyk et al., 2016).

Bacillus Licheniformis plays an important role in the biotechnology field as a strain for expression platform, compound producer, environmental applicant, and finally as a probiotic (Muras et al., 2021). This latter application has included products for human health, veterinary application, and aquaculture, alone or combined with other probiotic strains (Muras et al., 2021). Different species of *Bacillus licheniformis* probiotics have been shown and analyzed to adapt to the human gastrointestinal tract. However, some probiotics carrying *B. licheniformis*, moreover, remain considered unsafe due to their antibiotic resistance and the possibility of spreading resistance to other pathogenic bacteria. As a result, before using a strain in a procedure, it is essential to check for antibiotic resistance genes (Sorokulova et al., 2008; Muras et al., 2021).

To the best of our knowledge, an exhaustive review of *Bacillus licheniformis* focused on its uses as a probiotic, spore formation, mechanism of action, and experimental results centered on different diseases is still lacking in the literature. Therefore, this review provides an exhaust summary of the recent literature and our analysis of the data provided on the current state of knowledge about experimental and clinical research that may allow a more comprehensive

perspective of the therapeutic potential of *B. licheniformis* alone or in combination.

Bacillus spore formation and germination

Under environmental stress, such as nutritional restriction, *Bacillus spp*. bacteria produce spores (Todorov et al., 2022). Spores are a specific cell type made up of metabolically inactive cells that can withstand chemical and physical challenges like air drying, high temperatures, high pressure, UV light, and acidity. The presence of numerous distinct layers and the spore core's high dehydration level contributes to this resistance. *Bacillus spp*. begin sporulation near the conclusion of the exponential–stationary growth phases, when nutrients are limited, and the formation of heat-resistant spores requires around 8 h (Elisashvili et al., 2019). Sporulation can be caused by nutritional stress and by exposing the cells to harsh environmental conditions such as pH and temperature extremes. This life cycle event represents a great opportunity for increasing probiotic strain development, storage, and distribution to customers from a biotechnological standpoint (Mingmongkolchai and Panbangred, 2018).

However, although these spores can potentially resist harsh conditions, there is a specific limit. For the case of *Bacillus licheniformis*, combining very high pressures and temperatures resulted in a combined impact on spore germination and inactivation and the involvement of specific germination receptors in its processes (Borch-Pedersen et al., 2017). Also, during germination, it may have specific enzymatic activities compared to other *Bacillus* species, resulting under specific nutritional optimum conditions. For example, the bacterial spore cortex is essential for spore latency and stability, and germination-specific lytic enzymes must hydrolyze it to allow proper germination and cell outgrowth (Giebel et al., 2009). The breakdown of the cortex is an important stage in spore germination. The cortex-lytic enzymes involved in the depolymerization of cortical peptidoglycan in *B. licheniformis* spores are close to those found in other *Bacillus* species; however, some investigations indicate that the primary enzymatic activity found during germination is about a lytic transglycosylase, probably SleB, and this protein seems to play a more significant role in *B. licheniformis* spore germination than in other *Bacillus* species (Aspholm et al., 2019). Each probiotic has positive characteristics on the organism depending on its specific strain and this impacts through various mechanisms (Kechagia et al., 2012). The benefits of *B. licheniformis* as a probiotic when compared to other sources of natural and encapsulated probiotics mainly lie in sporulation, since this can contribute to greater survival in the adverse conditions found in the stomach and allow its arrival to the small intestine (Casula and Cutting, 2002).

Clinical trials of *Bacillus licheniformis*

The Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines were followed when conducting this review (Page et al., 2021). PubMed platform was browsed for "*Bacillus licheniformis*" as a first approach for the number of articles of this specie and then followed by "*Bacillus Licheniformis* probiotic." The data covered the period from 2016 to mid-2022 (July 1st). The eligibility criteria were the explicit use of *Bacillus Licheniformis* alone or in combination with other strains and focused on studies on diseases and disorders with scientific proof of their correlation with the probiotics. Experimental models (humans, animals, and *in vitro*) were considered. Some limitations include the possible articles that are not on this database and the studies before 2016. However, the aim of this review is to provide a general perspective of the current state of the art. **Figure 1** shows the PRISMA identification of studies via database and registers. A total of 157 articles were reviewed and represent almost 70% of the articles focused on BL as a probiotic since 1994 on this search engine. Except for 2020, which may have been mowed due to the COVID-19 outrage, there has been a noticeable increase in the number of articles since 2016.

There has been a surge in interest in using probiotic supplements as mediators in health and disease in recent years. This appeal is primarily motivated by mounting the evidence of the relationship between microbiota and disease

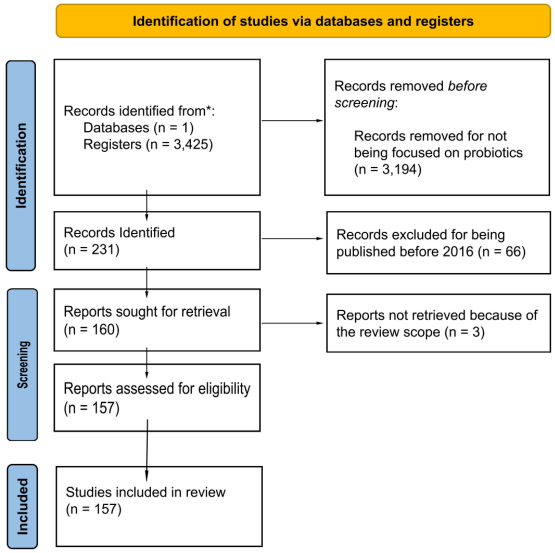


FIGURE 1
PRISMA Identification studies via database and registers. This figure illustrates the methodology followed in the development of the research on this review based on the PRISMA 2020 statement (Page et al., 2021).

pathophysiology within the human body (Day et al., 2019). For the case of *B. licheniformis* (Figure 2), this is not an exception, its spore-forming advantages mentioned before, and the harmful conditions that can resist making it a more interesting and novel probiotic against the different diseases studied. As shown in Figure 3, its potential effect on 12 different groups of diseases and disorders was studied. The proportion of scientific articles published revealed the completeness that this probiotic could give parting from general homeostasis to a specific organ or disease. Although many of the articles combine it with other probiotic strains, its lone effect has also been deepened. Most of the studies include animal trials or *in vivo* models, almost 75% of the total were found. The distribution of studies is shown in Figure 4, and this proportion could be because of the ease to deepen results in animals compared to human models, or for being more exhaustive than the *in vitro* studies. Human models have limitations like difficulty in interpreting

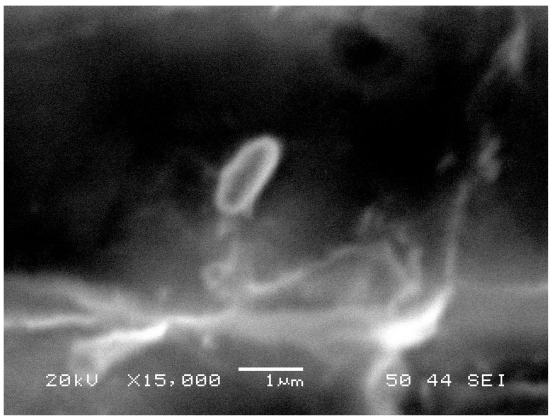


FIGURE 2
Scanning Electron Microscopy of Isolated *Bacillus Licheniformis*. The photography was taken by a JEOL JSM-6360LV using a magnification of 15,000X and an accelerating voltage of 20 kV.

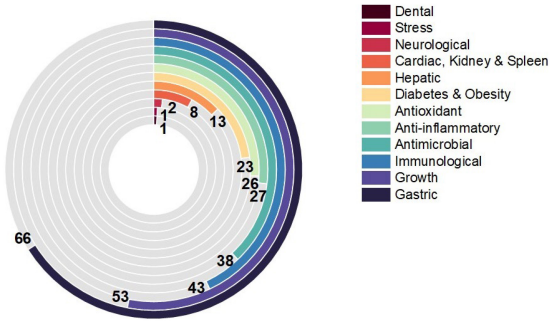


FIGURE 3
Proportion of reviewed articles on the evaluation of BL in 12 disorders. The number of articles that evaluated the effect of *Bacillus licheniformis* alone or in combination for each of the diseases is shown, and the appearance order is descending.

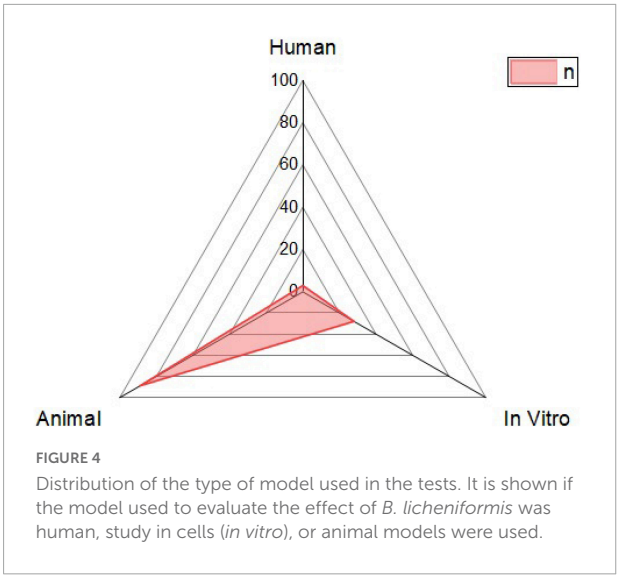


FIGURE 4
Distribution of the type of model used in the tests. It is shown if the model used to evaluate the effect of *B. licheniformis* was human, study in cells (*in vitro*), or animal models were used.

or generalizing the results, since the population investigated differs significantly from the one treated in daily life; also, participation in a study may have an impact on the outcome, since the restricted viewpoint of many trials leaves out critical information linked to the repercussions of the therapy on life quality, contentment, or expenses (Collet, 2000). The human trials of this review and their main outcomes are shown in Table 1. Most of the *in vitro* studies are a complement to animal studies, which may include *in vitro* biochemical assays or resected tissues. Herein, the results are shown by the group of diseases.

Gastrointestinal tract diseases

The most addressed area in the study of probiotics is the gastrointestinal system since they proliferate in it. Therefore, many benefits have been demonstrated. For this section, a total of 68 articles showed different uses for the gastrointestinal tract when administrated *B. licheniformis*, mainly findings on microbiota modulation, followed by approaches to gastrointestinal benefits in livestock animals and finally in specific diseases such as enteritis, colitis, diarrhea, etc. One of the most relevant studies showed that in combination with *B. subtilis* could achieve an increased villus height in the ileum and a decrease in the crypt depth in the jejunum as well as the ratio of both, which can improve nutrient absorption and general digestion (Wang et al., 2021). Another study using the same combination of probiotics found that they secrete the enzymes protease, lactase, lipase, and amylase, which also provide benefits in digestion (Yang et al., 2021). Moreover, working individually with the diversity of the microbiota, an increase in *Lactobacillus* and *Firmicutes* was obtained (Chen and Yu, 2020). Also when induced colitis in rats, it lowered

TABLE 1 Human articles summary.

Assay	Model	Relevant information	Year	Reference
Triglyceride level lowering	Human	Twelve-week trial using *Bacillus licheniformis* in combination with another spore forming bacilli probiotics have remarkable triglyceride lowering	2020	Campbell et al., 2020
Radiotherapy side effects protection	Human	In pediatric patients with central nervous system tumor, probiotics may have a critical preventive function in the etiology of gastrointestinal symptoms caused by radiotherapy. Gut barrier function, innate immunity, and intestinal repairmen can all be influenced by *Bacillus licheniformis* probiotics. Probiotics given prophylactically during irradiation therapy for CNS tumor patients can alleviate RT-related symptoms and improve cancer patients' quality of life, in part by lowering inflammatory reactions and gastrointestinal toxicity.	2017	Du et al., 2018
Endotoxemia, Triglyceride lowering and inflammatory biomarkers	Human	After 30 days of *B. licheniformis* with other spore-based probiotic supplementation, dietary endotoxin, triglycerides, and possibly systemic inflammation were reduced. Changes in the gut microbiome, gut permeability, or a combination of the two could be the underlying cause of the observed reductions in post-prandial endotoxemia. More research is needed to confirm if a longer period of treatment with a spore-based probiotic leads to further health benefits.	2017	McFarlin et al., 2017

Related to human clinical trials of *B. licheniformis*, assay focuses, and main outcomes.

parameters of inflammation, weight loss, severity, and colon shortening (Li et al., 2019).

From the revised study models, the various types of studies focus on birds, mainly broilers, principally hens, and chickens, followed by those derived from pigs and marine animals, and finally rodents. Therefore, we can visualize that current gastrointestinal studies of *B. licheniformis* are more focused on the benefits for farm animals, and they seek to avoid the consumption of antibiotics by replacing them with probiotics. However, with the results on inflammation and gut microbiota regulation, it should be considered in further studies like irritable bowel disease, constipation, and colorectal diseases in animal models and humans.

Growth promoter

Probiotics have already been proven to enhance the absorption of some nutrients (calcium, zinc, and vitamin B12) and lower the incidence of anemia, which may help children grow by preventing infections and micronutrient shortages. Previous research has explored the effects of probiotics on the diet in terms of weight and height gain in malnourished children, as well as the possibility of weight gain in well-nourished children in underdeveloped nations (Onubi et al., 2015). It has been proposed that supplementing locally accessible foods with probiotics could be a useful intervention for improving child growth, particularly in underdeveloped nations (Onubi et al., 2015).

However, for the specific application of *B. licheniformis* in animals and growth, a total of 55 studies were found, being one of the most relevant uses for this probiotic. Our study observed that most animal publications reported positive results on growth, mass gain, and feed conversion against weight gain. These publications also show that the number of viable units of *B. licheniformis* and its markers are associated with overall homeostasis. It was found that supplementing broilers with this probiotic significantly improved their weight and gain (Rodrigues et al., 2020), as well as their average daily feed intake. The growth was also associated with *B. licheniformis* competitive growth against pathogens (Chen and Yu, 2020). In lambs, the supplementation shows a significantly low feed efficiency (dry matter intake/average daily gain) (Jia et al., 2018). Also, results from a study on the pathogenicity of GFP-tagged *Vibrio parahaemolyticus* Dahv2 and the protective impact of the probiotic strain, *Bacillus licheniformis* Dahb1, on Asian catfish indicated that these organisms could be employed to manage aquatic illnesses and benefit the aquaculture sector (Gobi et al., 2016). This can potentially contribute to improving not only the health and fitness of animals intended for human consumption but also to human studies and child growth.

Anti-inflammatory and immunostimulatory effects

Regarding the close relationship that the immune system and its inflammatory process have with the digestive system, a total of 44 studies for immune response parameters and trials were found and 27 studies had different inflammatory markers on different models. Current information showed that in combination with *B. subtilis*, a slight increase in IL-10 can be obtained, a decrease in TNF-α, and a protective effect when exposed to a specific antigen (Deng et al., 2017). Also, it has been reported that the intake of *B. licheniformis* is related to a reduction of pro-inflammatory cytokine IL-8 and an increase in of IgM and, IgG (Sun et al., 2021), while IgA antibodies, and higher concentrations of total serum proteins and globulins was also found (Gong et al., 2018). In a report about the challenge of the pathogen *E. coli* and in combination with *B. subtilis*

in pigs, the authors found upregulation in the expression of TLR4, NOD2, iNOS, IL-8, and IL-22, CCL28 chemokines and its CCR10 receptor mRNA genes, and an increase of subpopulations of CD4-CD8- T-cells and no changes in the number of IL-7Rα-expressing cells (Yang et al., 2016). A specific summary of the most relevant articles on the anti-inflammatory effect of *Bacillus licheniformis* is shown in **Table 2**. In the aquaculture field, the enhancement of immunity is frequently mentioned as a reason why probiotics employed in the fishery are efficient at mediating protection against pathogenic diseases. Long-term feeding could maintain the persistent activation of immune cells throughout the feeding time. An application of BL probiotics was successful in sustaining systemic and mucosal immunity, as well as resistance to *A. hydrophila* (Gobi et al., 2018).

When compiling the data, it was observed that the deepest and most available studies on mediators and effectors related to the immune system are found in mice and broilers, but there are still many areas of the immune system associated with this probiotic pending to be explored, since its participation on multiple metabolic pathways could aid on immune-related diseases and disorders, principally the ones related to reported inflammatory mediators.

Antimicrobial activity

Scientific research has centered on studying pathogenic microorganisms and developing methods to prevent and treat human illnesses for many years. Conversely, in a symbiotic connection, other bacterial species may benefit the host. Antibiotics are not only the most common method used to treat infections currently (Silva et al., 2020) but also it may affect commensal bacteria in the host. Antibiotic overuse could have negative consequences both for patients and public health, such as drug-specific adverse effects and the selection of multidrug-resistant microorganisms. Probiotic formulations with immunostimulatory effects or inter-bacterial competition between beneficial and harmful bacteria are among the many that claim to benefit human health. Probiotics have been suggested as a novel and viable technique for controlling and preventing a variety of infectious diseases in this specific topic (Yang et al., 2020). For this probiotic, a current research found that *B. licheniformis* produces proticin, an antibiotic of phosphorus-containing triene (Todorov et al., 2022).

For the antimicrobial results in *B. licheniformis*, a total of 27 studies demonstrated its potential effect. In aquaculture, a study on zebrafish (*Danio rerio*) challenged the potential probiotic *Bacillus licheniformis* protective effects and *in vitro* antagonistic activities against GFP-tagged *Vibrio parahaemolyticus*. Zebrafish infected with it had 100% death, but zebrafish treated with *B. licheniformis* experienced total survival after 30 days (Girija et al., 2018). Also, in combination with *B. Breve*, an *in vitro* study showed significant inhibition against the adhesion of the pathogenic *K. rhizophila* (Rohith and Halami, 2021) and anti-vibrio activity (Sekar et al., 2019). The crude extract reveals antiviral activities against porcine epidemic diarrhea virus in Vero cells, and lower the viral shedding in piglets (Peng et al., 2019), Also in piglets, the sodium butyrate generated by *B. licheniformis* improves *Salmonella* shedding (Barba-Vidal et al., 2017). Other studies show that this probiotic has antimicrobial proteins, and high auto- and co-aggregation capabilities against pathogenic bacteria (Pahumunto et al., 2021). In the biomedical fields, the biosynthesis of silver nanoparticles employing the probiotic *Bacillus licheniformis* may be applied to manage bacterial populations that create biofilms (Shanthi et al., 2016). Multiple studies show that intestinal eubiosis is attributable to the inhibition of pathogenic

TABLE 2 Anti-inflammatory main articles.

BL strain/brand	Model	Relevant information	Year	Reference
Bacillus licheniformis Zhengchangsheng® (BL20386)	Rats	A significant decrease in serum LPS level in the BL & Prebiotic group compared with the high-fat diet group, implying the alleviation of endotoxemia and systemic chronic inflammation. Rat liver was also evaluated for the levels of LPS after treatment. The liver LPS was significantly lower in the BL group compared to the HF group.	2020	Li et al., 2020
MegaSporeBiotic™	Rats	Pretreatment with probiotics BL with other spores resulted in a significant reduction in serum AST, ALT, proinflammatory cytokines (TNF-α, IL-1β), ZO-1 and TAC, as well as hepatocyte necrosis, which was similar to the well-known hepatoprotective agent, silymarin.	2020	Neag et al., 2020
Bacillus licheniformis Zhengchangsheng® (BL20386)	Mice	Studies on the mouse model show that Dextran Sulfate Sodium induced colitis changed the diversity of the intestinal microbial composition and diversity led to an increase of inflammation in colon, which was counteracted by BL administration.	2019	Li et al., 2019
Not specified	Human	The radiotherapy treatment in experiment group markedly upregulated the serum level of ET, CRP, and TNF-α, IL-1β, IL-6.	2018	Du et al., 2018
Not specified	Rats	The treatment of probiotics decreased systematic inflammatory responses as evidenced by the decrease of TNF-α	2017	Deng et al., 2017

Most relevant articles and outcomes of *B. licheniformis* supplementation in anti-inflammatory effects.

microorganisms, so the aforementioned factors can achieve a reduction in infections. Although most of the studies focus on animals, multiple studies show that probiotics from the *Bacillus* family have antimicrobial properties also in humans and therefore comparable results can be expected (Hallaj-Nezhadi et al., 2022). Despite the promise advantages of probiotics for intestinal health, there is still no agreement or standardization on delivery techniques or the use of probiotic dosage forms for antimicrobial therapy; however, *B. licheniformis*, because of its potential effect as an antimicrobial agent and its survival through the gastrointestinal tract, could be a novel strain for the research of it.

Antioxidant capacity

Oxygen species, mainly referred to as free radicals, and oxidative stress are a matter of concern nowadays. Recent studies have reported that antioxidants are produced by probiotic strains that scavenge hydroxyl radicals and superoxide anions. Molecular pathways of diabetes, atherosclerosis, inflammatory bowel disease (IBD), and damage to the heart, brain, or transplanted organs have all been linked to oxidative stress. The most acceptable species and strains for a probiotic antioxidative intervention for a certain clinical condition must be carefully considered (Hoffmann et al., 2019).

For the specific results of *B. licheniformis*, 27 studies revealed its potential effect. In a study with fish, it was shown that in combination with *B. subtilis*, they improved the levels of glutathione s-transferase (GST), glutathione reductase (GR) (Salehi et al., 2022), and combined decrease in the T-BARS marker was obtained, which indicates an increase in antioxidant enzymes (Guardiola et al., 2017). This probiotic alone had a positive impact on antioxidant capacity in the liver, serum, and intestine in birds (Zhao et al., 2020). Another study revealed that dietary supplementation with *B. Licheniformis* Dahb1 could improve innate immune function by reducing the oxidative stress linked to ammonia accumulation in tissues and blood (Gopi et al., 2022). The study for specific probiotic strains that give the most effective prevention and mitigation of oxidative stress must be continued to produce novel products with the potential to prevent oxidative stress. Further research is required to fully understand the antioxidative capabilities of prospective probiotics. Although most of the studies focus on animals, multiple studies show that probiotics from the *Bacillus* family have antioxidant properties also in humans and therefore comparable results can be expected.

Diabetes and obesity modulatory effect

Metabolic disorders can encompass a set of diseases that lead to different routes and mechanisms that affect many vital organs; that is why for purposes of this review we have specifically limited the metabolic section to diabetes and obesity, since other parts could address gastrointestinal, hepatic, cardiovascular, and neurological problems and are deepened on the other sections or have not been studied yet. Gut microbiome regulation and probiotic beneficial metabolic effects have been investigated in patients with type 2 diabetes mellitus. Probiotics have lower total cholesterol, triglyceride levels, CRP, inflammatory biomarkers, glucose, insulin, and blood pressure regulation. Also, they have shown an improvement in HDL levels without affecting BMI or LDL levels (Kocsis et al., 2020).

Table 3 summarizes the most relevant articles on this topic. A total of 23 studies revealed the potential effect of *B. licheniformis*, alone or in combination with other probiotics or prebiotics in parameters related to diabetes and obesity, such as glucose levels, lipidic profiles, etc. Some of the mechanisms involved with *Bacillus licheniformis* are activating the AMPK pathway and suppressing the NF-κB (Lu et al., 2021). These effects specifically for *B. licheniformis* and its potential role as a supplementary therapeutic method were demonstrated in mice with a high-fat diet induced, since they showed a reduction in body weight, while it improved glucose tolerance, obesity, and insulin resistance (Cao G. T. et al., 2019). Also in high-fat diet rats, a reduction in total cholesterol, triglyceride, LDL levels, and body weight gain was observed at the same time that strains linked to obesity were reduced in microbiota composition (Li et al., 2020). Finally in humans, a 12-week trial of BL in combination with other spore-forming bacilli probiotics showed significant triglyceride reduction in patients with hypertriglyceridemia (Campbell et al., 2020). Further studies on long-term administration need to be done to complement the effect on this type of metabolic disease.

Liver diseases

The gut–liver axis in most liver diseases has been proved, from the simple pathogenesis of fatty liver diseases (both alcoholic and non-alcoholic) to liver failure and, finally cirrhosis (Wiest et al., 2017). In *Bacillus licheniformis* specifically, a total of 13 studies showed its potential effect to prevent liver damage. Some of the results include the modulation of the expression of genes linked to fatty acid production and oxidation in the liver (Zhao et al., 2020), prevention of mild fibrosis and piecemeal necrosis in the liver (Wang et al., 2020), acute liver toxicity induced with acetaminophen, one of the most used analgesics and antipyretic agents in the world (Neag et al., 2020), reduction of liver weight, hepatic steatosis and effective alleviation of liver inflammation, possibly by modulating the NF-κB signaling pathway (Lu et al., 2021), and many others.

In the most relevant articles, BL interaction with gut bacteria showed a positive influence on liver damage, and a study on sheep and lambs has resulted in a significant decrease

TABLE 3 Diabetes and obesity main articles.

BL strain/brand	Model	Relevant information	Year	Reference
Bacillus licheniformis Zhengchangsheng® (BL20386)	Mice	Decreased weight gain, fat formation, serum lipid profiles, and proinflammatory cytokine values. Improved lipid and glucose metabolism. Nuclear factor-B activation was inhibited, phosphorylated AMP-activated protein kinase activity was enhanced in the liver, and the expression of genes involved in lipid metabolism was modulated.	2021	Lu et al., 2021
Bacillus licheniformis N17-02	Vitro	When compared eight different strains of *Bacillus*, *Bacillales* and *Lactobacillus*; though all had different cholesterol-removal abilities, *Bacillus licheniformis* N17-02 had the best result and presence of bile salt hydrolase gene, as well as most beneficial probiotic characteristics. As a result, it might be a suitable hypocholesterolemic probiotic candidate.	2021	He et al., 2021
Not specified	Humans	Twelve-week trial using *Bacillus licheniformis* in combination with other spore forming bacilli probiotics have remarkable triglyceride lowering.	2020	Campbell et al., 2020
Bacillus licheniformis YB9	Mice	Deoxynivalenol could be degraded by BL (YB9), which also had a high survival rate. Supplementing with *Bacillus Licheniformis* prevented or reduced the harm. BL could be employed as a potential probiotic supplement for increasing food and feed safety by regulating the intestinal microbiota of both animals and humans, as well as repairing intestinal dysbiosis.	2020	Wang et al., 2020
Bacillus licheniformis Zhengchangsheng® (BL20386)	Rats	Combining *Bacillus licheniformis* with Xylooligosaccharides could be a dietary approach to alleviate gut dysbiosis, improve inflammatory status, and thereby reduce disorders linked with high fat diet obesity.	2020	Li et al., 2020
Bacillus licheniformis KT921419	Vitro	For 8 chosen bacterial strains from traditional fermented brine mango pickle, antioxidative, antidiabetic, and antityrosinase properties were investigated. *Bacillus licheniformis* KT921419 strain showed one of the best results on *in vitro* experiments and might be used as a new starter or auxiliary culture in a food system to impart health benefits.	2019	Ragul et al., 2020
Not specified	Mice	Without affecting food intake, *B. licheniformis* or a mixture of *Bacillus* stains reduced final body weight, improved glucose intolerance, and minimized hepatic fat accumulation in mice. Furthermore, the colonic microbiota of the *Bacillus*-supplemented and high-fat diet-fed mice differed dramatically. Probiotics derived from *B. licheniformis* could be effective in the management of a variety of metabolic disorders.	2019	Cao G. T. et al., 2019
Bacillus licheniformis MCC2512	Rats	The probiotics *B. flexus* MCC2427 and *B. licheniformis* MCC2512 had no negative effects on the health or behavior of the rats. Additional benefits of probiotic cultures include normal hematological parameters, lower blood cholesterol, enhanced HDL-cholesterol, increased cholic acid excretion in the stool, and higher Polyunsaturated fatty acids content in the liver. *Bacillus* bacteria in the feces was increased, whereas harmful bacteria were decreased. Overall, these probiotic cultures studied are safe and effective, and that they are likely to be safe for human ingestion.	2018	Shobharani et al., 2019
Bacillus licheniformis PUFSTP35	Vitro	When compared eight different strains of *Bacillus*, the most promising candidate for use as a helpful probiotic appears to be *B. licheniformis* PUFSTP35 from fermented mango pickle. *In vivo* investigations to confirm the probiotic potential of the tested isolates are required.	2017	Ragul et al., 2017

Most relevant articles and outcomes of *B. licheniformis* supplementation in diabetes and obesity modulation.

in serum levels of total bilirubin and cholesterol, parameters that point out a boost in transference from liver to bile, and leading indirectly to an improvement in liver function (Devyatkin et al., 2021). Also, the interaction of *B. flexus* and *B. licheniformis* showed a reduction in serum cholesterol, and improve in HDL-cholesterol, respectively, along with other biochemical parameters and microbiota studies that indirectly validate its efficacy and propose its use for human consumption (Shobharani et al., 2019). Another study revealed the role of BL in the homeostasis of gut microbiota and the modulation of bile acid; and in combination with *Lactobacillus salivarius* and *Pediococcus pentosaceus*, it prevented liver fibrosis and downregulated the hepatic expression of profibrogenic genes in rats (Shi et al., 2017). These results demonstrate one of the most relevant applications of *B. licheniformis* and could lead to novel applications in human hepatic diseases, both alcoholic and non-alcoholic.

Cardioprotective effect

The microbiota in humans has been recognized as a new prospective risk factor for cardiovascular diseases. Atherosclerosis, heart failure risks, and influence of the gut microbiota in them have been previously reported. Even though animal research has revealed that gut microorganisms

may influence heart disease risk, no such relation has been observed in humans (Forkosh and Ilan, 2019). For the case of *Bacillus licheniformis*, even though animal studies have not reported benefits on specific cardiovascular diseases yet, results obtained in eight studies demonstrate that not only significant improvement in hematological parameters, in general, could be achieved in combination with other *Bacillus* species (Adorian et al., 2019) but also regulation of other disorders that indirectly could be related to cardiocirculatory problems, such as triglycerides regulation (Campbell et al., 2020) for atherosclerosis, and its role in risk reduction of heart attacks, coronary diseases, cardiopathies, and many other heart illnesses (Peng et al., 2017). Also in humans, a study of 30-day probiotic supplementation of BL with other oral spore-based could reduce dietary endotoxemia (McFarlin et al., 2017). Even though endotoxemia is the result of a translocation of LPS into the circulation, studies revealed its link to an elevated risk of many cardiovascular diseases (Moludi et al., 2020).

Another application in heart failure prevention could be *Bacillus licheniformis* potential role in microbiota regulation. A recent study linked microcirculatory abnormalities in heart failure patients with anatomical and functional alterations in the gut. Emerging data suggest that gut bacteria may play a role in the pathogenesis of heart failure (Kamo et al., 2017). The breach in the intestinal epithelial barrier might allow microbial compounds to enter the bloodstream, exacerbating this disease by triggering inflammatory responses, an effect this probiotic has previously been reported to prevent in many articles and further elaborated upon. When looking for up-to-date information on cardiovascular disease, it is important to focus on the microbiota as a pathway for treatment of heart failure and other diseases. Additional research is necessary; however, the notion of the heart–gut axis might pave the way for advances in the development of novel diagnostics and therapy techniques focused on cardiovascular health.

Neurological diseases

The gastrointestinal physiology, including digestion and gut bacteria composition, is influenced by abnormal brain activities. The gut microbiota has a strong robust bidirectional interaction with the central nervous system and impacts its outcome and mechanism of it. According to several neurological findings on the gut–brain axis, this enhances gut homeostasis. The mechanisms underlying this axis are diverse, with multiple routes involved both directly and indirectly (Suganya and Koo, 2020). Probiotic supplementation as an aid for biochemical signaling of the microbiota–gut–brain pathway, in which the intestinal microbiota, enteric nervous system, and central nervous system get connected, could have a positive influence against dysbiosis and enhancement of neuroactive substances such as serotonin or dopamine. New terms such as psychobiotics, also known as live biotherapeutics or substances with bacterially mediated beneficial effects on the brain, are currently being studied as a single or combination therapy for psychiatric and neurodevelopmental disorders, as well as possibly neurodegenerative diseases, as they could become novel treatment alternatives toward the prevention and control of brain disorders (Long-Smith et al., 2020).

Although it was found that only two studies for the specific application of *B. licheniformis* in neurological effects (apart from the psychological mentioned later), this could be mainly because of the recent attention given to this field. A 28-day trial in weaning piglets showed beneficial effects of neurotransmitters in serum and hypothalamus, serum γ-aminobutyric acid, and higher colonic concentrations of butyrate and valerate in combined probiotic supplementation (BL, *B. subtilis*, and *Clostridium butyricum*) in comparison with control and antibiotic-treated groups (Cao G. et al., 2019). An application in humans in the neurological field involved pediatric central system tumor's side effects study caused by radiotherapy including mouth ulcer, nausea, vomiting, abdominal pain, and diarrhea. Although the effect of BL preparation on children's survival rates and tumor recurrence was not evaluated in this investigation, it showed an improvement in intestinal function and repairment, inflammatory responses, and immunity that could lead to a better efficacy in the final treatment (Du et al., 2018). These results encourage to improve further investigations of this probiotic in neurological diseases, such as Alzheimer's, Parkinson's, multiple and amyotrophic lateral sclerosis, etc.

Psychological disorders

Probiotics have been used recently in investigations to assist in negative emotions, altered behaviors, cognitive performance, and stress relief. Many scientific investigations are underway to see if probiotic supplements might assist those who are suffering from psychological stress. For *Bacillus licheniformis*, a total of two studies were found, one focused on aquaculture and the other on rats. For the purpose of this review, psychological disorders have been separated from the rest of neurological diseases to delve deeper into them.

The first one revealed that in combination with *B. Amyloliquefaciens*, results showed an improvement in larval fish survival and transport stress resistance (Tarnecki et al., 2019). One of the primary goals of any aqua farmer or entrepreneur seeking to maximize output is to reduce stress on farmed fish. This major challenge in aquaculture has prompted extensive research on reducing or eliminating the impact of stress on cultivated animals. Among the several stress reduction treatments used in aquaculture, dietary probiotic interventions have emerged as promising, empirical, and long-term solution (Ciji and Akhtar, 2021).

The second one, which focused on *B. licheniformis* alone exhibited, an improvement in behavioral changes, nervous system metabolites, neurotransmitters, and gut microbiota changes in the rat model, and demonstrated a possible new mechanism of subhealth status alleviation in psychology and behaviors, specifically because of the gut microbiome that could consume more propionic acid, resulting in alterations in brain neurotransmitters as glutamic acid (Glu), γ-aminobutyric acid (GABA), and 5-hydroxytryptophan (5-HT). At the same time, it could contribute to the reduction of norepinephrine in the brain, corticosterone, and TNF-α in the blood, as well as the inhibition of hyperactivity on the hypothalamic–pituitary–adrenal (HPA) axis and lead to anxiety reduction (Feng et al., 2022). These investigations provide new possibilities for further research on emotional disorders, their pathogenesis, and the development of their therapeutic approaches in animals and humans.

Dental care and bone health

Periodontal healthcare and oral cavity mechanisms seem to be far from the application of probiotics interaction with the host; however, some of the topics mentioned before such as the stimulation of immune responses, inhibition of pathogens in the gastrointestinal tract, and synthesis of antimicrobial compounds could aid on the prevention and treatment of dental care diseases. In the context of disease pathogenesis, the microbiological relationship between these two mucosal locations may be linked. Several studies have found that oral bacteria can travel to the gastrointestinal tract via hematogenous and enteral axis (Kitamoto et al., 2020). It has been postulated that an oral–gut communication axis exists, but its role in the development of neurodegenerative illnesses has yet to be discovered. However, the use of probiotics for the control of various oral health disorders, like dental caries, periodontitis, gingivitis, halitosis, burning mouth syndrome, and oral cancer has been previously studied on many probiotic strains (Mishra et al., 2020). For *Bacillus licheniformis* and dental care, only one study has been reported focused on periodontitis.

Periodontitis has been linked in a lot of research to other chronic non-communicable diseases such as cardiovascular and neurological diseases (Sansores-España et al., 2021). Both deepened the beneficial effect that *B. licheniformis* has on them. In this specific study, the combined effect of BL with *B. subtillis* in rats with an experimental periodontitis-induced model was done. The main results showed a reduction in alveolar bone loss and the number of peripheral blood eosinophils in probiotic therapy concluding that with this study, further research on human clinical trials could be applied (Messora et al., 2016). This study opens new perspectives of *B. licheniformis* potential effect, not only on the whole oral healthcare applications mentioned before but also on the ones related to bone-loss diseases such as osteoporosis, a disease which has also been suggested to be approached with probiotics (Collins et al., 2018).

Safety of *Bacillus licheniformis*

The safety of this probiotic has been tested in animal and human studies, and it can even be found over the counter as a supplement. From a study by PCR and ELISA to search for enterotoxin genes and molecules directly, none of those analyzed were found. In addition, when evaluated in BALB/c mice, rabbits, and pigs, there were no significant changes, at the histopathological, behavioral, or hematic level with chronic consumption (Sorokulova et al., 2008). The absence of genotoxicity with chronic consumption is also reported from a micronucleus assay in mice. When used topically and ophthalmic, no redness or edema was observed (Nithya et al., 2012). Some infections caused by this bacterium have been described; however, they are related to a previous lesion in tissues and/or organs, so their use in healthy patients can be considered safe (de Boer et al., 1994). The absence of antibiotic resistance in humans and animals is also observed (EFSA Panel on Additives and Products or Substances used in Animal Feed [FEEDAP] et al., 2019).

Concluding remarks

The use of probiotics in the medical and veterinary environment has increased. Although information updates over time, the benefits of the consumption of probiotics cannot be refuted. In the case of *Bacillus licheniformis*, at this moment, most of their studies are focused on animal tests in comparison to humans. *B. licheniformis* has proven to be a probiotic for safe consumption with the ability to resist the conditions of the entire gastrointestinal system since it is an organism that has the ability to form spores and this, in turn, benefits its industrialization and handling in less than the optimal conditions for its production, getting better proliferation without losing its vitality.

Multiple benefits are observed in various pathologies and their prevention is mainly focused on the gastrointestinal and immune systems, in which the pursuit of a substitute and therapy for antibiotics after they have been used or even to replace them, stands out. In addition to the modulation of the immune response, it was found antimicrobial properties, enzyme secretion improvement, and the enhancement of eubiosis by improving the diversity in the microbiota. These benefits correlate with the improvement of diseases such as enteritis, colitis, infectious diarrhea, etc. Also, benefits associated with the circulatory system are shown, such as the modulation of markers associated with diabetes, CRP, glucose, insulin, lipid profile, and blood pressure regulation, etc. It was shown that this probiotic

is associated with hepatoprotection and cardioprotection since it shows reduced dietary endotoxemia and modulation of liver toxicity and other molecules with a direct or indirect relation.

A few studies focused on Neurological and Psychological disorders were obtained, in which an improvement in the modulation of serum γ-aminobutyric acid, glutamic acid, 5-HT, and higher colonic concentrations of butyrate and valerate was observed. Moreover, better stress and anxiety response and reduction of norepinephrine could lead to a promising therapy aid in the treatment of this type of disorder. Improvements in periodontitis and other diseases associated with the uptake of nutrients for bone health, besides an improvement in antioxidant enzymes, were reported.

More studies are necessary to give a more comprehensive perspective of this probiotic in each field, but still, it is remarkable that *B. licheniformis* could be exploded not only for the diseases mentioned before but also to others such as the ones related to other fields such as dermatological, endocrine, muscle and joint, respiratory, genitourinary, etc. Although it could have some benefits for each of them, it is important to consider that this probiotic, and there are many others that could enhance its effects on a specific disease more than *B. licheniformis*.

Comparing the potential effects between probiotics and species is a complicated but necessary task to ensure the best selection of them, alone or in combination. As can be observed, many of the diseases present are the result of dysbiosis and its collateral effects; understanding the cause of it. Together with the correct probiotic treatment could be a new method to modulate the signs and symptoms that determine each disease out of range, before, after, or instead of the current treatment. With this review, we trust that further studies of each probiotic and its strains are deepened to fulfill information gaps as well as promote the study in humans and therefore achieve a better selection of them for a specific disease or disorder.

Author contributions

HR-O and RC-S devised the review article, the main conceptual ideas, proof outline, and worked on editing and reviewing of the article. HR-O and BR-B made the systematic review and wrote the first draft of the article. All authors discussed the results and contributed to the final 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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Assessment of shelf-life and metabolic viability of a multi-strain synbiotic using standard and innovative enumeration technologies

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The number of live bacterial cells is the most used parameter to assess the quality of finished probiotic products. Plate counting (PC) is the standard method in industry to enumerate cells. Application of PC implies critical aspects related to the selection of optimal nutrient media and growth conditions and underestimation of viable but not cultivable (VBNC) cells. Flow-cytometry (FC) is a culture-independent methodology having the potential to selectively enumerate live, damaged, and dead cells representing a powerful tool for in-depth monitoring of probiotic products. We monitored the shelf life of a clinical batch of a synbiotic composition PDS-08 targeting the pediatric population by means of PC and FC according to International Conference on Harmonization (ICH) pharma guidelines testing the Arrhenius model as predictive tool; PC enumeration revealed higher destruction rate than FC suggesting a faster reduction in cultivability than membrane integrity and thus a possible shift of the bacteria into a VBNC status. PDS-08 maintained acidification capability over time, when re-suspended in nutrient medium, even in samples tested sub-optimally for CFU detection (below 1 billion cells/dose). Due to similar kinetics described by the study of metabolic activity and membrane integrity, FC might be suggested as a valid tool for the study of functional stability of a probiotic product.

KEYWORDS

plate counts, VBNC, flow-cytometry, Arrhenius model, probiotic, functional stability

Introduction

Probiotics were defined in 2001 by the World Health Organization (WHO) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Gibson et al., 2017). Consequently, international bodies such as the Food and Agriculture Organization (FAO) and WHO recommend that the product labels should

include information on "minimum viable numbers of each probiotic strain at the end of the shelf-life" (FAO/WHO Guidelines, 2002). Enumeration of live probiotic bacteria may thus be considered the main evaluation tool for product feasibility, formulation, stability and quality control. Accordingly, the number of cells in finished products represents the dominant parameter used in commercial agreements, quality, and regulatory assessments. Therefore, the accurate determination of the viability of probiotics is of fundamental importance to meet the FAO/WHO guidelines (Jackson et al., 2019). In the probiotic industry, measuring viability is usually limited to enumeration of the bacteria through culture-dependent plate counting (PC) technique on nutrient agar medium (Davis, 2014). The number of colonies detected on the appropriated plates after incubation are then used to express the microbial counts as colony forming units (CFUs). Unfortunately, different probiotic strains have different optimal growth media and conditions, making the accurate enumeration of both single-and multi-strain probiotic products a challenging task. Notably, cultivability encompasses only a subset of cells considered to have the "viable" status. Indeed, evaluating cell "viability" by the PC technique likely underestimates microbial potency when cells enter the viable but not cultivable state (VBNC) during shelf life and the total number of cells present in the sample. The application of flow cytometry (FC) might overcome PC limitations, providing data on different structural and functional properties of cells which describe the viability status of the bacterial population. This can enable quantification of viability beyond cells reproductive capacity on nutrient media, providing deeper insight into the functional strain-related responses to various applications (Wilkinson, 2018; Foglia et al., 2020). The International Standard ISO 19344 – IDF 232 "Milk and milk products - Starter cultures, probiotics and fermented products - Quantification of lactic acid bacteria by flow cytometry" (ISO 19344) currently represents a benchmark for FC staining protocols based on membrane integrity, intracellular enzymatic activity, and membrane potential for the determination of bacterial cell viability. To date, the industrial use of FC for probiotics is still limited, but new evidence in methods validation (Pane et al., 2018; Foglia et al., 2020; Michelutti et al., 2020) and comparison with other possible methodologies (Gorsuch et al., 2019) is growing. The current study aimed at generating a thorough comparison of PC and FC methodologies using a Good Manufacturing Practice (GMP) clinical batch of the synbiotic formulation PDS-08 targeting the pediatric population. PDS-08 contains 9 probiotic strains and an oligofructan-enriched inulin. We monitored the shelf life of the clinical batch of PDS-08 during one-year of storage at different temperatures. According to our previously described approach (Foglia et al., 2020), the Arrhenius model for predictive microbiology was applied to data generated by FC and PC to predict the mortality of probiotic cells during storage. Alongside the evaluation of cell density through PC and FC, we introduced the study of probiotic functional stability evaluating the metabolic capacity of the lyophilized cells to acidify when inoculated in growth media. Data from enumeration by PC and FC were then compared with acidification profiles, showing that acidification trends were more comparable to FC survival kinetics than PC ones. The coupled use of PC and FC combined with metabolic activity measurements suggest that FC data provide a more accurate representation of functional stability of a probiotic product during its production and on the shelf, and consequently might be considered as valid alternative for enumeration and quality assessment method in the probiotic industry.

Materials and methods

Probiotic product formula

Probiotic product formula PDS-08 contain 6.3 g inulin Orafti®Synergy1 (Beneo, Mannheim, Germany) and the following nine probiotic strains: *Bifidobacterium animalis* subsp. *lactis* SD-Bi07-US, *Bifidobacterium animalis* subsp. *lactis* SD-CECT8145-SP, *Bifidobacterium breve* SD-BR03-IT, *Bifidobacterium breve* SD-BR632-IT, *Bifidobacterium longum* SD-CECT7347-SP, *Lactobacillus acidophilus* SD-NCFM-US, *Lacticaseibacillus casei* SD-CECT9104-SP, *Lacticaseibacillus rhamnosus* SD-GG-BE, *Ligilactobacillus salivarius* SD-LS1-IT. Labeled probiotic potency was $>20\times10^9$ viable cells per dose (6.5 g). Finished product PDS-08 was analyzed (Biolab srl, Novara, Italy) at the moment of batch release *via* flow cytometry (ISO 19344, 2015: IDF 232: 2015) which resulted in a cell count of $>15\times10^9$ Total Fluorescent Unit (TFU)/g and $>11\times10^9$ Active Fluorescent Unit (AFU)/g; and plate count method (Internal Method 014–06) which resulted in a target cell count of $>6.5\times10^9$ CFU/g. To exclude product sample heterogeneity three random samples, withdrawn in triplicate during product manufacturing, were analyzed for total fluorescent units (TFU), and Relative Standard Deviation was <10%. Water activity of the product (a_w) was monitored during the study to exclude any possible detrimental effects on probiotic cells or spoilage of the product due to the increase of a_w.

Storage conditions and stability testing according to ICH guidelines

Samples were stored and checked according to the pharma International Conference on Harmonization (ICH) Guideline Q1A (Table 1) over a period of 12 months. At each checkpoint, the number of cells (TFU, AFU and CFU), as well as the pH over 24 h and a_w were determined.

Plate counting

Samples (dose of 6.5 g) were serially diluted in peptone saline water solution. Each dilution was plated on De Man, Rogosa and Sharpe (MRS) agar (BD Difco™ San Josè, CA) by inclusion technique and plates were incubated at 37°C for 72 h under microaerophilic conditions. Sample preparation and enumeration

were performed following Weitzel et al. (2021) guidance for probiotic bacteria enumeration.

Flow cytometry

FC analyses were performed using the BD Cell Viability Kit with liquid counting beads (distributed by BD Bioscience, San Josè, CA). The latter offers an easy-to-use dye combination to distinguish between live and dead cells based on assessment of cell membrane integrity. Thiazole Orange (TO) solution allows the staining of all cells while Propidium Iodide (PI) targeted damaged and dead cells. BD Liquid Counting Beads was always used as reference for the cell enumeration. Cell staining and analysis were preformed according to the ISO 19344:IDF 232 protocol with custom integration described by the method reported by Foglia et al. (2020). In order to guarantee result accuracy during the 12 months study, the flow cytometry gating procedure was kept constant at each time point. To have a reference test for enumeration, samples were preserved at-20°C and were enumerated during the study at each time point.

Calculation and expression of results of cell enumeration

Plate count results were expressed as CFU/g (detailed in the Supplementary Material). Plates with less than 30 and more than 300 colonies were not considered in the count and only clearly visible colonies were enumerated (i.e., colonies grown on the border of the plate were not counted). Flow Cytometry results were expressed as Active Fluorescent Unit (AFU/g), non-Active Fluorescent Unit (n-AFU/g) and Total Fluorescent Unit (TFU/g). n-AFU represents the damaged and dead cells stained with PI, the non-permeant dye which only enters cells with a non-intact membrane and binds to DNA. The Total Fluorescent Unit (TFU) represents the total number of cells obtained by the sum of AFU and n-AFU cells. Damaged and dead cells (n-AFU) can be calculated also as TFU – AFU. For the stability testing, only TFU and AFU parameters have been considered. Principles for determinations of AFU and n-AFU in FC protocols using BD Liquid Counting Beads are reported in Supplementary Material.

TABLE 1 Storage conditions according to International Conference on Harmonization (ICH) pharma guidelines Q1A for the development of new drug products.

Study	Storage condition	Minimum time needed for data submission	Control points (months)
Refrigerated condition	5°C ± 3°C	12 months	0, 3, 6, 12
Long Term	Zone II 25°C ± 2°C 60% ± 5% RH Zone IVb 30°C ± 2°C/ 75% ± 5% RH	12 months	0, 3, 6, 12
Accelerated	40°C ± 2°C 75% ± 5% RH	12 months	0, 1, 2, 3, 6

RH: Relative Humidity.

Evaluation of acidification activity

At each analysis time point, samples were reconstituted in 100 ml of MRS broth and incubated in a thermostatic bath at 37°C for 8 h. The acid fermentation metabolites generated serve as an indicator of metabolic or functional stability of the bacteria. Measurements of pH were recorded at the starting point (T0) and at different time points during incubation (after 2, 4, 6, 8 and 24 h) using a SevenCompact pH meter S220 (Mettler Toledo, Columbus, Ohio).

Prediction of microbial stability using the linear Arrhenius model

A standard two-step method was used to obtain the Arrhenius model and to assess the influence of temperature on the stability PDS-08. Predictive microbiology describes the exponential loss of bacterial viability (as TFU, AFU and CFU) over time by the following equation (first order low):

$$Nt = N0e - kt \quad (1)$$

Nt: number of viable microorganisms at t = t(x);
N0: number of viable microorganisms at t = 0;
k: destruction rate;
t: time;

By plotting ln(Nt/N0) over time (t), the destruction rate k can be determined for each stability temperature. The effect of temperature on k is described by the Arrhenius equation:

$$k = Ae - Ea / RT \quad (2)$$

k: destruction rate (time-1);
A: frequency factor (time-1);
Ea: activation energy (J.mol-1);
R: gas constant (8,314 J.mol-1, K-1);
T: temperature (Kelvin).

By plotting ln(k) versus 1/T, a straight line is obtained, which leads to the parameters of equation (2). By extrapolation, k is obtained for any temperature included between the extremes of the stability plan. Equation (1) can be used to predict the number of viable microorganisms at any time and for any storage condition. Finally, k is related to the decimal reduction time in months (D1), the time needed for the bacterial population to be reduced by 90% that can be calculated as follows:

$$D1 = \ln 10 / k \quad (3)$$

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Results

Stability testing by plate count enumeration

We initially set out to perform a routine, CFU-based analysis of product shelf-life stability according to the general industry practice of PC enumeration. PC results are shown in Figure 1. Next to enumeration, Water Activity (a_w) levels were monitored and were found below 0.25 at the extreme condition of storage at Zone IVb (30°C, 75% RH) after 12 months (data not shown). As expected, best performances were observed for lower storage temperature; sample stored at 5°C and 25°C had a survival rate of 76.9 and 61.5%, respectively, after 12 months, resulting in a total number of live cells well above 1×10^9 cells which is conventionally considered the minimum effective dose for a probiotic product (Hill et al., 2014). At 30°C, cell numbers exceeded after 6 months of storage but dropped slightly below this threshold at the 12-month measurement. This drop in CFU-based viability was even more pronounced at 40°C resulting in detrimental effects after 3 months with further deterioration as time progressed (Figure 1). Taken together, the conventional industrial analysis method suggests PDS-08 can be stored for at least a year when either refrigerated or stored at room temperature, providing this does not exceed 25°C.

Stability testing by flow cytometry enumeration

When stability was monitored using membrane integrity, sample stored at 5°C, 25°C, 30°C and 40°C showed 86.3, 93.8, 67.0 and 29.7% viability, respectively, after 12 months. Overall, all samples were above the cut-off limit of 1×10^9 cells (Figure 2).

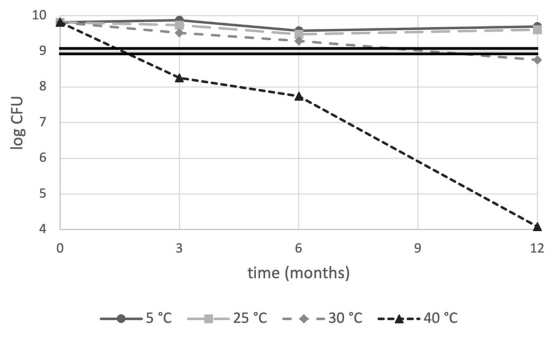

FIGURE 1
Plate count stability trends of PDS-08 over time (months) at 5, 25, 30 and 40°C. CFU data are expressed as Log numbers. The double straight line indicates the threshold value for the effective dose of 1×10^9 CFU/dose, as per probiotic food guidelines.

CFU vs. AFU

Predictive microbiology was applied to describe the phenomenon of lyophilized cell decay during storage at various conditions. Decrease in CFU/g (cell cultivability) and TFU/g or AFU/g (cell membrane integrity) were described by two parameters; the destruction rate (k) and the decimal reduction time (D1; section 2.6). For all tested temperatures, k values from equation (2) were substantially higher in the case of PC compared to FC enumerations (Table 2; Supplementary Figure S1), suggesting the existence of a significant subpopulation of cells that are alive but unable to form colonies. When expressed as the decimal reduction time, D1 values from equation (3) confirmed that the trend of loss in viability by CFU counts was faster than loss in membrane integrity by AFU determination. These results clearly indicate that the reduction of cultivability over time is faster than the reduction of membrane integrity at the same temperature, and that higher storage temperature are more detrimental, leading to a faster reduction of both cultivability and membrane integrity. As an example, to visualize the differences between data generated with FC and PC respectively, we plotted CFU, AFU and n-AFU over time at the temperature of 30°C in function of TFU values used as a reference (Figure 3).

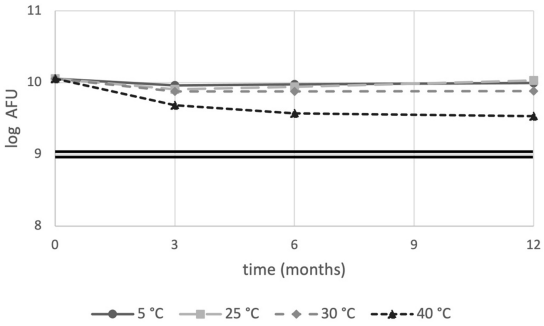

FIGURE 2
Flow cytometry stability trends of PDS-08 over time (months) at 5, 25, 30 and 40°C. AFU data are expressed as Log numbers. The double straight line indicates the threshold value of 1×10^9 AFU/dose.

TABLE 2 Bacterial/probiotic cultivability and membrane integrity destruction rate (k) and decimal reduction time (D_1) of synbiotic samples at different temperatures.

Temperature	Destruction rate k (months-1)			Decimal reduction time D1 (months)		
	CFU	AFU	TFU	CFU	AFU	TFU
5°C (278.15°K)	0.0247	0.0025	0.0020	93.2	921.0	1151.3
25°C (298.15°K)	0.0468	0.0034	0.0025	49.2	677.2	932.0
30°C (303.15°K)	0.1844	0.0161	0.0141	12.5	143.0	163.3
40°C (313.15°K)	0.6983	0.1417	0.1212	3.3	16.2	19.0

CFU = Colony Forming Units; AFU = Active Fluorescent Units; TFU = Total Fluorescent Units.

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Acidification activity of lyophilized cells during storage

We introduced the study of acidification capability of cells in long-term storage as a parameter to assess the functional stability of the probiotics in the PDS-08 product. After incubating the lyophilized cells stored at different temperatures in MRS broth, pH reduction was determined at each timepoint considered for viability assessment (Table 1). To provide an efficient overview of acidification trends during time, pH kinetics obtained in the early (3rd month) and late stage (12th month) of shelf life are reported in Figures 4, 5, respectively. After 3 months of storage, samples stored at 5, 25 and 30°C retained a robust acidification capability (Figure 4). After 12 months of storage, only samples stored at 30°C recorded a marked delayed acidification while a ΔpH of 1.26 was anyway detected (Figure 5). Samples stored at 40°C showed at both the timepoints a weak acidification ability with a ΔpH of 0.2 at 3 months and 0.14 at 12 months. However, when fermentation was prolonged up to 24 h, samples stored at 40°C were able to acidify to pH 3.89 and 4.96 after 6 and 12 months of storage, respectively. To investigate the possible correlation between metabolic activity (acidification) and CFU and AFU trends, the kinetics of CFU, AFU, TFU and pH reduction were graphically represented by plotting the slopes for each parameter evaluated at 12-month control point at each temperature of storage (Figure 6). Overall, this comparison suggests that thermal stress over time affected all the parameters investigated, with higher impact on CFU followed by AFU and TFU. However, all samples retained the acidification ability during 8-24 h of fermentation in MRS even at 40°C after 12 months. From data reported in graphs represented in Figure 4, 5, regression curves have been generated to extrapolate the slopes of each pH trend (Supplementary Table S1). Plotting slopes (Supplementary Table S1) allowed to visualize the impact of temperature on the pH production by the probiotic bacteria (Supplementary Figure S2). As expected, higher temperature and longer storage time, slow down the metabolic capability of the probiotic product to acidify the medium over 8-h course (Supplementary Figure S2). Considering all the acquired information, we gathered all the enumeration and acidification slopes (Supplementary Table S2) to finally generate the graphical representation of the effect of temperature on different kinetics after 12 months of storage at different temperature (Figure 6). Finally, these group of results indicate that pH trends were more comparable to AFU trends than to the decay in CFU, suggesting the plausible contribution of a partial re-activation of VBNC cells contributing to the acidification activity used in this case as a functional marker.

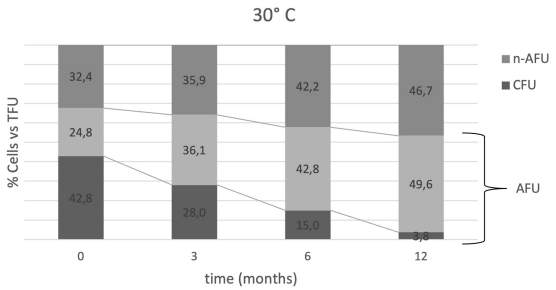

FIGURE 3
Bacterial heterogeneity distribution over time at 30°C expressed as the % of cells (CFU, AFU and n-AFU) vs. TFU (100% of the population at each time point). Relative % of AFU is the sum of AFU+CFU values; for example, t0 is represented by 42.8% CFU vs. TFU, 67.6% AFU vs. TFU and 32.4% n-AFU vs. TFU. First line from the top is representative of the AFU trend vs. time. Second line from the top is representative of CFU trends vs. time.

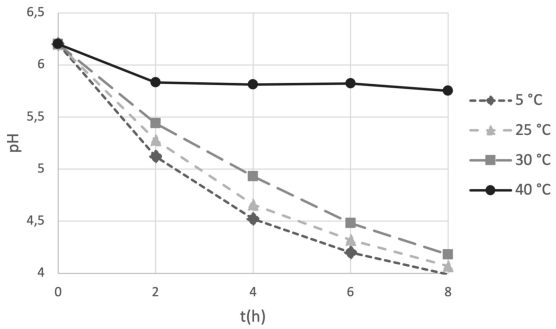

FIGURE 4
pH trends over 8h fermentation in MRS broth by PDS-08 stored for 3months at 5, 25, 30 and 40°C.

Discussion

To meet the industry guidelines (IPA 2018),[1] probiotics products are expected to be viable at end of the declared shelf-life with the

1 https://ipaeurope.org/wp-content/uploads/2020/03/20180523-IPA-Europe-Probiotic-Criteria-Document.pdf

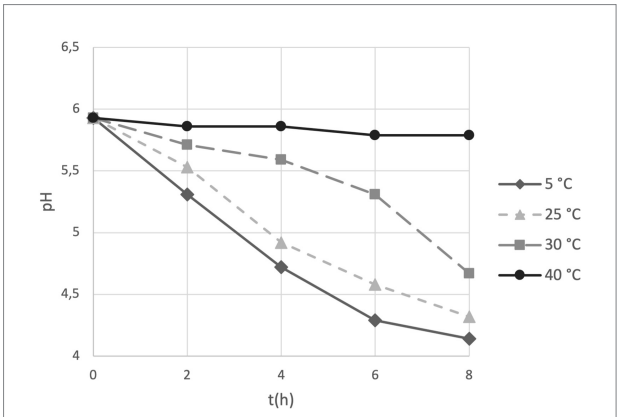

FIGURE 5
pH trends over 8h fermentation in MRS broth by PDS-08 stored for 12months at 5, 25, 30 and 40°C.

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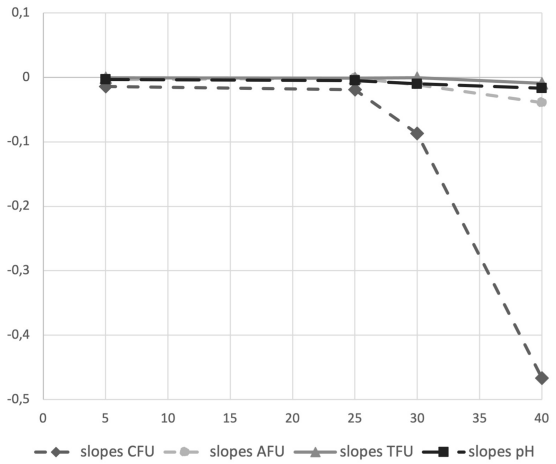

FIGURE 6
Graphical representation of acidification and enumeration (CFU, AFU and TFU) slopes obtained on clinical batch sample of synbiotic formula (PDS-08) after 12months of storage at different temperature (5, 25, 30 and 40°C).

minimum documented efficacious dose (Hill et al., 2014). Many regulatory agencies rely only on culture-dependent methodologies (e.g., plate counts) to evaluate the "viability" and ensure the accuracy of label claims. However, these assays are known to underestimate microbial potency for several reasons, including the lack of a growth medium that is optimal for the target microorganisms (especially in a multi strain/species/genera consortia), and lowered cultivability when cells enter the VBNC state, and long time before data acquisition for sample incubation. The significant drop in recovered bacteria over time using plating methods could be interpreted as a loss of viable cells and is rarely interpreted as an increase in VBNC cells within the same sample. As previously reported by our group (Foglia et al., 2020), the kinetics between cultivability and a potential progression to VBNC state were monitored using a combined approach of plate counts (PC) and flow cytometry (FC) enumeration, where the first targeted the viability in terms of colonies generation and the second measured the viability in terms of cell membrane integrity depending on fluorochromes used for the analysis. Results of that study (Foglia et al., 2020) illustrate that the loss of cultivability is faster than the loss of membrane integrity within the heterogeneity of a freeze-dried single strain bacterial population. Accordingly, the hypothesis risen is that AFU cells are likely representative of the VBNC and/or dormant population plus the subgroup of cultivable cells (CFU). Consequently, VBNCs may constitute a significant portion of live cells that are consumed by the end user with potential health benefits (Blinkova et al., 2014). In this paper, we strengthen the combined use of PC and FC enumeration adding pH acidification as third pillar for monitoring functional stability (i.e., the capability of the product to acidify over time), in order to evaluate if cellular metabolism was more preferentially descriptive of FC or PC enumeration data. Since the product was constituted by fermentative micro-organisms (mainly producing acetic and lactic acids) we opted for pH as the main metabolic indicator. The synbiotic product PDS-08 was able to express primary metabolic activity up to 12 months and at different storage condition (5, 25, 30 and 40°C; Figure 4). This evidence, even in its methodological simplicity, suggests that the metabolic and functional stability is retained by the synbiotic product throughout shelf life. Interestingly, CFU decay is faster than the decreases of AFU, TFU and pH which showed similar kinetics. Besides cells viability, expression of metabolic pathways or specific effector molecules that directly interact with the host are additional mechanisms by which probiotics exert their beneficial effects (L-Chiao et al., 2013). Further research is required to verify if probiotics or candidate bacterial strains as live biotherapeutic products (LBPs) that lose cultivability (CFU) but maintain membrane integrity (AFU) can still express these molecules and to what extent. The probiotic market it is expected to exceed 85 billion USD by 2026 (Research and Markets, Probiotics – Global Market Outlook 2017–2026); the current discovery of Next Generation Probiotics (NGPs) and of LBPs reasonably will give an important boost to the market of therapeutical microorganisms in the coming years with the presence of different novel bacterial species, often strictly anerobic. In this scenario, we strongly recommend that more methodologies for cell enumeration (see Davis, 2014 for an extensive review) and function are investigated and validated. PC methodology is arduous to implement on these new species due to often-unknown growth requirements and necessity to operate in oxygen-free environment (Bircher et al., 2018). In this context, FC approach can represent a culture-independent valid tool for a real-time check of bacterial population from the early phase of a product design (e.g., strain characterization) to the latest phase (e.g., cells administration in clinical trial, finished product stability; Muller et al., 2013). Moreover, future investigation will allow the use of FC for bacterial enumeration and identification taking advantage of species-specific or even strain-specific primers (Chiron et al., 2018). As matter of fact, this work intends to open a wider research line where the first goal is the design of strain-specific and even molecule-specific markers (primers and/or antibodies). This will permit the application of our concept for the improvement of the Quality Control standards of probiotic finished products and the tailoring of probiotic formulas under the functional perspective. What we seek for the future in the Quality Control context is to be able to discriminate and quantify the single strains within a blend over time to boost to the Market of High-Quality Probiotic products; next to this, we aim at individuating probiotic-effector molecules or traits to consolidate the concept of functional stability monitoring during time both in single-and multi-strain blend. In conclusion, this paper demonstrates that a comparison of PC and FC provides a comprehensive study of the heterogeneity of a probiotic population which would be impossible by PC alone; by including a third block in the scheme that was the analysis of acidification capability used as parameter of the metabolic activity, and its similarity with FC trends, we can suggest the FC as a valid tool for the analysis of functional stability of probiotics. Accordingly, the acquisition and collection of data on bacterial samples at different stages of their cycle by means of flow cytometry is strongly encouraged. Finally, the use of the Arrhenius mathematical model was proven to be reliable also for predictive microbiology.

Conclusion

In our previous work we presented the use of Plate Count (PC) and Flow-cytomety (FC) enumeration in a comparative approach to investigate the heterogeneity status of probiotic cells in finished products. We concluded that FC represents a valid and innovative tool for bacterial population study and enumeration and that the Arrhenius model can work as predictive model for bacterial survival during time. However, our broad scope is to elucidate the missing link between the number of probiotic cells, their status and importantly their function over a shelf-life period. In this optic, we proposed in this paper the innovative concept of functional stability, to be applied to probiotic finished product along with the monitoring of their cellular viability. The acidification capability of commercial lyophilized probiotics was selected as first and intuitive parameter to investigate. At the same time, we monitored bacterial shelf life by PC and FC followed by data elaboration with the Arrhenius model. Interestingly, we discovered that acidification kinetics by cells in storage at different temperatures were more comparable to FC survival kinetics than the ones described by PC. These results suggest that FC is a reliable tool to study the numerosity and the functional status of a probiotic population when, differently, PC limits the observation of a bacterial population purely to its cultivability. We are planning further experiments to extend this concept to the monitoring during time of probiotic effector molecules or beneficial pathways both in conventional and novel strains.

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

MP conceived the study. AV performed the enumeration and acidification analysis on samples in storage. SA and AA designed the set-up of the experiments and witnessed the cytofluorimetric evaluation. ADP, PB, and RD collaborated in the interpretation of the results, contributed to the writing and critically review of the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AV, SA, AA, ADP and MP are employees of Probiotical Research s.r.l. RD is co-founder and co-CEO of Seed Health and PB is employee of Seed Health.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.989563/full#supplementary-material

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Probiotic assessment and antioxidant characterization of *Lactobacillus plantarum* GXL94 isolated from fermented chili

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Oxidative stress is caused by an imbalance between prooxidants and antioxidants, which is the cause of various chronic human diseases. Lactic acid bacteria (LAB) have been considered as an effective antioxidant to alleviate oxidative stress in the host. To obtain bacterium resources with good antioxidant properties, in the present study, 113 LAB strains were isolated from 24 spontaneously fermented chili samples and screened by tolerance to hydrogen peroxide (H_2O_2). Among them, *Lactobacillus plantarum* GXL94 showed the best antioxidant characteristics and the *in vitro* antioxidant activities of this strain was evaluated extensively. The results showed that *L. plantarum* GXL94 can tolerate hydrogen peroxide up to 22mM, and it could normally grow in MRS with 5mM H_2O_2. Its fermentate (fermented supernatant, intact cell and cell-free extract) also had strong reducing capacities and various free radical scavenging capacities. Meanwhile, eight antioxidant-related genes were found to up-regulate with varying degrees under H_2O_2 challenge. Furthermore, we evaluated the probiotic properties by using *in vitro* assessment. It was showed that GXL94 could maintain a high survival rate at pH 2.5% or 2% bile salt or 8.0% NaCl, live through simulated gastrointestinal tract (GIT) to colonizing the GIT of host, and also show higher abilities of auto-aggregation and hydrophobicity. Additionally, the usual antibiotic susceptible profile and non-hemolytic activity indicated the safety of the strain. In conclusion, this study demonstrated that *L. plantarum* GXL94 could be a potential probiotic candidate for producing functional foods with antioxidant properties.

KEYWORDS

lactic acid bacteria, *Lactobacillus plantarum*, antioxidant activity, probiotic, hydrogen peroxide

Introduction

Probiotics are defined as "live microorganisms which when administered in adequate amounts, confer health benefits to the host" (Hill et al., 2014). The majority of probiotics belong to the lactic acid bacteria (LAB) group. Lactic acid bacteria (LAB) are a diverse group of gram-positive bacteria that widely exist in nature including plants, animals and

fermented foods, which was generally recognized as safe (GRAS) microorganisms, have a wide range of industrial applications, such as food fermentation, pharmaceutical, chemical and other industries (Feng and Wang, 2020). *Lactobacillus*, *Bifidobacteria*, and *Streptococcus* are the most famous genera that are described (Kiani et al., 2021). *Lactobacillus* is a fundamental group of LAB and is generally regard as safe. It is also the most common LAB strains in fermented foods (Das et al., 2016). The therapeutic effect of *Lactobacillus* fermented milk on gastrointestinal diseases has been found for a long time, and increasing experimental evidences in recent years have also made human understanding of *Lactobacillus* to a new height (Kok and Hutkins, 2018). Numerous studies have shown that some *Lactobacillus* strains benefit the host by improving balancing intestinal microbiota (Schnupf et al., 2018; He et al., 2020), regulating immunity (Galdeano and Perdigón, 2006; Håkansson et al., 2019), reducing cholesterol (Ebel et al., 2014; Fuentes et al., 2016), alleviating diabetes (Lee et al., 2013; Li et al., 2019), regulating lactase activity (Hajare and Bekele, 2017), and producing vitamins (Greppi et al., 2017; Li et al., 2017). As a symbiotic organism, it may play an important role in the host health maintenance and disease control. Oral *Lactobacillus* to support physiological and physical functions, thereby reducing the risk of disease or shortening the duration or severity of disease, has become an important means of treatment or prevention of disease. There are numerous preclinical and clinical studies confirming the gastrointestinal benefits of *Lactobacillus* in healthy individuals and in a wide range of both minor and serious health conditions (de Simone, 2019). As a result, sales of probiotic-based health products, including pharmaceuticals, dietary supplements, and functional foods, are growing rapidly. Meanwhile, *Lactobacillus* has become the most common probiotic preparation in the market, with good development prospects (Mazzantini et al., 2021).

Adverse environments such as high or low temperature, low pH, bile salts, oxygen, or limited nutrition can cause stress inducing that affect LAB survival during processing and storage, as well as survival, proliferation, and function in the gastrointestinal tract (GIT; Bron and Kleerebezem, 2011; Mills et al., 2011). Among them, oxidative stress is critical important, which can greatly affect the survival ability of LAB. High oxygen levels can lead to the formation and accumulation of reactive oxygen species (ROS), including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and highly reactive hydroxyl radicals (HO•), and cause cell damage, affecting their physiological functions (Amaretti et al., 2013). Therefore, improving the oxidative stress ability of LAB cells is crucial to ensure high bacterial activity in storage and gastrointestinal tract (Feng and Wang, 2020).

In addition, a great quantity of researches reported that some LAB strains have good antioxidant potential in recent years and can be used as a high-quality natural antioxidant (Mishra et al., 2015). Ingestion of these probiotics and their fermented products can help remove ROS from the host gut, reduce the risk of oxidative damage to host cells and the incidence of chronic diseases (Amaretti et al., 2013). For instance, pretreatment with *L. plantarum* ZLP001 could protected IPEC-J2 cells against H_2O_2-induced oxidative damage as indicated by cell viability assays and significantly alleviated apoptosis elicited by H_2O_2 (Wang et al., 2021). Therefore, the antioxidant capacity of LAB is gradually attracting people's attention. Various methodologies have been used to assess the antioxidative properties of LAB *in vitro* and *in vivo*. The common assays *in vitro* were based on determining O_2 tolerance (Li et al., 2010), resistance to H_2O_2 (Bchmeier et al., 1997), the reducing power (Tang et al., 2017), radical production and scavenging capacity including ABTS scavenging assay, DPPH scavenging assay (Mu et al., 2019), superoxide radical scavenging assay (Balcazar et al., 2007), hydroxyl radical scavenging assay (Gao et al., 2013), and oxygen radical absorbance capacity (ORAC) assay (Persichetti et al., 2014). The *in vivo* experiments involved establishing animal models, such as the D-galactose-induced aging mouse model (Tang et al., 2016; Lin et al., 2018) and UV-irradiated hairless mouse model (Ishii et al., 2014), and were based on determining physiological and biochemical indexes of experimental animal blood or tissues to evaluate the antioxidants' AOCs. At present, plenty of LAB strains with antioxidant effects through these experiments was screened out, such as *L. plantarum* 21, *Lactobacillus* sp. SBT-2028, *L. fermentum* ME-3, *L. plantarum* AR113 and *L. casei* KCTC 3260 (Kaizu et al., 1993; Lee et al., 2005; Ou et al., 2009; Achuthan et al., 2012; Lin et al., 2020). However, the antioxidant mechanisms of probiotic LAB are complex, and different strains use different mechanisms. It has been suggested that LAB may play antioxidant roles through scavenging ROS, chelating metals, increasing antioxidant enzymes levels, and modulating the microbiota (Feng and Wang, 2020). It is necessary to study the antioxidant properties of different strains for systematically revealing the antioxidant mechanism of LAB.

L. plantarum strain GXL94 with potential antioxidant capacity was isolated by hydrogen peroxide tolerance assay of 113 LAB strains obtained from fermented food. In the present study, we report the *in vitro* evaluation of probiotic properties and antioxidant activity of this isolate. The purpose of this research was to screen the functional LAB with good quality and lay a foundation for rational development and utilization of LAB.

Materials and methods

Bacterial strains and culture condition

Twenty-four samples of traditional fermented chili were collected from the domestic producers in Guangxi province in China. These samples were transferred to the laboratory and stored at refrigerator 4°C. The samples were enriched by adding 1% volume to 50 ml of sterile de Man Rogosa Sharpe (MRS) broth and incubated at 37°C for 48 h. 100 μl of each sample were spread over solidified MRS medium and incubated 24 h at 37°C. Then, five single colonies with distinct morphology were picked from each sample and subjected to initial morphological and

biochemical tests. One hundred and thirteen cultures were obtained in total and they were identified using the 16S rDNA sequences. The genomic DNA of the strain was extracted and then performed PCR amplification with universal primers. Finally, the PCR product was recovered and sequenced. The sequencing results came from the Changsha Qingke Biological Co., Ltd. Sequencing results were compared on the NCBI website for homology comparison. All strains were stored in de Man, Rogosa, and Sharpe (MRS) broth with 40% glycerol at −80°C and isolated on MRS agar plates. Before all experiment, they were transferred three times for high activity in MRS broth after activated at 37°C for 16 h.

Tolerance analysis to hydrogen peroxide of LAB

The previously reported method was used with some modifications (Bchmeier et al., 1997). The overnight cultures were adjusted to the same bacterial concentration and inoculated at 1% v/v into MRS broth containing 13 mM H_2O_2. After incubation at 37°C for 2 h, the cultures were plated onto MRS agar and incubated at 37°C for 48 h. The bacterial colonies were counted.

Three LAB strains were harvested by centrifugation after cultured for 18 h. GXL94, GXL50, SCL43 cells were resuspended and cultivated at 37°C for 2 h in fresh sterilized MRS broth containing different concentrations of H_2O_2 (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 mM) after adjusted OD_{600} to 0.80. Plate count method was used to count the LAB cells immediately after cultivated. The survival rate was calculated according to Guo et al. (2009).

Bioscreen assay

To analyze the biomass proliferation, pure cultures were inoculated (3%, v/v) into MRS broth supplemented with different concentrations of H_2O_2 (1.0, 2, 3, 4, 5, and 6 mM) and incubated at 37°C for 60 h. The control medium contained no H_2O_2. Every 1 h, the plate was shaken briefly, and the OD_{600} was measured by microplate reader.

Expression levels of genes involved in antioxidation

Lactobacillus plantarum GXL94 activated was inoculated (3%, v/v) in MRS broth containing different concentrations of H_2O_2 (0, 2.0, and 3.0 mM), and the LAB cells were harvested at different phase (lag phase, mid-logarithmic phase, primary stationary phase or middle-stationary phase) for RNA extraction. The total RNA of LAB cells were extracted and reversed into cDNA. These cDNAs were used as the template for determined antioxidant related gene expression level. The primers used were according to previous reported (Tang et al., 2017) and 16S rRNA was used as a house-keeping gene. The real-time PCR data was analyzed by using the $2^{-\Delta\Delta CT}$ assay and expressed as n-fold change relative to the experimental control (0 mM H_2O_2).

Detection of antioxidation in fermented supernatant, intact cells, and cell-free extract of GXL94

GXL94 was cultured in MRS broth at 37°C and the intact cells and fermented supernatant were harvested by centrifugation (6,000 g for 10 min at 4°C) after incubation for 18 h. The cells were washed three times with isotonic saline (0.9%) and resuspended in an equal volume of isotonic saline. The cell pellet was adjusted to 1×10^{10} CFU/ml. Cell-free extracts were obtained from cell suspensions containing 1×10^{10} CFU/ml that were subjected to ultrasonic disruption (ten 5-s strokes at 0°C with 5-s intervals, 10 min; Scientz-IID, China) and centrifuged (6,000 g for 10 min at 4°C) to remove the cell debris. *In vitro* antioxidative activities of these bacterial samples were evaluated in terms of the scavenging rates of 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), superoxide anion, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ABTS•), hydroxyl radicals and superoxide anion radical as described (Tang et al., 2017; Mu et al., 2019). The total reducing power assay was performed according to a reported method (Tang et al., 2017), and ascorbic acid was used as the standard to determine the reducing activity.

Growth of LAB under acid, bile salts, and NaCl

The LAB strains were inoculated (2%, v/v) into MRS broth and the cells were harvested by centrifugation after cultured for 18 h. Then the collected cells were treated with different initial pH (2.5, 3.0, 4.0, 5.0), various bile concentration (0.3%–2%, w/v) or various sodium chloride (NaCl) concentration (2.0%–8.0%, w/v) for 3 h, the survival rates were measured after treatments. The viable count before incubation was measured as control (CK).

Survival to simulated gastrointestinal tract transit

Survival to simulated gastrointestinal tract (GIT) conditions was evaluated according to the previous study (Lin et al., 2018). Intact cells (OD_{600} = 1.0) were resuspended in simulated gastric juice (SGJ:25 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g/l pepsin, pH 3.0) and incubated at 37°C for 3 h at 200 rpm. SGJ-treated cells were harvested by centrifugation (10,000 ×g for 5 min at 4°C), resuspended in equal volume of simulated pancreatic juice (0.15 g/100 ml bile salt, 1 g/L trypsin, pH 8.0), and incubated at 37°C for 3 h at 200 rpm. The cells incubated in normal saline were

used as controls. The survivors were counted by pour plating on MRS agar (incubation at 37°C for 48 h).

Auto-aggregation assay

The auto-aggregation ability was performed according to Lin et al. (2020). The stain of LAB cultured for 18 h were harvested by centrifugation (10,000 rpm, 10 min at 4°C). The sediment was re-suspended in sterile normal saline and adjusted to 10^9 CFU/ml after washed thrice with sterile normal saline. Next, the suspensions of LAB were incubated at 37°C for 5 h. The optical density at 600 nm of upper layer suspension was measured at 0, 1, and 5 h by a UV spectrophotometer without hanging the microbial suspension. Auto-aggregation ability was calculated as follows: Auto-aggregation ability (%) = $(1 - A_t/A_0) \times 100\%$.

Where A_t is OD_{600} at 1, 3, or 5 h, and A_0 is OD_{600} at 0 h.

Co-aggregation assay

The co-aggregation ability was performed according to Lin et al. (2020) with modifications. The strain of LAB and *E. coli* O157: H7 were harvested as described above. The LAB and *E. coli* O157:H7 cells were adjusted to 0.25 and 0.60 at OD_{600} after washed three times with normal saline, respectively. Equal volumes (5 ml) of different LAB and *E. coli* cells suspension were mixed together by vortexing for 10 s. The absorbance (A_{mix}) of upper layer of the suspension at 600 nm was measured after incubation at 37°C for 5 h. The percentage of co-aggregation was calculated as follows:

Co-aggregation ability (%) = $[((A_1 + A_2)/2 - A_{mix})/(A_1 + A_2)/2] * 100\%$.

Where A_1 and A_2 represent absorbance of the two strains at 0 h, respectively. A_{mix} represent absorbance of the mixture after 5 h of incubation.

Cell surface hydrophobicity assay

The cell surface hydrophobicity of LAB was determined according to previous report with some modifications. The method for preparing the cell suspensions was the same as that in auto-aggregation assay. The mixture of LAB cells and xylene in equal volumes (2 ml) was incubated at room temperature (25°C) for 30 min after mixed by vortexing for 3 min. The adherence of LAB to hydrocarbons was calculated as follows:

Cell surface hydrophobicity (%) = $[(A_0\text{-}A_t)/A_0] \times 100\%$.

Where A_0 is OD_{600} before treatment with xylene, and A_t is the OD_{600} of the aqueous phase after treatment with xylene.

Antibiotic susceptibility

Antibiotic susceptibility of LAB strains was evaluated using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Ahire et al., 2021). Susceptibility to the following 10 antibiotics was measured: 5 mcg of ciprofloxacin, and rifampicin; 10 mcg of ampicillin, gentamicin, and penicillin G; 15 mcg of erythromycin; 30 mcg of tetracycline, chloramphenicol, vancomycin, and kanamycin. LAB strains (1×10^8 CFU/ml) were spread on MRS agar plate, and antibiotic disks were placed on these plates under sterile conditions. After incubation at 37°C for overnight, the diameter of clear zones surrounding the disks was measured in millimeters (mm).

Hemolytic activity

Hemolytic activity was performed as described by Ahire et al. (2021) with some modifications. Overnight grown culture of *L. plantarum* GXL94 was streaked on 7% defibrinated sheep blood agar plates and incubated at 37°C for 48 h. After incubation, the plates were observed for α-hemolysis (dark and greenish zones), β-hemolysis (lightened-yellow or transparent zones), and γ-hemolysis (no change or no zones).

Statistical analysis

The results were expressed as mean ± standard error. All experiments were carried out in triplicate. The procedure univariate in SAS was used to analyze data on relative expression value of each gene between different treatments. One-way analysis of variance (ANOVA) and Student's *t* test were used to test the significant differences ($p < 0.01$ and $p < 0.001$) of means.

Results

Lactobacillus plantarum GXL94 was resistant to hydrogen peroxide

In this study, three cultures exhibited a moderate-to-strong resistance to H_2O_2. *L. plantarum* GXL94 and GXL50 was the most resistant strain against H_2O_2 with more than 100 CFU in the plate. *Lactobacillus pentosus* SCL43 was less tolerance than GXL94 and GXL50, however it also showed a certain tolerance to H_2O_2 (Table 1). Thus, these three strains were chosen to examine the tolerance to different concentration of H_2O_2.

The tolerate ability of GXL94, GXL50 and SCL43 to different concentrations of H_2O_2 were presented in Table 2. The survival rate of three strains were decreased with the concentration of H_2O_2 increasing. The survival rates were over

50% at 14 mM H_2O_2 for GXL94 or GXL50 and at 12 mM H_2O_2 for SCL43. When concentration of H_2O_2 up to 22 mM, there was still a survival rate of 19.64% for GXL94. However, at the same concentration of H_2O_2, there was no viable LAB colonies for GXL50 and SCL43 after cultivated for 2 h. This result indicated that strain GXL94 possessed a stronger antioxidant property.

Growth of GXL94 at different concentrations of H_2O_2

As mentioned above, H_2O_2 could induced oxidative stress of LAB. The growth of SCL43, GXL50 and GXL94 was inhibited by H_2O_2 as shown in Figure 1. The addition of H_2O_2 inhibited the growth and the lag phase was obviously elongated with the concentration of H_2O_2 increasing, which indicating that the presence of H_2O_2 cause bacterial oxidative damage. The further results showed that the growth inhibition of GXL94 in the range from 0 to 5 mM, and the growth was ceased as concentration amount to 6 mM H_2O_2. These results showed that GXL94 could survived the challenge of H_2O_2 up to 5 mM, higher than SCL43, GXL50 and other strains reported before.

TABLE 1 Resistance of LAB strains to hydrogen peroxide.

CFU/100 (μl)	Total	LAB strains number
<10	109	
10~49	1	GXL97
50~100	1	SCL43
>100	2	GXL50, GXL94

Analysis of the transcript levels of the antioxidant-related genes in strains exposed to H_2O_2

Relative expression levels of eight antioxidant-related genes were detected when exposed to different concentrations of H_2O_2 (Figure 2). The result showed that the expression levels of four genes including *GPX*, *gshR2*, *gshR3*, and *gshR4* in GXL94 at lag phase were increased with increasing of H_2O_2 concentration, and shown positive correlation, however *gshR1*, *npx*, *cat*, and *nox* gene with no apparent correlation between H_2O_2 and expression levels. The expression levels of *gshR1* and *nox* at the mid-logarithmic phase were positive correlation with the increasing of H_2O_2 concentration, while the genes expression levels of *npx* was decreased with the increasing H_2O_2 concentration, moreover there are no apparent correlation between H_2O_2 and mRNA level of other six genes. At primary stationary phase, all of eight antioxidant-related genes were significantly upregulated with H_2O_2 treatment, but they were no apparent correlation. At middle stationary phase, the expression level of *gshR1*, *gshR3* and *nox* were significantly induced, while only the gene *nox* expression level showed an apparent correlation with H_2O_2 concentration.

Among the induced transcription of eight genes related to antioxidation at primary stationary phase, expression of *GPX* was increased by 11.73-fold and 6.26-fold under 2.0 mM and 3.0 mM H_2O_2 challenge, respectively. Expression level of *gshR1* was increased by 3.99-fold and 3.22-fold under 2.0 mM and 3.0 mM H_2O_2 challenge, respectively. The expression level of *gshR2* was increased by 122.28-fold and 112.22-fold treated with 2.0 mM and 3.0 mM H_2O_2, respectively. In addition, *gshR3* and *gshR4* mRNA expression was elevated by 40.32-fold/18.86-fold and 46.71-fold/14.67-fold in response to the treatment with 2.0 mM and 3.0 mM H_2O_2. The cumulative values of the eight genes' expression at primary stationary phase were largest

TABLE 2 Survival rates (%) of LAB in different concentrations of hydrogen peroxide.

Concentrations of H_2O_2	GXL94		GXL50		SCL43	
	CFU/ml	Survived rate (%)	CFU/ml	Survived rate (%)	CFU/ml	Survived rate (%)
0 mM	$(5.43 \pm 0.21) \times 10^8$	100%	$(5.5 \pm 0.26) \times 10^8$	100%	$(1.36 \pm 0.01) \times 10^9$	100%
2 mM	$(5.33 \pm 0.58) \times 10^8$	99.91%	$(5 \pm 0.1) \times 10^8$	99.53%	$(1.06 \pm 0.06) \times 10^9$	98.81%
4 mM	$(4.93 \pm 0.8) \times 10^8$	99.52%	$(5.4 \pm 0.26) \times 10^8$	99.91%	$(7.1 \pm 0.3) \times 10^8$	96.91%
6 mM	$(4.29 \pm 0.79) \times 10^8$	98.83%	$(4.03 \pm 0.86) \times 10^8$	98.45%	$(1.26 \pm 0.17) \times 10^8$	88.69%
8 mM	$(1.65 \pm 0.15) \times 10^8$	94.08%	$(2.53 \pm 0.51) \times 10^8$	96.13%	$(8.81 \pm 0.82) \times 10^7$	86.99%
10 mM	$(1.12 \pm 0.19) \times 10^7$	80.72%	$(9.3 \pm 0.36) \times 10^6$	79.73%	$(6.18 \pm 1.7) \times 10^7$	85.30%
12 mM	$(1.73 \pm 0.15) \times 10^6$	71.40%	$(1.81 \pm 0.29) \times 10^6$	71.60%	$(2.75 \pm 0.05) \times 10^6$	70.50%
14 mM	$(4.13 \pm 0.55) \times 10^4$	52.85%	$(3.64 \pm 0.84) \times 10^4$	52.18%	$(1.42 \pm 0.01) \times 10^4$	45.47%
16 mM	$(1.19 \pm 0.07) \times 10^4$	46.66%	$(5.51 \pm 1.32) \times 10^3$	42.80%	$(3.63 \pm 0.24) \times 10^3$	38.98%
18 mM	$(1.1 \pm 0.17) \times 10^3$	34.82%	$(3.53 \pm 0.62) \times 10^2$	29.15%	$(4.74 \pm 0.04) \times 10^2$	29.29%
20 mM	$(1.45 \pm 0.24) \times 10^2$	24.74%	$(2.73 \pm 0.64) \times 10^1$	16.44%	$(4.45 \pm 0.45) \times 10^1$	18.05%
22 mM	$(5.2 \pm 0.8) \times 10^1$	19.64%	0	0.00%	0	0.00%

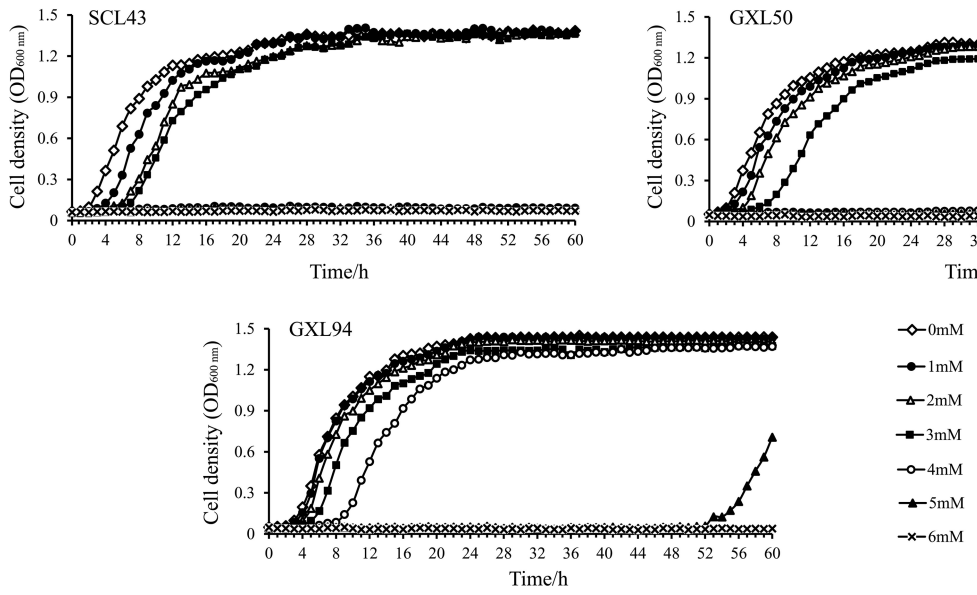

FIGURE 1
Growth density of *Lactobacillus pentosus* SCL43, *Lactobacillus plantarum* GXL50 and GXL94 under oxidative stress. Experiments were conducted in MRS containing different concentrations of H_2O_2 (1, 2, 3, 4, 5, and 6mM).

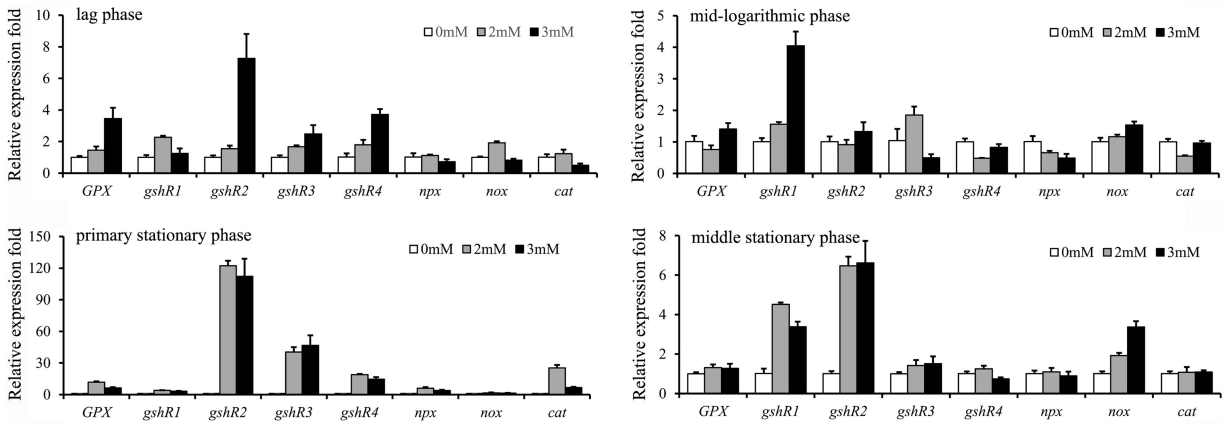

FIGURE 2
Effects of the addition of hydrogen peroxide on the expression of 8 antioxidant-related genes in *Lactobacillus plantarum* GXL94. The graphs show the relative mRNA levels of 8 genes of cells grown in MRS medium with different concentrations of hydrogen peroxide at the lag phase, mid-logarithmic phase, primary stationary phase and middle stationary phase.

of the four cultivation stages, which indicate that GXL94 exhibited higher antioxidant activity in the primary stationary, the GSH system was supposed to have played a major role in antioxidant activity.

Antioxidant activity *in vitro*

The antioxidant mechanisms of probiotic LAB are complex, and different strains use different mechanisms. Various approaches have to be combined in order to identify and characterize the antioxidant activity of LAB. In this study, five commonly used antioxidant indexes of GXL94 *in vitro* were analyzed (Table 3).

Reducing activity of different samples of the fermentate of GXL94 was measured and expressed as an equivalent amount of ascorbic acid. The cell-free extract exhibited the stronger reducing activity (262.88 ± 12.30 μmol/L ascorbic acid equivalent) than the intact cells (185.66 ± 5.94 μmol/L ascorbic acid equivalent).

Scavenging of DPPH free radical is attributed to the hydrogen donating ability of antioxidants and is routinely used

TABLE 3 Antioxidant activity of different samples of *Lactobacillus plantarum* GXL94.

Subjects	Fermented supernatant	Intact cell	Cell-free extract
Reducing activity (μmol/L ascorbic acid equivalent)	-	185.66 ± 5.94	262.88 ± 12.30
Scavenging of DPPH•(%)	95.98 ± 1.59	83.82 ± 0.71	38.84 ± 1.93
Scavenging of ABTS (%)	94.63 ± 0.54	89.47 ± 0.36	89.61 ± 0.31
Scavenging of •OH (%)	88.47 ± 0.32	61.43 ± 0.82	86.01 ± 0.28
Scavenging of $\bullet O_2^-$ (%)	34.41 ± 1.4	10.53 ± 3.1	14.47 ± 1.73

for antioxidant assay. Experiment result indicated that the fermented supernatant (95.98% ± 1.59%) and intact cells (83.82% ± 0.71%) of *L. plantarum* GXL94 exhibited more stronger DPPH scavenging ability compared to the cell-free extract (38.84% ± 1.93%; $p < 0.05$).

ABTS radical cation-scavenging activity was also an important index for antioxidant capacity analysis. In this study, ABTS scavenging ability was evaluated, and the result showed that the intact cells and cell-free extract exhibited a high scavenging activity, with ratios at 89.47% ± 0.36% and 89.61% ± 0.31%, respectively (Table 2). However, which were still slightly weaker than fermented supernatant (94.63% ± 0.54%).

Fenton reaction was used to measure the scavenging activities of hydroxyl radicals. The fermented supernatant (88.47% ± 0.32%) and cell-free extract (86.01% ± 0.28%) demonstrated a significantly higher scavenging capacity, compared to that of intact cells (61.43% ± 0.82%)($p < 0.05$).

Through the improved pyrogallol autoxidation method, it was found that all of samples exhibited $\bullet O_2^-$-scavenging activity, and the clearance rate of fermentation supernatant was highest, reached 34.41% ± 1.4%. In conclusion, *L. plantarum* GXL94 has a great antioxidant activity *in vitro*, and has promising antioxidant potentials in therapeutic benefits for human health.

Tolerances to acid, bile salt, and NaCl

Tolerances to low acid and high bile stresses are significant properties for any potential probiotic bacteria. The abilities of LAB to tolerate the pH and bile salt were presented in Figure 3. The gradual decreased survival of *L. plantarum* GXL94 (pH 5.0 98.07%; pH 4.0 98.04%; pH 3.0 97.8%; pH 2.5 96.7%) was noticed when the cells were incubated at different pHs as compared with control (Figure 3A). Moreover, a similar decreased pattern (97.34%; 97.09%; 95.37%; 95.09%) was observed with the concentration of bile salt treatment increases. Besides this, the viable cells of GXL94 was increased under the condition of 2% NaCl due to proliferation, but decreased significantly as osmotic pressure increasing (4%: 97.4%; 6%: 96.5%; 8%: 95.4%) as compared with the control (Figure 3). These results indicated that GXL94 has good tolerance to low acid and high concentration of bile salts and NaCl treatment.

Survival to simulated gastrointestinal tract transit

The isolates were further subjected to survival in simulated gastrointestinal tract (GIT) conditions, and the survivors (log N/N_0) to SGJ and simulated GIT juices are shown in Figure 4. GXL94 showed good survival percentage in both conditions for 3 h, with the survival rate above 99% in 3 h in SGJ and above 95% in 3 h in GIT juices. Comparatively, GXL94 exhibited the strongest resistance to gastric treatment, but the lowest survival to pancreatic juice. Thus, the intact cells of GXL94 could live through simulated GIT in colonizing the GIT of the host.

Adhesion potential

Bacterial adhesion to hydrocarbon was evaluated, and as shown in Table 4, the percentage hydrophobicity for GXL94 cells showed 33.01 ± 3.65% for xylene. The co-aggregation ability of GXL94 with the pathogen *E. coli* O157:H7 was only 30.87% ± 0.21% after incubation at 37°C for 5 h. In addition, GXL94 showed an auto-aggregation percentage of 10.63%, 29.48%, and 50.45% after 1, 3, and 5 h of incubation, respectively.

Antibiotic susceptibility and hemolytic activity

Tolerance of the strain GXL94 to 10 kinds of antibiotics was shown in Table 5. As is known to all, the susceptibility and resistance of LAB to various antibiotics are variable depending on the species (Li et al., 2020). *L. plantarum* GXL94 showed sensitivity toward antibiotics belonging to the class of β lactam, macrolactams, nitrobenzenes (Table 5). Meanwhile, GXL94 was resistant to quinolones, aminocyclitol glycosides, polyketide, glycopeptides, aminoglycosides class of tested antibiotics (Table 5). Besides this, GXL94 also was found intermediary sensitive to macrolides. Our results indicated that this LAB strain exhibited a good antibiotic susceptibility.

In addition, GXL94 showed no zone of hemolysis when streaked onto sheep blood agar plates, which was indicative of γ-hemolytic activity (non-hemolytic). And these result further confirmed the biosafety of this strain.

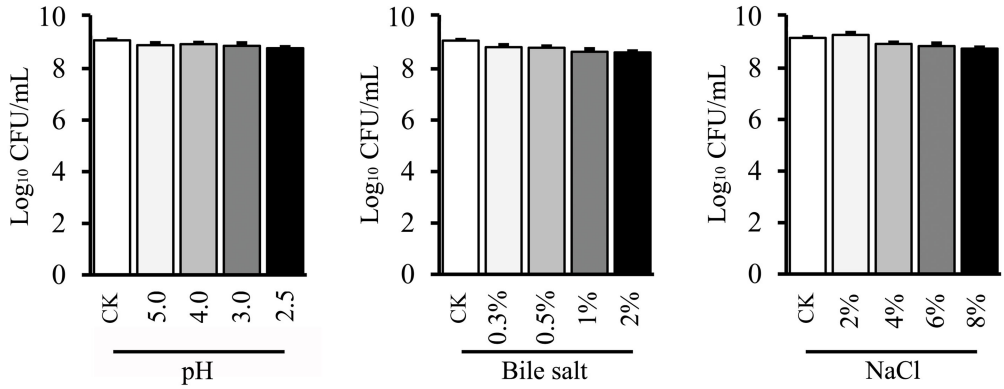

FIGURE 3
Effect of low pH, bile salt, and NaCl on viable count of *Lactobacillus plantarum* GXL94.

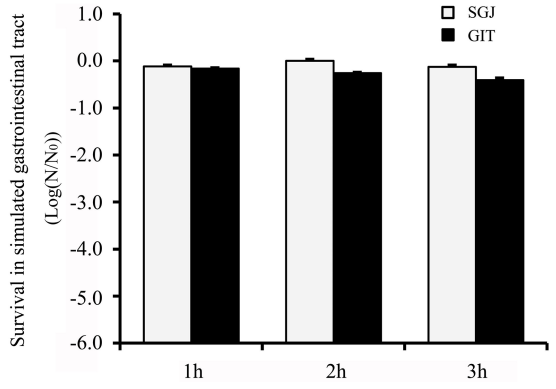

FIGURE 4
Tolerance of simulated gastrointestinal tract (GIT) transit of the cells of *Lactobacillus plantarum* GXL94. Survival was expressed as reduction of log (N/N0) cycles, where N_0 and N are the number of viable cells, respectively, before and after exposure to stress; standard error bars are shown.

Discussion

H_2O_2 is a weak oxidant, but can permeate the cell membrane and form more active ROS, such as hydroxyl radicals, *via* the Fenton reaction, auto-oxidation and other reactions thereby causing oxidative damage subsequently (Mishra et al., 2015). It is worth mentioning that lactic acid bacteria are regarded as catalase-negative microorganisms used to produce fermented foods (Mokoena et al., 2021). In recent years, a plenty of LAB strains resistant to hydrogen peroxide have been reported. For instance, *L. plantarum* KCC-24 showed moderate resistance to H_2O_2 as which can grow normally under the condition of 1.5 mM hydrogen peroxide (Vijayakumara et al., 2015). *L. plantarum* Y44 had a higher survival rate (>55%) when exposed to 1.0 mM hydrogen peroxide (Mu et al., 2018). Moreover, the latest research confirms that *L. plantarum* AR113 could tolerance against 8.0 mM H_2O_2, and it turned into exponential phase at 48 h under 3.5 mM H_2O_2 (Lin et al., 2020). In this study, we confirmed that *L. plantarum* GXL94 has a stronger hydrogen peroxide resistance than these antioxidant strains mentioned above, as it can tolerate up to 22 mM hydrogen peroxide concentration.

Most antioxidant evaluation methods based on reactive species can be applied to probiotics, including intact cells, cell-free extracts and cell lysates or their metabolic products to evaluate their antioxidant capacity *in vitro* (Lin and Chang, 2000; Zolotukhin et al., 2018; Noureen et al., 2019). In this study, we evaluated the antioxidant effects of this three GXL94 samples *in vitro* respectively, and found both fermented supernatant and intact cells have a good antioxidant potential capacity. Previous research results show that antioxidant activity of LAB strains could be attributed to their production of cell-surface active compounds including proteins, lipoteichoic acid or extracellular polysaccharides (Li et al., 2012; Polak-Berecka et al., 2013), carotenoids (Kim et al., 2019), ferulic acid (Bhathena et al., 2008), or histamine (Azuma et al., 2001). Therefore, whether GXL94 can produce antioxidant molecules to improve its antioxidant performance remains to be further verified.

Antioxidant enzymes including SOD and catalase, thioredoxin, NADH oxidase-NADH peroxidase system, and glutathione system are regarded as three main enzymatic defense mechanisms against oxidative stress in LAB (Serrano et al., 2007; Feng and Wang, 2020). Catalase plays an important role in reducing oxidative stress by decomposing H_2O_2. LAB have been classified for a long time as unable to produce catalase because of the lack of heme (Ricciardi et al., 2018). However, several researches in recent years have demonstrated that at least some LAB strains are able to synthesis a heme-containing catalase, such *L. plantarum* CNRZ 1228, *L. sakei* YSI8, *L. brevis* CGMCC1306 (Abriouel et al., 2004; An et al., 2010; Lyu et al., 2016). The presence of this gene can significantly improve LAB antioxidant activity. We also cloned heme-containing catalase gene (*cat*) in strain GXL94, and the transcription of this gene was elevated by 25.3-fold and 6.8, when treated with 2 mM and 3 mM H_2O_2 at primary stationary phase, respectively. Coupled NADH

TABLE 4 Auto-aggregation, Co-aggregation and cell surface hydrophobicity of the LAB.

Strains	Auto-aggregation (%)			Co-aggregation (%)	Cell surface hydrophobicity (%)
	1 h	3 h	5 h		
GXL94	10.63 ± 2.36	29.48 ± 0.34	50.45 ± 4.16	30.87 ± 0.21	33.01 ± 3.65

TABLE 5 Antibiotic susceptibility of *Lactobacillus plantarum* GXL94.

Class of antibiotic	Antibiotic	Concentration	Zone of inhibition in millimeter (mm)	Breakpoints as per CLSI and EUCAST
β lactam	Ampicillin	10 mcg	29.2	S
	Penicillin G	10 mcg	30.35	S
Quinolones	Ciprofloxacin	5 mcg	8.68	R
Aminocyclitol glycosides	Gentamicin	10 mcg	6	R
Polyketide	Tetracycline	30 mcg	10.6	I
Macrolactams	Rifampicin	5 mcg	26.6	S
Nitrobenzenes	Chloramphenicol	30 mcg	27.13	S
Glycopeptides	Vancomycin	30 mcg	6	R
Macrolides	Erythromycin	15 mcg	17.55	I
Aminoglycosides	Kanamycin	30 mcg	6	R

oxidase—NADH peroxidase system was a common oxidative stress resistance mechanism found in LAB. In these coupled reactions, intracellular oxygen is first used to oxidize NADH into NAD^+ by NADH oxidase, thereby releasing H_2O_2. Subsequently, H_2O_2 is reduced to H_2O by NADH peroxidase (Miyoshi et al., 2003). In this study, we found that two key genes (*npx* and *nox*) of these system were present in GXL94, and the *npx* gene was significantly up-regulated 3.8–6.1-fold at primary stationary phase stage in the presence of hydrogen peroxide. To some extent, the antioxidant capacity of bacteria is related to the biosynthesis or accumulation of glutathione. Bacteria with fully operational glutathione system can directly detoxify or eliminate H_2O_2 and lipid peroxyl radicals, and thus have defense against H_2O_2 accumulation (Kullisaar et al., 2010). Previously, it was thought that gram-positive bacteria cannot synthesize GSH and thus do not have the GSH-glutaredoxin system. However, later studies revealed that some LAB, such as and *Lactobacillus fermentum* E3 and E18, naturally synthesize GSH at a high level (Kullisaar et al., 2002). Whereafter, a fully functional GSH system comprising both GSH peroxidase and GSH reductase in *L. fermentum* strain ME-3 was first time discovered by Kullisaar and colleagues (Kullisaar et al., 2010). We also identified a functional GSH system in GXL94 in present study. It was noteworthy that the transcription of the four *gshR* genes were elevated by 3.22-112-fold when GXL94 was challenged with high concentration of H_2O_2 (3.0 mM). Moreover, *GPX* function as a H_2O_2 receptor and redox transducer, which can directly detoxify or eliminate hydrogen peroxide and peroxyl radicals (Kullisaar et al., 2010). Our results also confirmed that the gene was significantly up-regulated under hydrogen peroxide stress. Therefore, we speculate that the GSH system played a major role to ensure the survival of GXL94 under hydrogen peroxide pressure. In recent years, some researchers have used omics studies to reveal the molecular mechanism of the antioxidant performance of *L. plantarum*. The results showed that various metabolic pathways such as base excision repair system, recombinational DNA repair pathway, pyruvate metabolism, carbon metabolism, trichloroacetic acid cycle, amino acid metabolism, and microbial metabolism were closely related to these antioxidant properties (Zhai et al., 2020; Tian et al., 2022). In future studies, we can use these methods to further explore the molecular mechanism of GXL94 antioxidant properties.

Lactic acid bacteria mainly play a probiotic role in the intestinal tract. After oral ingestion, it needs to experience adverse survival factors in the gastrointestinal tract, such as low pH of gastric acid, bile salts, digestive enzymes, etc. Tolerance to gastrointestinal tract is an important indicator for screening and evaluating effective probiotics. *Lactobacillus paracasei* FM-LP-4 could survive pH 2.5 for 3 h, but the log CFU/ml was greatly reduced from initial 9 to 7 after 3 h and also showed tolerance to 0.5% bile (Wang et al., 2016). Adesulu-Dahunsi et al. (2018) reported the survival rate of *L. plantarum* strain OF101 was 98.4% and 96.9% at pH 2.5% and 0.3% bile, respectively. The results of probiotic characteristic tests showed that MA2 could survive at pH 2.5% and 0.3% bile salt for 3 h and maintained a survival of 70% (Tang et al., 2017). In addition, commercial probiotic *L. casei* Zhang showed survival of 82.7%/97.4% in simulated gastric juice or intestinal juice (Guo et al., 2009). In another study, the survival percentage of *L. plantarum* AR113 in simulated fluids were only 86.7% (Lin et al., 2018). In view of these reports, our isolate GXL94 were found to show better performance in terms of survival in GIT.

The adhesion ability of lactic acid bacteria to host intestinal cells is another important indicator to evaluate its probiotic property. Auto-aggregation, co-aggregation and adhesion to solvents are the measures to estimate bacterial ability to colonize the intestinal wall. In present study, the auto-aggregation

percentage observed with the strain GXL94 (50.45%, 5 h) was higher as compared with the *L. plantarum* AR113 (30.1%), 1KMT (45.63%), 4 BC (39.56%; Kaktcham et al., 2018; Lin et al., 2020). The adhesion of GXL94 to xylene was comparatively higher than *L. plantarum* strains UBLP40 (20.4%) and 1KMT (23.71%) reported for xylene adhesion in 1 h (Kaktcham et al., 2018; Ahire et al., 2021). These results indicate that our strain are capable of adhering to epithelial cells and mucosal surfaces. Moreover, GXL94 also showed 30.87% co-aggregation with *E. coli*. Co-aggregation of probiotic LAB strains with pathogenic bacteria enables them to form a barrier that may facilitate the colonization of the pathogen in the gastrointestinal tract. Low levels of co-aggregation may play an important role in preventing the formation of biofilms and preventing the persistence of pathogenic species in the GIT (Todorov et al., 2017).

One of the important characteristic of probiotic is the safety for human consumption and the absence of acquired and transferable antibiotic resistance. In this study, we observed that *L. plantarum* GXL94 showed sensitive to ampicillin, penicillin G, tetracycline, rifampicin, chloramphenicol and erythromycin, which was similar to the recent finding of Lin et al. (2020). Some researchers have tested the drug-resistant phenotypes of selected probiotics and found that most LABs are intrinsic resistant to kanamycin, gentamicin, streptomycin, vancomycin and ciprofloxacin (Li et al., 2020). Our result corroborate well with these results. Actually, some probiotic strains with intrinsic antibiotic resistance can help restore the intestinal microbiota after antibiotic treatment (Gueimonde et al., 2013). Some *Lactobacillus* strains could acquired tetracycline resistance through horizontal gene transfer (Campedelli et al., 2019). GXL94 showed moderate tetracycline resistance in this study, therefore, we need to evaluate whether there are mobile genetic elements with horizontal transfer potential on both sides of tetracycline resistance related genes in future studies. In addition, the hemolytic activity was also evaluated as a criteria for selecting probiotic strains. Our result indicated that GXL94 showed γ-hemolytic or no hemolytic activity, and that it is consistent with found in previous studies (Bhushan et al., 2021). Overall, based on the antibiotic susceptibility profile and hemolytic activity *in vitro*, GXL94 could fulfill the primary requirements of a probiotic and make this strain a safe candidate for further validation in future probiotic studies *in vivo*.

Oxidative stress is the cause of various chronic human diseases, such as cancer, diabetes, heart disease, stroke, Alzheimer's disease, rheumatoid arthritis, cataract, and aging (Mishra et al., 2015). Antioxidants are molecules that interact with free radicals generated in cells and terminate the chain reaction to relieve this oxidative stress before damage is done to the vital molecules. However, in the process, antioxidants are themselves oxidized. Thus, there is a constant need to replenish antioxidant resources as one antioxidant molecule can react with only a single free radical (Halliwell and Gutteridge, 1990). Consequently, it is essential to search for and develop natural nontoxic antioxidants to protect the human body from free radicals and slow the progress of many chronic diseases. LABs have been considered as an emerging source of effective antioxidants in recent years due to their long tradition of safe use and potential intestinal benefits (Nardone et al., 2010). It is a general consensus that LAB supplementation can regulate intestinal microbiota, and this microbiota composition reconstruction has the potential to improve the host redox state (Qiao et al., 2012; Xin et al., 2014). Therefore, it is important to consume dietary supplements or fermented foods that contain high antioxidant activity lactic acid bacteria to delay the development of chronic diseases. In view of its good antioxidant properties and beneficial properties, we believe that GXL94 has a good prospect in the application of functional foods with antioxidant properties.

Conclusion

In this study, a LAB strain demonstrated good *in vitro* antioxidative effect was screened out through a series of experimental evaluation. *L. plantarum* GXL94 showed good tolerance to high concentration of hydrogen peroxide pressure and strong scavenging capacities to various free radical. After analyzing the eight antioxidant related genes, *GPX* and *gshR* were found to up-regulated greatly after H_2O_2 treated, which may provide the evidence for revealing the molecular mechanism of antioxidant activity of GXL94. Besides this, GXL94 also exhibited promising survivability to acid-, bile salt-, osmotic-, gastric juice-, intestinal juice-tolerance tests, and high auto-aggregation. The non-hemolytic activity and antibiotic susceptibility profile of the strain indicated the safety of probiotic for use. Overall, based on the above analysis for antioxidative and probiotic properties, the strain *L. plantarum* GXL94 could be considered as potential antioxidant probiotic for commercial use.

Data availability statement

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

Author contributions

YZ, WG, CXi, and YP were responsible for the study design, supervision, and manuscript preparation. CXu and ZZ were responsible for the experiment and analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Consumption of the cell-free or heat-treated fractions of a pitched kefir confers some but not all positive impacts of the corresponding whole kefir

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Introduction: Kefir consumption can have many metabolic health benefits, including, in the case of specific kefirs, improvements in plasma and liver lipid profiles. Our group has previously shown that these health benefits are dependent on the microbial composition of the kefir fermentation, and that a pitched kefir (PK1) containing specific traditional microbes can recapitulate the health benefits of a traditional kefir. In this study we investigated how different preparations of kefir impact cholesterol and lipid metabolism and circulating markers of cardiovascular disease risk and determine if freeze-drying impacts health benefits relative to past studies.

Materials and methods: Eight-week-old male and female C57Bl/6 mice were fed a high fat diet (40% kcal from fat) supplemented with one of 3 freeze-dried kefir preparations (whole kefir, cell-free kefir, or heat-treated kefir) for 8 weeks prior to analysis of plasma and liver lipid profiles, circulating cardiovascular disease (CVD) biomarkers, cecal microbiome composition, and cecal short-chain fatty acid levels. These groups of mice were compared to others that were fed a control low-fat diet, control high fat diet or high fat diet supplemented with milk, respectively.

Results: All kefir preparations lowered plasma cholesterol in both male and female mice, while only whole kefir lowered liver cholesterol and triglycerides. Plasma vascular cell adhesion molecule 1 (VCAM-1) was lowered by both whole kefir and heat-treated kefir in male mice but not females, while c-reactive protein (CRP) was unchanged across all high fat diet fed groups in males and females.

Conclusion: These results indicate that some of the metabolic benefits of consumption of this kefir do not require whole kefir while also indicating that there are multiple compounds or components responsible for the different benefits observed.

KEYWORDS

kefir, cholesterol, metabolic health, cardiovascular disease, non-alcoholic fatty liver disease

Introduction

Humans have been utilizing fermentation as a means of food preservation for thousands of years (Hutkins, 2018). One of the most common substrates for fermentation is milk, with foods such as yogurt and kefir among the oldest fermented foods known (Bourrie et al., 2016; Hutkins, 2018). Kefir in particular is gaining popularity, largely due to an increased appreciation of the potential health benefits. Benefits that have been attributed to specific kefirs include anti-cancer (de Moreno de LeBlanc et al., 2006; Maalouf et al., 2011), immunomodulatory (Vinderola et al., 2005; Lee et al., 2007), cholesterol lowering (Bourrie et al., 2018, 2021), and other metabolic health improvements (Al-Shemmari et al., 2018; Ghizi et al., 2021).

Recently, interest in the health benefits of fermented foods has extended beyond the whole foods to consider the contribution of different fractions and even individual components of the food matrix. Indeed, many food fermentations result in the transformation of food constituents and the production of bioactive components (Marco et al., 2017). Kefir is one of the fermented foods that has had health benefits attributed to multiple different components, which not only includes the fermentation by-products but also a consortium of live microorganisms (yeast and bacteria) that could directly affect the gastrointestinal landscape. For instance, the exopolysaccharides produced by kefir specific microorganisms have been shown to have anti-obesity and immunomodulatory activity (Vinderola et al., 2006a; Lim et al., 2017), while cell-free fractions of kefir have also been shown to have immunomodulatory, lipid lowering, and anti-cancer effects (Vinderola et al., 2006b; Gao et al., 2013; Santanna et al., 2017). Additionally, kefir peptides and peptide fractions have been shown to have a wide range of health impacts including reductions in hyperlipidemia, atherosclerosis, improved neurological function, and reduced non-alcoholic fatty liver disease (NAFLD) risk (Quiros et al., 2005; Chen et al., 2016; Tung et al., 2017, 2020; Malta et al., 2022). There is also some evidence that kefir or kefir components may improve factors associated with atherosclerosis and cardiovascular disease (CVD) development, both of which are associated with obesity and hyperlipidemia (Santanna et al., 2017; Tung et al., 2020; Ghizi et al., 2021).

While traditional kefir is made using kefir grains, pitched kefir is produced by pitching pure cultures microorganisms into milk prior to fermentation. Our group has previously shown that a pitched kefir produced using microorganisms isolated from a traditional kefir could recapitulate the health benefits of traditional beverage in a mouse model of diet-induced obesity (Bourrie et al., 2021). Given that our previous work only examined the impact of whole kefir, we set out to determine whether the microorganisms present in our pitched kefir (PK1), or their components are required for lipid lowering, and if this lipid lowering effect was accompanied by concomitant improvements in CVD and atherosclerosis biomarkers such as vascular cell adhesion molecule 1 (VCAM-1) and C-reactive protein (CRP). To examine this, we fed a high-fat/high-cholesterol diet supplemented with PK1, a centrifuged and filter sterilized cell-free fraction (CFK), and a heat-treated fraction (HK) that underwent a high heat treatment to kill the kefir microbes. We also wanted to determine if freeze-dried kefir consumption would result is health benefits similar to those observed using liquid kefir in our past work. The CFK preparation allowed us to remove all microbes from the kefir and test a fraction that contained no microbes or microbial components, while the HK preparation allowed us to test a fraction in which the microbes had been killed but their microbial components were still present. By comparing how these different kefir preparations impacted weight gain, plasma and liver lipid profiles, short chain fatty acid production, gastrointestinal microbiome composition, and circulating biomarkers for CVD, we hoped to gain insight into how microbial fermentation byproducts may impact the previously described health benefits of our pitched kefir product. Sex differences that may alter the health impacts of PK1were determined by including both male and female mice. We hypothesized that the whole, heat-treated, and filter-sterilized kefir fractions share a common fermentation metabolite that improves plasma and liver lipid profiles, as well as VCAM-1 and CRP.

Materials and methods

Kefir grain sourcing, microbial composition, and production

Kefir fermentation was carried out as previously described for PK1 pitched kefir (Bourrie et al., 2021). Briefly, bacterial and yeast cultures were pitched into 2% fat milk and fermented at room temperature (22°C) for 18 h. Cell-free kefir was produced through centrifugation (3,000 x g for 20 min at 4°C) followed by filter sterilization using a 0.22 µm filter, while heat-treated kefir was produced by heating kefir at 85°C for 40 min. Both cell-free and heat-treated kefir were produced from freshly fermented PK1 kefir.

Animals and treatments

A 48-week-old C57BL/6 female and 48-week-old C57BL/6 male mice were obtained from Jackson Labs. Mice were separated by sex and randomly grouped into 20 cages with 4 mice per cage by a blinded lab animal technician and groups were balanced for average body weight. All mice were housed on aspen wood chip bedding in filter-topped cages with nestlets, tunnels, and nesting material as enhancements. Room conditions, and access to food and water were carried out as previously described (5). Cages were allocated to one of 5 groups ($n = 8$) consisting of low-fat diet + milk (LFD), high fat diet (HFD) + milk (HFD), HFD + PK1 (PK1), HFD + cell-free PK1 (CFK), and HFD + heat-treated PK1 (HK). The LFD group received standard rodent chow (PicoLab Rodent Diet 5L0D), while for HFD treatment, mice received a diet consisting of 40% calories from fat supplemented with 1.25%

cholesterol by weight (Research Diets D12108C). All food was provided in powdered form and supplied in food cups. Freeze-dried kefir was mixed into the food daily at a ratio of 200 mg freeze-dried kefir, equal to 2 g of liquid kefir, to 20 g of food. This would result in mice consuming kefir at a rate which equates to approximately 1 cup of kefir for a human on a 2000 kcal per day diet (a physiologically relevant dose for human consumption). This would represent approximately 0.4 kcal, or 2.5% of the daily calorie intake of the mice. Body weights were taken weekly for the duration of the study. After 8 weeks, the animals were euthanized by CO_2 asphyxiation and blood, tissues, and cecal content were collected, snap-frozen, and stored at -80°C until further analysis. All experiments were carried out following the Canadian Council on Animal Care guidelines with approval from the Animal Care and Use Committee at the University of Alberta (AUP 00000671).

Freeze-drying of kefir and microbial viability

Kefir preparations and milk underwent freeze-drying in a VirTis Ultra 35l Freeze Dryer at an average pressure of 13mTorr and condenser temperature of −86°C for 48 h. Freeze-dried kefir and milk powder was stored at 4°C until use. Freeze-dried kefir powder was reconstituted in water and plated weekly to assess microbial survival and consistency across the trial. Bacterial enumeration was carried out using De Man, Rogosa and Sharpe (MRS) agar supplemented with 200 ppm cycloheximide while yeasts were quantified using yeast extract, glucose, and chloramphenicol (YEGC) agar. Both bacterial and yeast enumeration were carried out weekly in triplicate. Reconstituted freeze-dried milk, cell-free kefir, and heat-treated kefir had no colony growth present when plated, while the microbial density of reconstituted freeze-dried PK1 was $2.6 \pm 0.4 \times 10^7$ CFU/ml for bacteria and $7.0 \pm 2.4 \times 10^5$ CFU/ml for yeast and fresh PK1 control had $3.1 \pm 0.8 \times 10^8$ CFU/ml bacteria and $5.4 \pm 1.9 \times 10^6$ yeast. No significant differences were observed in CFU/mL of either bacteria or yeast in the freeze-dried PK1 for the duration of the study.

Plasma cholesterol and CVD biomarker measurements

Plasma was separated from whole blood and total cholesterol was determined as previously described (Bourrie et al., 2018). High density lipoprotein-cholesterol (HDL-C) was determined using an enzyme-linked immunosorbent assay (ELISA) following manufacturer's recommendations (MyBioSource, San Diego, CA). Non-HDL cholesterol was determined by subtracting HDL-C from total cholesterol. Plasma CRP and VCAM-1 were analyzed using commercial kits following manufacturer's recommendations (Meso Scale Discovery, Rockville, MD; and Millipore Sigma, Burlington, MA respectively). Plasma was diluted 2,500 fold for CRP quantification and 100,000 fold for VCAM-1 quantification. Samples were measured in duplicate with CV values under 3% for both CRP and VCAM-1.

Liver triglyceride analysis

Liver lipids were extracted using a chloroform methanol extraction, and triglycerides and cholesterol were quantified using commercial kits as previously described (Bourrie et al., 2018).

Short-chain fatty acid analysis

Cecal content samples were thawed on ice, weighed (30 mg/sample) and homogenized in 600 µl of 25% phosphoric acid. Samples were centrifuged at 15,000 rpm at 4°C for 10 min and the supernatant was syringe filtered using a 0.45 µm filter (Fisher Scientific, Ottawa, ON). A 200 µl aliquot of filtered sample was combined with 50 µl of internal standard (23 mmol/ml, isocaproic acid) and analyzed on a Scion 456-GC instrument (Forgie et al., 2019).

Cecal microbiota analysis

Total DNA was extracted from cecal content samples using the QIAamp fast DNA mini stool kit (Qiagen, Valencia, CA). Bacterial 16S rRNA gene amplicons were constructed using the V3V4 primer region as described by Illumina (Illumina Inc., San Diego, CA): 341F (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG - 3′) and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). Sequencing was accomplished using a paired-end MiSeq platform (2 × 300 cycles; Illumina Inc.) and raw sequences were filtered, trimmed (forward – 270 bp; reverse – 220 bp) and merged with the divisive amplicon denoising algorithm (DADA2) into amplicon sequence variants (ASVs) using the Quantitative Insight into Microbial Ecology 2 (QIIME2) pipeline (Bokulich et al., 2018; Bolyen et al., 2019); ~10,000 merged ASVs were generated per sample. Phylogenetic trees were constructed using qiime alignment (mafft; mask) and phylogeny (fastree; midpoint-root) functions. Taxonomy was assigned using the SILVA v138 database (Quast et al., 2013). QIIME2 files were transferred into R using the qiime2R (version 0.99.4) package and analyzed with the phyloseq (version 1.34.0) package (McMurdie and Holmes, 2013). Additional filtering was done to remove "Chloroplast" and "Mitochondria" assigned ASVs. The ASVs were numbered from most to least abundant. Samples were rarefied for alpha and beta diversity analyzes for females at 13,706 and males at 10,255 reads. Statistical significance was evaluated using an ANOVA with Tukey correction for Chao1, Shannon, and PD whole tree indices. Principal coordinates analysis of weighted and unweighted UniFrac indices were plotted and analyzed using the 'betadisper' and pairwise Adonis2 functions.

Statistical analyzes

All data are presented as mean values with their standard errors. The level of significance for all analyzes was set at $p < 0.05$, and p values between 0.05 and 0.1 were considered as trends. Sample size was determined by power analysis with a statistical power of 80% and a two-sided significance level of 0.05 using an effect size of a 20% reduction in plasma total cholesterol. This analysis resulted in a total population of $N = 40$ mice with a sample size of $n = 8$ mice per group for each sex. Data were tested for normality using the Shapiro–Wilk test, while homogeneity of variance was tested using the Bartlett test. Data were tested for cage effect using the PRCOMP function in SAS and no significant cage effects were observed. Plasma cholesterol, VCAM-1, CRP, and liver lipid data was analyzed using Analysis of Variance (ANOVA) with Tukey post-hoc for multiple comparisons utilizing the R packages multcompView, ggplot2, plyr, and lmPerm. Effect of treatment on microbiota was determined using permutational multivariate analysis of variance (ADONIS).

Results

Whole, cell-free, and heat-treated kefir all reduced weight gain in male but not female mice

As previous studies from our group showed inconsistent results regarding weight gain in female HFD fed mice, we wanted to examine if there were any sex specific differences in how kefir consumption and the microbial fraction of kefir impacted weight gain. After 8 weeks of high fat diet feeding, each of the PK1, CFK, and HK groups showed a reduction in weight gain in male C57Bl/6 mice ($p < 0.05$; Figure 1A) when compared to the HFD control group. In female mice, HFD and CFK mice gained more weight than LFD control, while kefir and HK had intermediate weight gain and were not different from LFD or HFD (Figure 1B).

All kefir fractions improved plasma cholesterol profiles

Total cholesterol levels were examined in order to determine how different kefir fractions impact cholesterol metabolism. In male mice, each of the PK1, CFK, and HK groups had lower total cholesterol levels than the HFD control group (Figure 2A), while in female mice the PK1 and CFK groups had lower levels of total cholesterol and HK mice showed a trend for a reduction ($p = 0.054$; Figure 2D). HDL cholesterol was unchanged among all high-fat diet fed groups of female mice (Figure 2E). In male mice, while there was no difference between HFD, PK1, and HK groups, the HDL cholesterol levels in the CFK group were lower than both HFD and PK1 (Figure 2B). Non-HDL cholesterol followed the same pattern as total cholesterol in both sexes with all treatment groups being lower in the male mice (Figure 2C), while PK1 and CFK were lower in females; however, HK mice no longer showed a trend to be lower than HFD (Figure 2F).

Only whole kefir lowered liver lipid levels

Liver triglyceride and cholesterol levels are common indicators of metabolic health and NAFLD and as such, we sought to determine how consumption of different kefir fractions impacted these measures. In male mice, PK1 consumption lowered both liver cholesterol (Figure 3A) and triglyceride (Figure 3B) content when compared to HFD control, while CFK showed a trend to lower triglyceride levels ($p = 0.090$; Figure 3B) and HK was not different for either measure. In female mice, PK1 once again resulted in a decrease in liver cholesterol (Figure 3C) and triglycerides (Figure 3D), while HK and CFK consumption did not alter levels of liver triglycerides or cholesterol.

Kefir treatments have varied effects on circulating markers of CVD risk

As high plasma cholesterol levels are related to the development of CVD, we determined how different kefir preparations impacted circulating levels of VCAM-1 and CRP, which are important factors in the development of atherosclerosis and common biomarkers of CVD risk. In male mice, both PK1 and HK resulted in a reduction in plasma VCAM-1 (Figure 4A); however, this was not seen in female mice (Figure 4B). In male mice, plasma CRP was elevated in response to HFD ($p < 0.05$) relative to LFD, whereas all kefir treatments were intermediate and not different from LFD or HFD (Figure 4C). In female mice, all kefir treatments had higher plasma CRP than LFD, and were not different from HFD (Figure 4D).

Kefir supplementation did not alter short-chain fatty acid composition in mice cecum

Cecal content analysis of SCFAs found no difference in kefir supplemented treatment groups compared to HFD control (Table 1). The LFD group maintained the greatest amount of acetate, propionate, and butyrate concentrations compared to HFD control in both female and male mice. Total SCFA concentrations were significantly higher for both female ($p < 0.001$) and male ($p < 0.01$) mice of the LFD group compared to the HFD group.

Kefir had no impact on overall microbial community structure

Cecal microbial analysis revealed that supplementation with kefir preparations on a HFD (Kefir, HK, and CFK) had no

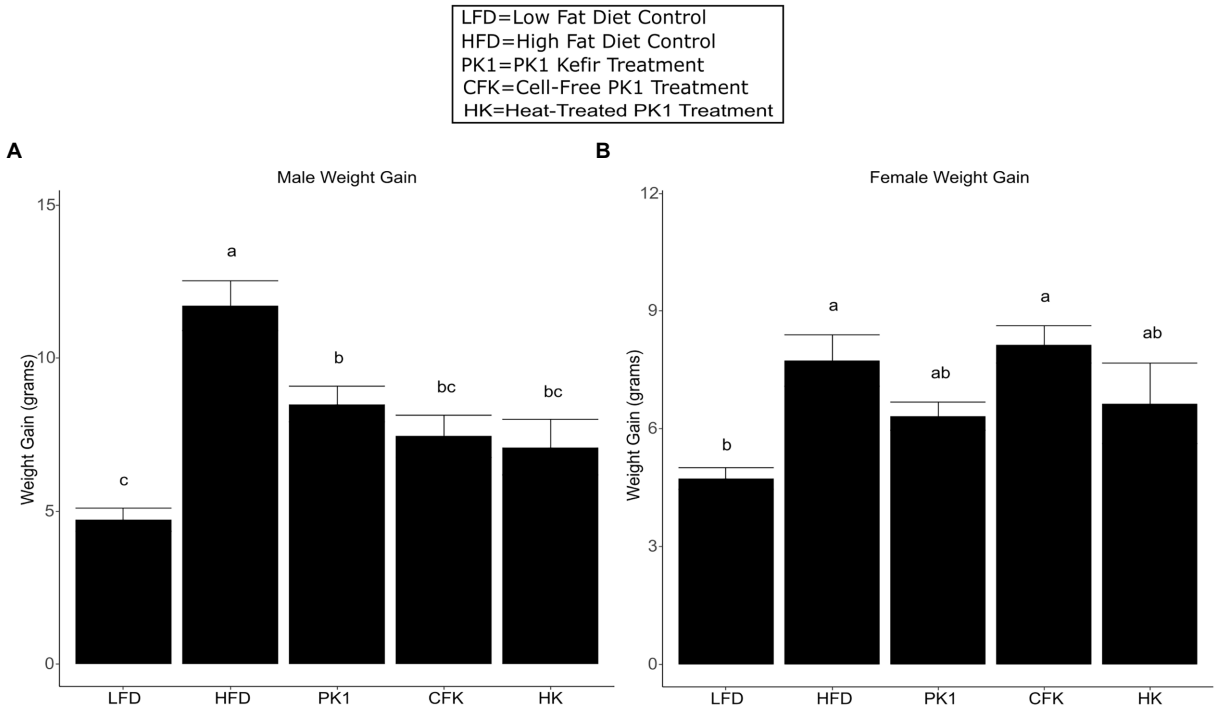


FIGURE 1
Weight gain of male **(A)** and female **(B)** C56Bl/6 mice fed a standard chow control (LFD), and (HFD) and kefir fractions on a HFD background (CFK, HK, and PK1). Data are expressed as mean values with their standard errors (n=7–8). Means that do not share a letter are significantly different (p<0.05). LFD, mice fed a low fat diet supplemented with freeze-dried milk for 8weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

appreciable impact on microbial community structure (Figures 5, 6). For female mice, alpha diversity metrics Chao1 and PD were significantly higher for the LFD group ($p < 0.05$) and lower for the PK1 group ($p < 0.05$) relative to the HFD, HK, and CFK groups. Shannon diversity was greatest for LFD, which was lower for HFD and PK1 groups (Figure 5A). The Chao1, Shannon and PD alpha diversity indices for male mice were not considerably impacted (Figure 5B). Weighted and unweighted UniFrac distance metrics all showed that the high fat diet accounted for the major shifts in microbial communities found in the cecum following kefir consumption when compared to the LFD group in both female and male mice (unweighted $p < 0.02$ and $p < 0.01$; weighted $p < 0.02$ and $p < 0.01$ respectively).

Discussion

This is the first study to examine how different kefir preparations consisting of whole kefir, cell-free kefir, and heat-treated kefir impact plasma and liver lipid profiles, the gastrointestinal microbiome, and plasma markers of CVD risk in both males and females using a mouse model of obesity. Given that previous work on traditional kefir had shown that multiple different fractions had positive health impacts (Bourrie et al., 2016), we expected that the CFK and HK treatments in our study would improve lipid and CVD biomarker profiles similarly to PK1 in both male and female mice. Male mice showed a reduction in weight gain across all three kefir treatment groups, while female mice did not have a reduction across any of the treatment groups relative to HFD. Interestingly, both plasma total cholesterol levels were lowered by all kefir preparations in both males and females, while HDL cholesterol was not different among all kefir treatments in females and was only lower in CFK male mice when compared to the HFD control. Non-HDL cholesterol levels were also lower in each kefir treatment in male mice, and in all but the HK group in female mice. This is important as elevated total and non-HDL cholesterol levels are associated with an increased risk of CVD and other metabolic diseases (Després and Lemieux, 2006; Brunner et al., 2019). We also observed that only PK1 and HK reduced VCAM-1 in males. However, this was not seen in the female mice. This may be due to the increased weight gain and generally more severe phenotypes observed in male C57Bl/6 mice when compared to their female counterparts receiving HFD. Indeed, male mice gained approximately 40% more weight than females when fed HFD and VCAM-1 levels in male HFD mice were nearly twice as high as in their female counterparts, while the male LFD group had VCAM-1 levels that were nearly identical to the HFD group in female mice.

In addition to metabolic syndrome and CVD, NAFLD is associated with hyperlipidemia and can lead to steatosis and liver

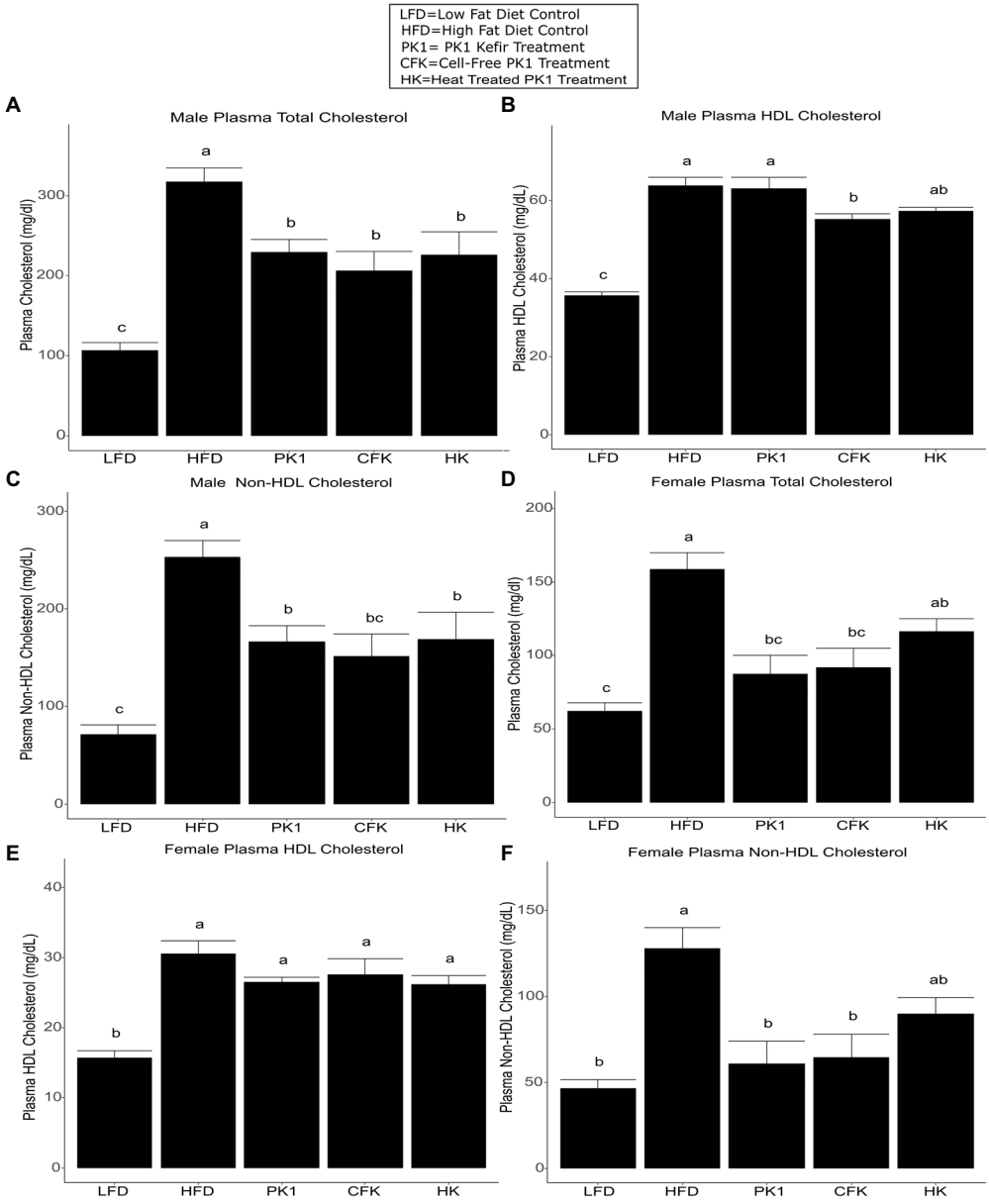


FIGURE 2
Concentration of total plasma cholesterol **(A,D)**, HDL cholesterol **(B,E)**, and non-HDL cholesterol **(C,F)** in male/female C57Bl/6 mice fed a control diet or high-fat diet supplemented with milk or different kefir preparations for 8weeks. Data are expressed as mean values with their standard errors (n=7–8). Means that do not share a letter are significantly different (p<0.05). LFD, mice fed a low fat diet supplemented with freeze-dried milk for 8weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

cancer (Loomba and Sanyal, 2013; Duan et al., 2014; Younossi et al., 2018), with increased levels of liver lipids associated with NAFLD risk (Angulo, 2002). We found that liver cholesterol and triglyceride levels were improved by PK1 in both male and female mice. The CFK treatment exhibited a trend for reduction of liver triglycerides in male mice only, and HK did not reduce liver cholesterol or triglycerides in either sex. These results are interesting, as they are contradictory to what we observed in plasma lipid profiles and may indicate that the component of kefir responsible for the observed reduction in liver lipids is somehow removed or reduced during the CFK and HK kefir preparation. One potential reason for these findings may relate to the angiotensin-converting enzyme (ACE) inhibitory action of kefir, which has been attributed to peptides produced from casein during fermentation (Quiros et al., 2005). ACE inhibitory drugs have been found to improve circulating cholesterol and lipid profiles in both human and animal studies (Ravid et al., 1995; Vasiljević et al., 1999; Dost et al., 2014). Some evidence suggests

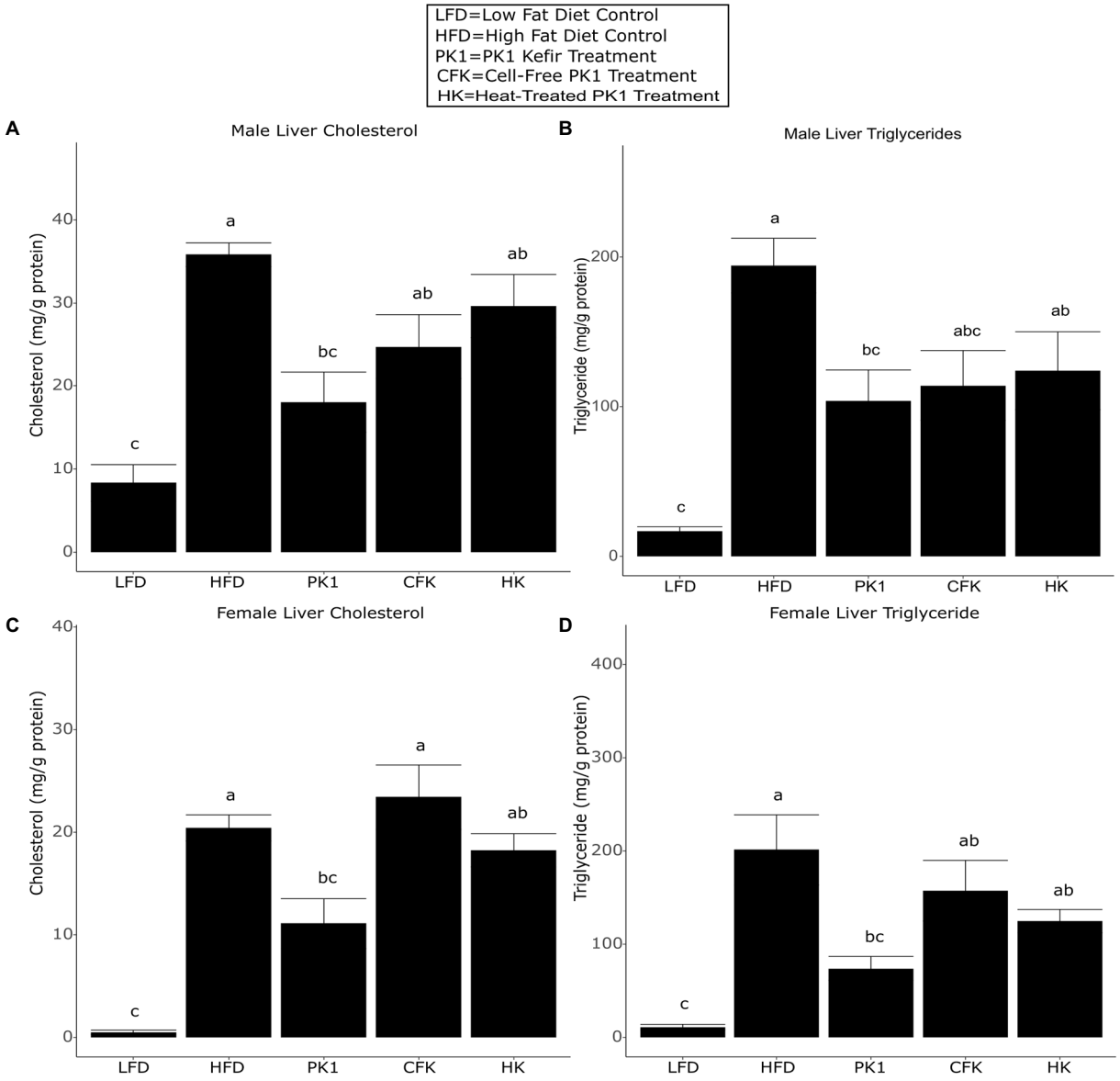


FIGURE 3
Concentration of liver cholesterol **(A,C)** and triglycerides **(B,D)** in male/female C57Bl/6 mice fed a control diet or high-fat diet supplemented with milk or different kefir preparations for 8weeks. Data are expressed as mean values with their standard errors (n=7–8). Means that do not share a letter are significantly different (p<0.05). LFD, mice fed a low fat diet supplemented with freeze-dried milk for 8weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

that these drugs may prevent fibrosis and cancer in existing NAFLD; however, more studies are needed, and no studies have examined if ACE inhibitors can prevent the development of NAFLD (Jonsson et al., 2001; Moreno et al., 2010; Li et al., 2018). It is therefore possible that the component of kefir responsible for lowering plasma cholesterol levels is some sort of heat stable peptide with ACE inhibitory activity, while there is a separate component responsible for the improvements in liver lipid levels that is lost or reduced during the preparation of CFK and HK.

Elevated plasma cholesterol, particularly elevated non-HDL cholesterol, has been associated with an increased risk of developing both CVD and diabetes (Scherer and Hill, 2016; Francula-Zaninovic and Nola, 2018; Brunner et al., 2019). One common underlying factor in CVD is the development of atherosclerosis and the associated plaque formation, which can increase an individual's risk of an adverse cardiovascular event (Francula-Zaninovic and Nola, 2018). Cell adhesion molecules, such as VCAM-1, are commonly used as biomarkers of endothelial function and have been used as indicators of increased CVD and atherosclerosis risk (Springer, 1994; Meng et al., 2021) and play an important role in plaque development (Cybulsky et al., 2001; Ley and Huo, 2001; Varona et al., 2019; Troncoso et al., 2021).

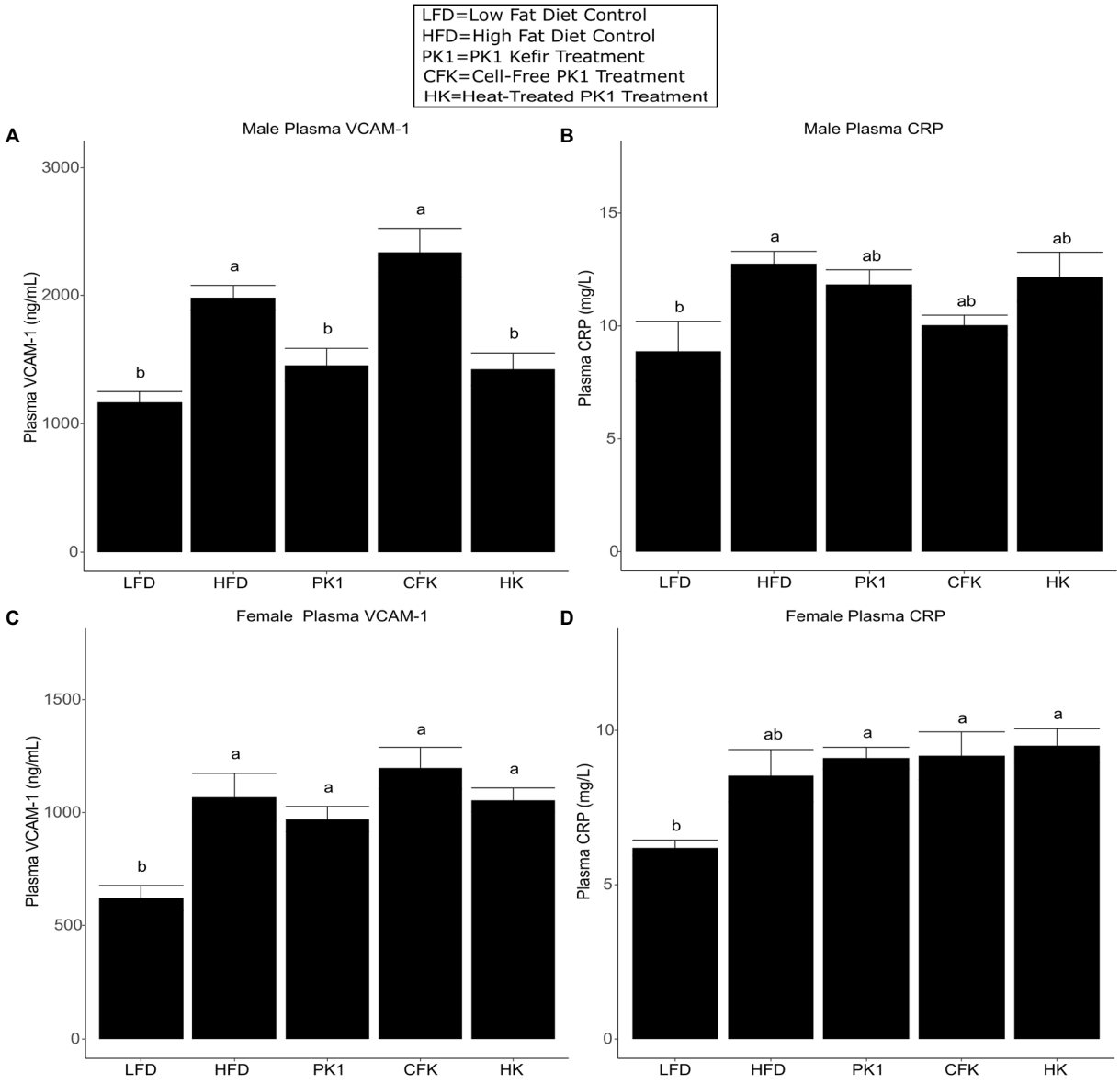


FIGURE 4
Concentration of plasma VCAM-1 **(A,B)** and CRP **(C,D)** in male/female C57Bl/6 mice fed a control diet or high-fat diet supplemented with milk or different kefir preparations for 8weeks. Data are expressed as mean values with their standard errors (n=7–8). Means that do not share a letter are significantly different (p<0.05). LFD, mice fed a low fat diet supplemented with freeze-dried milk for 8weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

We observed a reduction in plasma VCAM-1 in male mice fed both PK1 and HK, but not in the CFK while female mice showed no differences in VCAM-1 between any of the HFD fed groups. The sex difference is likely a result of the less severe atherosclerotic phenotype in female C57BL/6 mice (Paigen et al., 1987), with females in our trial having roughly half the level of VCAM-1 as males. A previous study found that VCAM-1 and plaque formation in the aorta were reduced by a peptide enriched spray dried kefir in HFD fed *ApoE*$^{-/-}$ mice (Tung et al., 2020). It is possible that the phenotypes observed in this study would have been more pronounced had we used a more severe phenotype, such as the *ApoE*$^{-/-}$ mouse. The lack of effect from CFK treatment may indicate that the compound responsible for this benefit is lost during the centrifugation and filtration process used to create cell-free kefir. Systemic inflammation is another important factor contributing to CVD risk (Vandanmagsar et al., 2011; Boulangé et al., 2016), with CRP being regarded as an important indicator of CVD event risk (Backes et al., 2004; Bisoendial et al., 2010; Avan et al., 2018). Our study found that CRP was not reduced by any of the kefir preparations in either male or female mice fed

TABLE 1 Concentration of SCFAs (μmol/g) in cecal content of male and female C57Bl/6 mice fed a control diet or high-fat diet supplemented with milk or different kefir preparations for 8weeks.

SCFA (μmol/g)	LFD	HFD	CFK	HK	PK1
Female					
Acetate	23.64 ± 1.56***	11.54 ± 1.68	13.17 ± 5.92	13.46 ± 3.58	12.14 ± 3.35
Propionate	12.39 ± 1.30***	7.35 ± 0.90	7.46 ± 2.42	8.04 ± 2.33	6.97 ± 1.58
Butyrate	6.08 ± 1.32***	1.67 ± 0.56	1.98 ± 1.73	2.13 ± 0.85	1.45 ± 0.84
Total SCFAs	42.92 ± 2.96***	20.9 ± 2.06	23.05 ± 10.16	24.04 ± 6.03	20.87 ± 5.14
Male					
Acetate	16.84 ± 5.31#	11.73 ± 1.23	12.63 ± 1.93	12.74 ± 2.71	13.87 ± 2.6
Propionate	10.29 ± 3.03	9.67 ± 0.89	9.08 ± 1.66	9.27 ± 1.35	11.23 ± 1.99
Butyrate	6.77 ± 1.34***	1.24 ± 0.26	1.54 ± 0.29	1.12 ± 0.25	1.33 ± 0.15
Total SCFAs	34.6 ± 8.76**	23.23 ± 1.89	23.71 ± 3.39	23.61 ± 3.73	27.07 ± 4.35

Data are expressed as mean values with their standard errors ($n = 7–8$). **Indicates a significantly different value from HFD ($p < 0.01$), ***indicates $p < 0.001$. LFD, mice fed a low-fat diet supplemented with freeze-dried milk for 8 weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8 weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8 weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

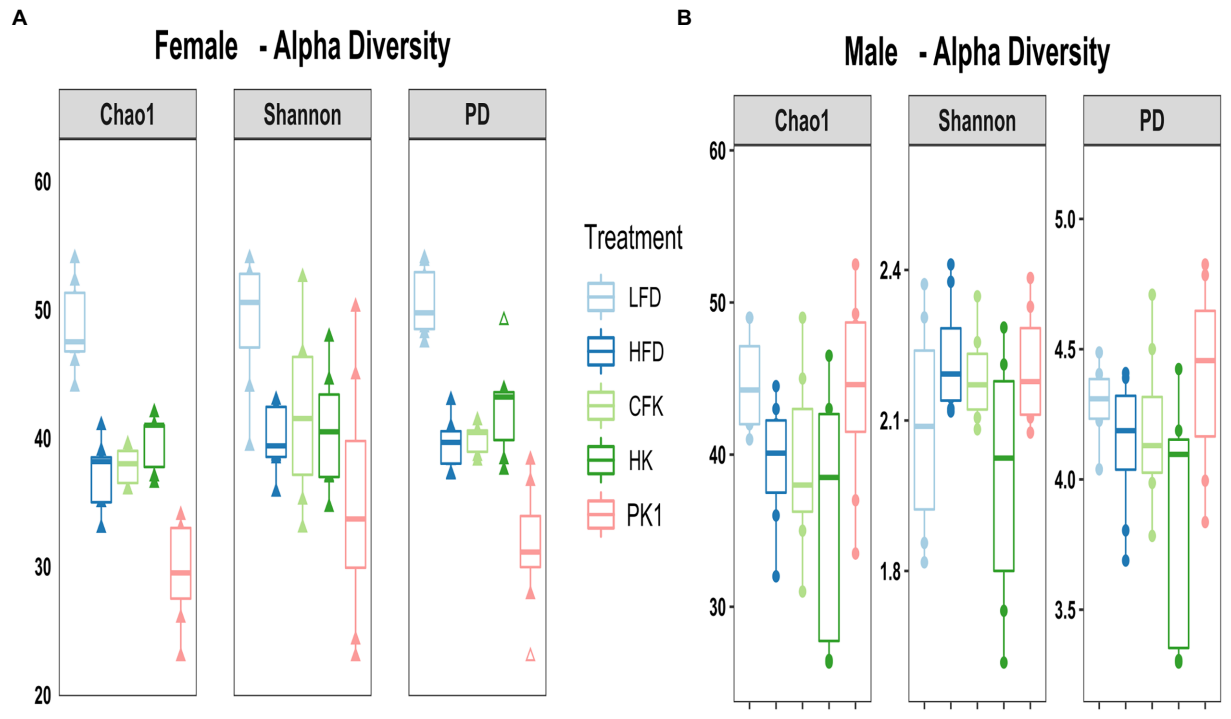
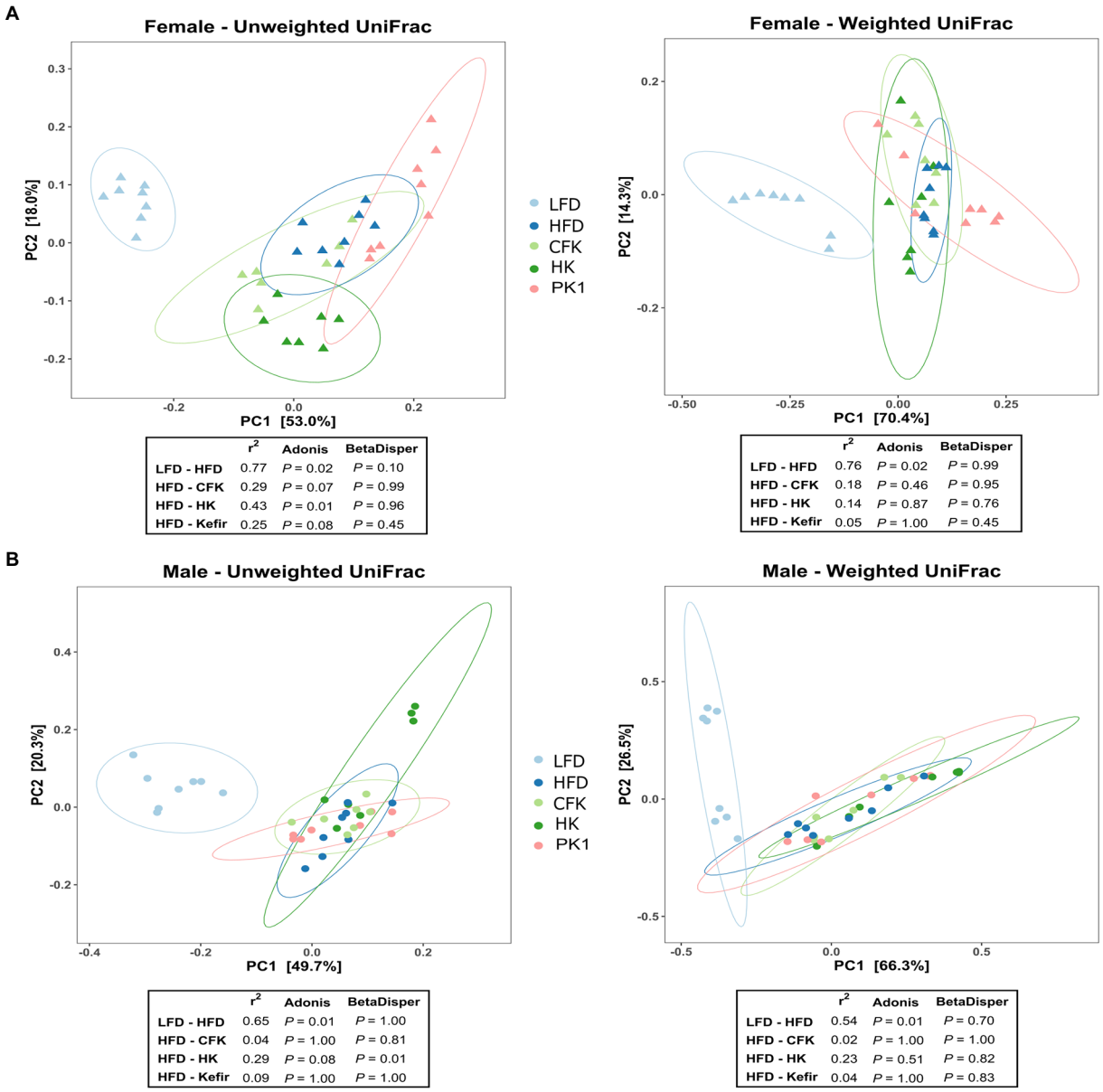


FIGURE 5
Alpha diversity metrics of the cecal microbiota in male/female C57Bl/6 mice fed a control diet or high-fat diet supplemented with milk or different kefir preparations for 8weeks. **(A)** Chao1 and PD indices indicated higher microbial diversity and richness in the LFD group ($p<0.05$) and lower diversity in the Kefir group ($p<0.05$) compared to HFD, CFK and HK groups for females with no difference with Shannon diversity index other than LFD maintained higher diversity. **(B)** No difference was found in male mice after kefir consumption for Chao1, Shannon or PD metrics compared to HFD group ($n=7–8$). Means that do not share a letter are significantly different ($p<0.05$). LFD, mice fed a low fat diet supplemented with freeze-dried milk for 8weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

HFD. This may indicate that, while kefir is able to impact endothelial function positively, the active components associated with these benefits do not impact chronic systemic inflammation as it relates to CRP levels. It is also important to note that, in mice, CRP is only a modest acute phase protein and may not respond to inflammation to the same degree as in humans (Torzewski et al., 2014; Huang et al., 2019). This may be why there has been evidence in human trials that kefir consumption can improve plasma CRP levels despite no corresponding evidence from mouse trials (Ghizi et al., 2021).



	r^2	Adonis	BetaDisper
LFD - HFD	0.77	$P = 0.02$	$P = 0.10$
HFD - CFK	0.29	$P = 0.07$	$P = 0.99$
HFD - HK	0.43	$P = 0.01$	$P = 0.96$
HFD - Kefir	0.25	$P = 0.08$	$P = 0.45$

	r^2	Adonis	BetaDisper
LFD - HFD	0.76	$P = 0.02$	$P = 0.99$
HFD - CFK	0.18	$P = 0.46$	$P = 0.95$
HFD - HK	0.14	$P = 0.87$	$P = 0.76$
HFD - Kefir	0.05	$P = 1.00$	$P = 0.45$

	r^2	Adonis	BetaDisper
LFD - HFD	0.65	$P = 0.01$	$P = 1.00$
HFD - CFK	0.04	$P = 1.00$	$P = 0.81$
HFD - HK	0.29	$P = 0.08$	$P = 0.01$
HFD - Kefir	0.09	$P = 1.00$	$P = 1.00$

	r^2	Adonis	BetaDisper
LFD - HFD	0.54	$P = 0.01$	$P = 0.70$
HFD - CFK	0.02	$P = 1.00$	$P = 1.00$
HFD - HK	0.23	$P = 0.51$	$P = 0.82$
HFD - Kefir	0.04	$P = 1.00$	$P = 0.83$

FIGURE 6
Principle coordinate analysis using unweighted and weighted UniFrac distance metric of the cecal microbiota of male/female C57Bl/6 mice fed a control diet or high-fat diet supplemented with milk or different kefir preparations for 8weeks. A major shift between LFD and HFD groups was determined in both **(A)** female and **(B)** male mice with no difference between HFD and kefir consumption groups (CFK, HK and Kefir; n=7–8). Means that do not share a letter are significantly different (p<0.05). LFD, mice fed a low fat diet supplemented with freeze-dried milk for 8weeks; HFD, mice fed a high fat diet supplemented with freeze-dried milk for 8weeks; PK1, mice fed a high fat diet supplemented with freeze-dried pitched kefir for 8weeks; CFK, mice fed a high-fat diet supplemented with freeze-dried cell-free kefir; HK, mice fed a high-fat diet supplemented freeze-dried heat-treated kefir.

All three kefir preparations supplemented to a high-fat and high-cholesterol diet minimally affected the cecal microbiota compared to the high-fat diet alone. PK1 consumption did not increase alpha diversity, as has been observed in a recent study exploring fermented food consumption in a human trial (Wastyk et al., 2021). Absence of an effect on alpha diversity in the current study could be explained by the background diet (HFD vs. uncontrolled) and the controlled environment used for mouse experiments. Due to the lack of fermentable carbohydrates, a high-fat diet can significantly alter gut microbial structure and decrease SCFA concentrations compared to a standard low-fat diet (Dalby et al., 2017). A change in SCFA levels is well documented to correlate with gastrointestinal health and microbiota (Makki et al., 2018; Cong

et al., 2022). In agreement, we found higher SCFAs concentrations in the cecum of LFD group with normal cardiovascular health profiles along with changes to the microbial community compared to HFD group. These differences may be due to fermentable fiber content of the low fat and high fat diets, as the low fat diet is made from whole food ingredients containing a variety of fibers while the high fat diet is semi-purified with cellulose as the only fiber. The fact that all three kefir fractions were unable to alter SCFA concentrations or the microbiota suggests that the beneficial effects on cholesterol levels in mice fed kefir is likely not related to SCFA production in the gut. The slight difference in the low abundance microbes in the cecum of female mice but not male mice, as determined by unweighted UniFrac analysis, is likely a result of sex differences in weight, food consumption, locomotor activity, energy expenditure and β cell adaptation upon dietary manipulation (Casimiro et al., 2021). It is also important to note that, while our results did not show significant changes to the cecal microbiome associated with kefir consumption, the composition of the gastrointestinal microbiome of C57BL/6 mice has been shown to differ throughout the gastrointestinal tract (Lkhagva et al., 2021). It is therefore possible that kefir consumption impacts microbiome composition in other sections of the gastrointestinal tract that may contribute to the physiological impacts observed in this study.

This study expands on previous work showing that a pitched kefir (PK1) containing traditional kefir microbes can improve plasma and liver lipid profiles in a mouse model of obesity and that different components of kefir can have beneficial impacts on host health (Maeda et al., 2005; Uchida et al., 2010; Bourrie et al., 2021), however this is the first study to examine different fractions of a pitched kefir in an attempt to differentiate the active components related to lipid control and atherosclerosis improvements. This is also the first study to our knowledge to examine these types of health benefits in both male and female mice, which is of particular importance given the sex differences that exist in both mice and humans with respect to the metabolic responses to obesity and the development of associated metabolic diseases (Paigen et al., 1987; Matyšková et al., 2008; Kautzky-Willer and Handisurya, 2009; Hwang et al., 2010; Sugiyama and Agellon, 2012; Yang et al., 2014). We found that while weight gain was lowered by PK1 consumption in male mice only, each of the kefir treatments lowered plasma total cholesterol and non-HDL cholesterol when compared to a HFD control in both sexes; however, only PK1 treatment resulted in a reduction in liver triglycerides and cholesterol levels in both male and females. These results are interesting as they show that consumption of either cell-free or heat-treated kefir results in similar improvements to plasma cholesterol profiles when compared to PK1 consumption but that improvements in liver lipids requires whole PK1 kefir. This may indicate that live kefir microorganisms are necessary to lower liver lipid content. Additionally, the impact of the kefir treatments for plasma and liver lipids was consistent across males and females, indicating that the mechanism of action is not sex dependent. VCAM-1 was only lowered in male mice receiving PK1 or heat-treated kefir, indicating a possible sex dependent response for this particular biomarker. It is also important to note that female mice had nearly half the levels of VCAM-1 when compared to male mice, and it is possible that with a more severe obesity phenotype there would have been a comparable decrease in plasma VCAM-1 between males and females. Additionally, the lack of improvement in VCAM-1 levels in CFK mice may indicate that, while live microbes are not necessary to lower VCAM-1 in plasma, their microbial components are. These results provide confirmation that reconstituted PK1 kefir containing specific kefir microbes can improve lipid profiles as well as circulating markers of atherosclerosis, and that the lipid lowering benefits are present in both males and females. This is encouraging, as the potential for functional foods that equally benefit male and female individuals is especially important given the increase in females developing metabolic diseases and the need for more research examining these conditions in female populations (Engin, 2017). Future work should focus on identifying how these fractions differ in the composition of their fermentation metabolites and, if possible, on the identification of an active component or components that are responsible for the health benefits observed in these animal trials.

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: BioProject, PRJNA885841.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee at the University of Alberta (AUP 00000671).

Author contributions

BCTB, PDC, CR, and BPW designed research. BCTB, AJF, and TJ conducted research. BCTB and AJF analyzed data. BCTB, AJF, and BPW wrote the paper. BPW had primary responsibility for final content. All authors have read and approved the final manuscript.

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Conflict of interest

BCTB, BPW, and PDC hold a patent for the method used to produce the pitched kefir used in the trial.

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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Effect of *Lactobacillus plantarum* KSFY01 on the exercise capacity of D-galactose-induced oxidative stress-aged mice

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Objectives: Aging is a process that involves comprehensive physiological changes throughout the body, and improvements in the exercise capacity of individuals may delay aging and relieve fatigue. Probiotics are subject to ongoing research to investigate their antioxidant properties. The purpose of this study was to investigate the effect of the probiotic *Lactobacillus plantarum* KSFY01 (*L. plantarum* KSFY01) on exercise tolerance in mice induced into a state of accelerated physiological aging by oxidative stress.

Methods: A mouse model of accelerated aging was established using D-galactose to induce oxidative stress. The bacteria *L. plantarum* KSFY01 was isolated from fermented yak yogurt. The effect of *L. plantarum* KSFY01 on the improvement of exercise capacity in aging-accelerated mice was evaluated by measuring their running time until exhaustion, histopathological sections, related biochemical indicators, and underlying gene expression.

Results: The oral administration of *L. plantarum* KSFY01 prolonged the running time of mice and reduced their creatine kinase (CK), alanine aminotransferase (ALT), and aspartate aminotransferasem (AST) levels. From this study, we observed that *L. plantarum* KSFY01 significantly improved the exercise capacity of mice and alleviated liver damage. Treatment with *L. plantarum* KSFY01 reduced the blood urea nitrogen (BUN), lactic acid (LD) accumulation, and lactate dehydrogenase (LDH) elevations produced by the accelerated aging state, and also reversed the changes in muscle glycogen (MG). Overall, *L. plantarum* KSFY01 could effectively improve metabolite accumulation, thereby relieving fatigue in exercised mice. The results of the antioxidant indices *in vivo* showed that *L. plantarum* KSFY01 intervention increased the activity of antioxidant enzymes, decreased the level of malondialdehyde (MDA), and restored the balance between the oxidative and antioxidant systems in fatigued mice. By investigating the underlying molecular mechanism, our results showed that *L. plantarum* KSFY01 intervention significantly reversed the decline in the expression levels of nuclear factor-erythroid 2 related

factor 2 (Nrf2) signaling pathway-related factors and improved the body's antioxidant capacity. We determined that the underlying molecular mechanism responsible for the antioxidant effect of *L. plantarum* KSFY01 mainly involves the activation of the Nrf2 pathway. The effect of *L. plantarum* KSFY01 was dose-dependent, and the expression level of Nrf2 increased with increasing dosage of the probiotic.

Conclusion: This study demonstrated that the probiotic *L. plantarum* KSFY01 exerts antioxidant effects and improved the athletic ability of mice. These findings are of significance to the development and utilization of probiotic resources.

KEYWORDS

***Lactobacillus plantarum*, D-galactose, oxidative, exercise, mRNA**

1. Introduction

Aging is a process that involves comprehensive physiological changes throughout the body, and is one that all biological individuals must experience (Wang W. et al., 2022; Wang L. et al., 2022). At present, the aging population is expanding, and it is estimated that the global population over the age of 60 will reach 2.1 billion by 2050 (Leeson, 2018). Aging reduces the metabolic rate, imbalances the antioxidant system, and results in an excess of free radicals due to their reduced clearance, thereby resulting in their accumulation in the body (Adetuyi et al., 2022). Simultaneously, the content of lipid peroxide increases in the plasma and tissues, and the anti-fatigue ability of the body also gradually decreases (Zheng et al., 2017). Studies have found that telomere length is preserved in healthy elderly people who practise vigorous aerobic exercise, being positively correlated with the maximum aerobic exercise capacity, and thereby indicating that a high capacity for exercise may contribute to delaying the process of aging (LaRocca et al., 2010). People who regularly exercise have slower heart rates, lower blood pressure, and lower cholesterol levels (Myers et al., 2007). These indicators directly reflect the association between exercise capacity and aging. The decline in organ and muscular function as well as the increase in fatigue caused by aging all contribute to the decline of exercise capacity. To counter this, maintaining an exercise regime can help improve bodily functions, alleviate fatigue, and maintain the overall vitality of the body. The process of aging interacts with the exercise capacity of an individual, whereby bodily health is not only the embodiment of a high capacity for exercise, but can also promote continuous exercise in a positive feedback loop, so as to further delay aging (Padilha et al., 2021).

Under normal physiological conditions, the aerobic metabolism of organisms continuously produces oxygen free radicals (ROS) which cause damage to cells. When the burden of damage caused by these ROS is greater than the repair capacity of the body, bodily aging results (Liu et al., 2022). During the aging process, the activities of antioxidant enzymes are significantly reduced. This causes the continuous accumulation of free radicals in the body, which consequently affects normal metabolism, increases bodily fatigue, and reduces the capacity for exercise, thereby ultimately resulting in the decline of the quality of life for the elderly (Tan et al., 2000). This trajectory illustrates a close relationship between oxidative stress, aging, and exercise capacity. Enhancing exercise function by bolstering the antioxidant processes within the body represents an effective way to delay aging. Slowing the process of aging, in turn, strengthens the vitality of the organs and muscles, thus promoting athletic function. This strategy comprises one effective method which can be used to delay physical fatigue; namely, by supplementing exogenous antioxidants to the body, thereby preventing the oxidation of easily-oxidized substrates in cells, inhibiting lipid peroxidation, and directly eliminating oxygen free radicals to reduce oxidative stress in the body (Kerksick and Willoughby, 2005; McLeay et al., 2017).

The development and utilization of probiotic lactic acid bacteria (LAB) comprises an active area of research in the field of food bioengineering. Numerous studies have been conducted on the prebiotic function of LAB to show that many employ antioxidant functions, though there exist differences in these functions between different strains. Zhang et al. (2021) Found that *Lactobacillus fermentum* HFY03 isolated from yak yogurt could significantly improve the running duration of mice, reduce their contents of urea nitrogen and lactic acid, and increase their levels of catalase and superoxide dismutase in the liver tissue, thereby indicating that *L. fermentum* HFY03 exerts an antifatigue effect through improving the antioxidant capacity of mice that run until exhaustion. Some studies have also found that the use of LAB that employ antioxidant functions could improve the antioxidant properties of fermentation-based products. Zhou et al. (2021) used *Lactobacillus plantarum* HFY09 to ferment soymilk and found that the resulting fermented soymilk could effectively alleviate the accelerated aging of mice induced by D-galactose (Zhou et al., 2021). There are many studies existing on probiotics, but at present, there are few reports existing on their impacts on

exercise capacity. In this study, a new lactic acid bacterium, *Lactobacillus plantarum* KSFY01 (*L. plantarum* KSFY01), was isolated from traditional fermented yak yogurt. It possessed high resistance to the conditions of pH 3.0, artificial gastric juice, and 0.3% bile salt. The results showed that *L. plantarum* KSFY01 was mostly unaffected by artificial gastric juice, and its survival rate was 86.26%. The growth rate in 0.3% bile salt was 54.24%. This study then constructed a mouse model of accelerated aging by the injection of D-galactose injection to investigate the effect of *L. plantarum* KSFY01 on the antioxidant levels and anti-fatigue response of mice. The results are intended to provide a reference for the application of *L. plantarum* in functional foods and for the development of health products.

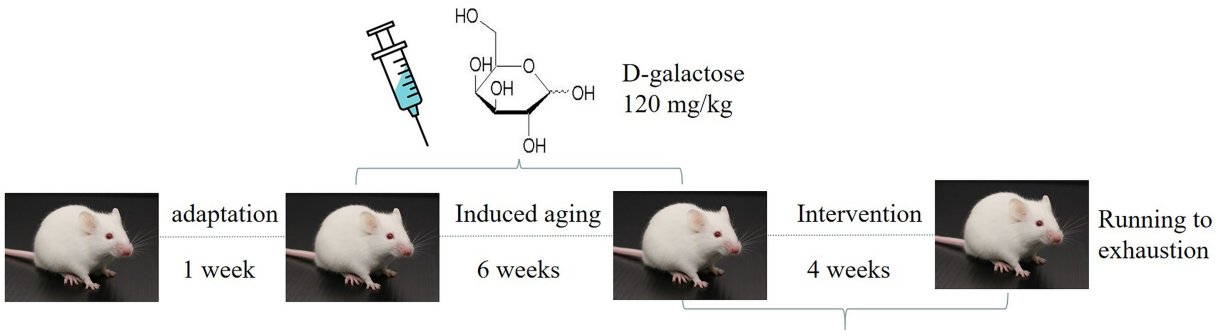
2. Materials and methods

2.1. Experimental microorganism strain

This study isolated a *L. plantarum* strain from yak yogurt obtained from Kashgar, Xinjiang, and named it *L. plantarum* KSFY01. The bacteria were stored frozen at-80°C and subsequently inoculated into sterilized MRS medium for resuscitation. The resuscitation conditions involved 37°C, culturing for 18–24 h, and the bacterial activation spanning two generations. The second generation of bacterial suspension was then taken and centrifuged at 5000 rpm/min for 10 min. The upper culture medium was discarded, while that which remained was added with the same volume of 0.9% physiological saline to make up the bacterial suspension. Using the gradient dilution method, the *L. plantarum* KSFY01 bacterial solution was diluted to 1.0×10^{10} and 1.0×10^{9} colony-forming units (CFU).

2.2. Animal experiment

Fifty 6-week-old male Kunming mice were purchased from the animal experiment center of Chongqing Medical University. Before the formal experiment commenced, the mice were placed under a 12-h light/dark cycle for 1 week of adaptive feeding with free access to food and water. At the end of the one-week adaptation period, the mice were equally divided into 5 groups (with 10 mice each): the normal group, model group, vitamin C group (Vit C), KSFY01 high-dose group (KSFY01-H), and KSFY01 low-dose group (KSFY01-L). The duration of the experimental period was 10 weeks (Figure 1). After the start of the experiment, the mice in all groups except the normal group were injected with D-galactose solution (120 mg/kg) intraperitoneally every day for 6 weeks, while the mice in the normal group were intraperitoneally injected with the same amount of saline. From the 7th week onwards, the mice in the normal group and the model group were gavaged with distilled water (0.02 ml/kg) every day; the mice in the Vit C group were gavaged with 200 mg/kg of vitamin C every day; the mice in the KSFY01-H group were gavaged every day with *L. plantarum* KSFY01 bacterial suspension at a dose of 1.0×10^{10} CFU/kg; mice in the KSFY01-L group were administered the *L. plantarum* KSFY01 bacterial suspension at a dose of 1.0×10^{9} CFU/kg for 4 weeks. After 10 weeks, the mice were subjected to the running exhaustion test, whereby the time to exhaustion of each group of mice was recorded. Then, the mice were immediately sacrificed, had their blood was sampled from



Normal	Saline 0.2mL
Model	Saline 0.2mL
Vit C	Vitamin C 200 mg/kg
KSFY01-H	*L. plantarum* KSFY01 1×10^{10} CFU/kg
KSFY01-L	*L. plantarum* KSFY01 1×10^{9} CFU/kg

FIGURE 1
Experimental design.

the eyeballs, and their liver and kidney tissues were promptly collected for subsequent experiments.

2.3. Running test

After 10 weeks, the mice were subjected to the running exhaustion test. The running wheel was set to 20 rpm/min, and the mice were forced to run on the wheel (YH-CS, Wuhan Yihong Technology Co., Ltd., Wuhan, Hubei, China). When the mice stopped running, electric shocks were performed 5 consecutive times until the mice did not run, thereby indicating exhaustion, and the running time was recorded.

2.4. Biochemical and energy metabolism indices

Mice eyeball blood was collected into 1.5 ml centrifuge tubes, placed in a 4°C refrigerator for 30 min, and then centrifuged (3,000 rpm at 4°C for 10 min). An appropriate amount of serum was then taken and analyzed with biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) to determine the contents of blood urea nitrogen (BUN), serum free fatty acid (NEFA), lactic acid (LA), lactate dehydrogenase (LDH), catalase (CAT), glutathione (GSH), malondialdehyde (MDA) levels, and muscle glycogen (MG). The enzyme-linked immunosorbent assay (ELISA; Shanghai Enzyme Link Biotechnology Co., Ltd., Shanghai, China) was used to determine the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase (CK) in the mouse serum.

2.5. Histopathological analysis

Immediately after the mice were dissected, portions of the mouse liver and kidney tissues were taken and placed in a 10% neutral formalin solution for fixation. The tissue samples were embedded, cut into 5–10 μm slices, and then stained with hematoxylin–eosin (H&E) dye (Li et al., 2021a). The pathological changes in the tissue were then observed under a light microscope.

2.6. Real-time quantitative PCR detection

The mRNA expression of SOD1, SOD2, CAT, interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), interleukin-10 (IL-10), heme oxygenase-1 (HO-1), nuclear factor-erythroid 2 related factor 2 (Nrf2), γ-glutamylcysteine synthetase (γ-GCS), and NAD(P)H dehydrogenase [quinone] 1 (NQO-1) were measured using real-time quantitative PCR (RT-qPCR; Wang et al., 2021). The extraction of mouse RNA from the liver and muscle tissue was conducted as follows. Mouse liver tissue and muscle tissue were removed from storage at −80°C, and 100 mg of each was then weighed into a homogenization tube, added with 1 ml of TRIzol reagent, and homogenized using beads for extraction. A nucleic acid analyzer was then used to measure the concentration of the extracted RNA to ensure that the purity value was between 1.8 and 2.0. The RNA was then reverse-transcribed into cDNA using a kit (Thermo Fisher Scientific, Inc., Waltham, MA, United States) according to the manufacturer's instructions, after which the resulting cDNA was aliquotted and stored at −80°C for later use. The reverse-transcribed cDNA was then used as a template whereby it was added with fluorescent dye and placed into a fluorescence quantitative PCR instrument to carry out the reaction. The volume used for the RT-PCR reaction system was 20 μl, and the reaction conditions were as follows: preheating at 95°C for 60 s, 40 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 35 s, and then ultimately tested at 95°C for 30 s and 55°C for 35 s. Each reaction was performed four times (Long et al., 2020). In the experiment, the β-actin gene was used as the internal reference gene, and the relative expression level of the target mRNA was determined by the $2^{-\Delta\Delta Ct}$ relative quantitative method (Li et al., 2021b). The respective primer sequences of the internal reference gene and target gene used in this study are shown in Table 1.

TABLE 1 Primer sequences used in the real-time quantitative PCR.

Gene names	Primer sequence
SOD1	Forward: 5′-AACCAGTTGTGTTGTCAGGAC-3′
	Reverse: 5′-CCACCATGTTTCTTAGAGTGAGG-3′
SOD2	Forward: 5′-CAGACCTGCCTTACGACTATGG-3′
	Reverse: 5′-CTCGGTGGCGTTGAGATTGTT-3′
CAT	Forward: 5′- GGAGGCGGGAACCCAATAG-3′
	Reverse: 5′-GTGTGCCATCTCGTCAGTGAA-3′
IL-1β	Forward: 5′-GAAATGCCACCTTTTGACAGTG-3′
	Reverse: 5′-TGGATGCTCTCATCAGGACAG-3′
IL-10	Forward: 5′-CTTACTGACTGGCATGAGGATCA-3′
	Reverse: 5′-GCAGCTCTAGGAGCATGTGG-3′
TNF-α	Forward: 5′-CAGGCGGTGCCTATGTCTC-3v
	Reverse: 5′-GCTGCAACAGGGGGTAACAT-3′
Nrf2	Forward: 5′-CAGTGCTCCTATGCGTGAA-3v
	Reverse: 5′-GCGGCTTGAATGTTTGTC-3′
HO-1	Forward: 5′-ACAGATGGCGTCACTTCG-3′
	Reverse: 5′-TGAGGACCCACTGGAGGA-3′
γ-GCS	Forward: 5′-GCACATCTACCACGCAGTCA-3′
	Reverse: 5′-CAGAGTCTCAAGAACATCGCC-3′
NQO1	Forward: 5′-CTTTAGGGTCGTCTTGGC-3′
	Reverse: 5′-CAATCAGGGCTCTTCTCG-3′
β-actin	Forward: 5′-CATGTACGTTGCTATCCAGGC-3′
	Reverse: 5′-CTCCTTAATGTCACGCACGAT-3′

2.7. Statistical analysis

All data are expressed as the mean ± standard deviation and plotted using GraphPad Prism (version 7.00) software. SPSS software (SPSS 22.0, SPSS Inc.) was used for statistical analysis of the data, and ANOVA and Duncan's test were used to evaluate the significance of the differences for each parameter of the samples ($p < 0.05$).

3. Results

3.1. Effects of *Lactobacillus plantarum* KSFY01 on exercise endurance In accelerated-aging mice

It can be seen from Figure 2 that the running time of the mice in the normal group was the greatest among all the groups, while the running time in the aging model group was the shortest.

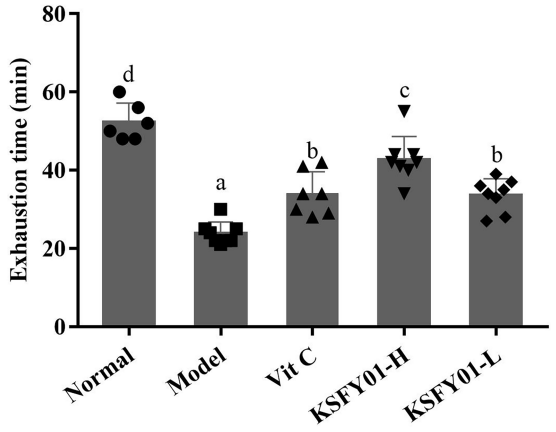


FIGURE 2
Running exhaustion time of mouse in each group. [a–d]Values in the same column with different letter superscripts indicate significant difference ($p<0.05$). Vit C, mice treated with vitamin C (200mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10}CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9}CFU/kg).

Compared with the aging model group, both *L. plantarum* KSFY01 and vitamin C could significantly ($p < 0.05$) prolong the running time of aging mice, and the *L. plantarum* KSFY01 high-dose group had the greatest prolongation effect, which was significantly better than that of either the low-dose *L. plantarum* KSFY01 group or the Vit C group. These experimental results show that *L. plantarum* KSFY01 can improve the exercise endurance of mice, prolong the exercise time of mice, and relieve their exercise-induced fatigue.

3.2. Energy metabolism in mice

As shown in Table 2, the serum levels of BUN, NEFA, and LD in the normal group were significantly ($p < 0.05$) lower than those in other groups, while the LDH and MG levels were significantly ($p < 0.05$) higher than those in other groups. Conversely, the above indicators in the serum of mice in the aging model group showed the opposite trend to that of the normal group. Compared with the aging model group, *L. plantarum* KSFY01 and vitamin C each reduced the levels of BUN, NEFA, and LD in the serum of aging mice, while having increased the levels of LDH and MG. These experimental results show that *L. plantarum* KSFY01 can reduce the BUN produced by mice following exercise, increase the activity of LDH, reduce the content of LD, and relieve the exercise-induced fatigue of mice.

3.3. Sports injuries in mice

It can be seen from Table 3 that the ALT, AST, and CK contents of the vitamin C group, *L. plantarum* KSFY01 high dose group, and *L. plantarum* KSFY01 low dose groups lay between the results of the model group and the normal group. Both the *L. plantarum* KSFY01 bacterial suspension and vitamin C had good alleviating effects by decreasing the high ALT, AST, and CK contents induced by D-galactose. The effects of the high-dose *L. plantarum* KSFY01 group and the vitamin C group were better than that of the low-dose *L. plantarum* KSFY01 group.

TABLE 2 The MG, BUN, NEFA, LD, and LDH levels in aging mouse.

Group	MG (mg/mL)	BUN (mmol/L)	NEFA (μmol/L)	LD (mmol/L)	LDH (U/L)
Normal	5.00 ± 0.17^{c}	3.27 ± 0.69^{a}	443.78 ± 32.89^{a}	2.12 ± 0.07^{a}	325.22 ± 24.94^{c}
Model	1.27 ± 0.17^{a}	7.23 ± 0.50^{c}	1459.14 ± 82.26^{e}	6.80 ± 1.00^{d}	195.43 ± 26.42^{a}
Vit C	2.67 ± 0.15^{b}	3.46 ± 0.81^{a}	853.55 ± 134.45^{c}	3.52 ± 0.62^{ab}	308.07 ± 68.53^{c}
KSFY01-H	2.84 ± 0.17^{b}	3.50 ± 0.79^{a}	710.75 ± 52.32^{b}	3.74 ± 0.38^{b}	298.88 ± 17.74^{bc}
KSFY01-L	2.66 ± 0.32^{b}	5.64 ± 0.71^{b}	1071.61 ± 71.53^{d}	5.00 ± 0.77^{c}	252.17 ± 70.00^{b}

The data are expressed as the mean ± SD. [a–d]Values in the same column with different letter superscripts indicate significant difference ($p < 0.05$). Vit C, mice treated with vitamin C (200 mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10} CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9} CFU/kg).

TABLE 3 The ALT, AST, and CK levels in aging mouse.

Group	ALT (ng/mL)	AST (ng/mL)	CK (ng/mL)
Normal	9.15 ± 0.62^{a}	17.24 ± 1.42^{a}	61.89 ± 5.65^{a}
Model	19.78 ± 1.77^{d}	39.83 ± 0.96^{e}	120.34 ± 3.25^{d}
Vit C	11.30 ± 1.66^{b}	24.52 ± 0.41^{c}	77.67 ± 8.45^{b}
KSFY01-H	10.87 ± 1.81^{b}	19.91 ± 1.36^{b}	72.26 ± 6.27^{b}
KSFY01-L	16.32 ± 0.75^{c}	30.51 ± 0.89^{d}	95.18 ± 3.95^{c}

The data are expressed as the mean ± SD. $^{a-d}$Values in the same column with different letter superscripts indicate significant difference ($p < 0.05$). Vit C, mice treated with vitamin C (200 mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10} CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9} CFU/kg).

TABLE 4 The CAT, GSH, and MDA levels in aging mouse.

Group	CAT (U/mL)	GSH (μmol/L)	MDA (nmol/mL)
Normal	11.34 ± 2.10^{b}	78.07 ± 8.37^{d}	4.36 ± 0.53^{a}
Model	5.45 ± 1.71^{a}	42.52 ± 8.88^{a}	15.68 ± 2.00^{e}
Vit C	7.27 ± 0.99^{a}	65.78 ± 12.06^{c}	9.73 ± 1.03^{c}
KSFY01-H	10.15 ± 0.75^{b}	72.87 ± 3.18^{cd}	6.71 ± 1.16^{b}
KSFY01-L	6.07 ± 1.39^{a}	55.04 ± 10.15^{b}	12.70 ± 1.57^{d}

The data are expressed as the mean ± SD. $^{a-d}$Values in the same column with different letter superscripts indicate significant difference ($p < 0.05$). Vit C, mice treated with vitamin C (200 mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10} CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9} CFU/kg).

3.4. Serum oxidation level of mice

The serum levels of CAT, GSH, and MDA were compared between groups, as shown in Table 4. Compared with the normal group, CAT enzyme activity in the model group was significantly decreased ($p < 0.05$), and the GSH content was also significantly decreased. After either vitamin C or *L. plantarum* KSFY01 intervention, the CAT enzyme activity and GSH level were both increased; among these, the high dose of *L. plantarum* KSFY01 was more effective than either vitamin C or the low dose of *L. plantarum* KSFY01. Compared with the model group, vitamin C and *L. plantarum* KSFY01 significantly reduced the MDA content ($p < 0.05$); among these, high-dose *L. plantarum* KSFY01 had the greatest effect.

3.5. Pathological evaluation

The morphology of liver tissue under the microscope in mice is shown in Figure 3. The liver lobules of the mice in the normal group exhibited a clear structure and no obvious changes in the intact liver cells, and there were no phenomena such as granular degeneration, vacuolar degeneration, hepatocyte enlargement, or necrosis. In the D-galactose-induced aging model group, the structure of the hepatic lobules of the mice was destroyed, the arrangement of hepatocytes was disordered, many vacuoles of different sizes appeared in the encapsulation, and there was inflammatory infiltration accompanied by cell necrosis. Both vitamin C and *L. plantarum* KSFY01 could reduce hepatocyte damage in aging mice. After the action of KSFY01-H, the structure of hepatic lobules was mainly intact, and the degeneration of hepatocytes was significantly improved. There were still vacuoles of different sizes in the liver tissue of mice observed in both the Vit C group and KSFY01-L group.

The morphology of kidney tissue under the microscope of mice is shown in Figure 4, and the glomerular vascular loops in the normal group were thin and clear. The number of endothelial and mesangial cells was normal. The surrounding renal tubules were also normal. The glomeruli in the kidney tissue of the mice in the aging model group were irregular in shape, some were ruptured or swollen, and there was inflammatory cell infiltration observed between the tissues. Both vitamin C and *L. plantarum* KSFY01 could reduce kidney tissue damage caused by aging, and the infiltration of inflammatory cells decreased to varying degrees.

3.6. Expression of mRNA in liver tissue

As shown in Figure 5, the expression of IL-1β and TNF-α mRNA in the liver tissue of the normal mice was the lowest, while the expression of IL-10, SOD1, SOD2, CAT, HO-1, Nrf2, γ-GCS, and NQO1 were the greatest. The model group showed the opposite trend. Vitamin C and *L. plantarum* KSFY01 could each downregulate IL-1β and TNF-α expression while upregulating IL-10, SOD1, SOD2, CAT, HO-1, Nrf2, γ-GCS, and NQO1 expression. The high-dose *L. plantarum* KSFY01 group had the strongest ability to cause these expression changes and gave the most similar values to those of the normal mice.

3.7. Expression of mRNA in mouse skeletal muscle tissue

We also analyzed Nrf2 pathway-related genes in mouse muscle tissue. IL-10, IL-1β, TNF-α, SOD1, SOD2, CAT, HO-1, Nrf2, γ-GCS, and NQO1 are oxidative genes involved in this pathway. These genes become abnormally expressed when the expression of Nrf2 changes. As shown in Figure 6, compared with the normal group, the expression levels of IL-10, Cu/Zn-SOD, Mn-SOD, CAT, HO-1, Nrf2, γ-GCS, and NQO1 mRNA in the muscle tissue of the model group were significantly decreased ($p < 0.05$). Conversely, the expression of IL-1β and TNF-α mRNA increased significantly ($p < 0.05$). Compared with the model group, the vitamin C and *L. plantarum* KSFY01 interventions each greatly improved the expression of these genes ($p < 0.05$) to the degree that they more resembled the normal group. The regulatory effect of *L. plantarum* KSFY01 was enhanced in a dose-dependent manner. The regulatory effect of high dose *L. plantarum* KSFY01 was greater than that of low dose *L. plantarum* KSFY01. These results indicate that *L. plantarum* KSFY01 has a positive effect on aging-induced muscle damage in mice.

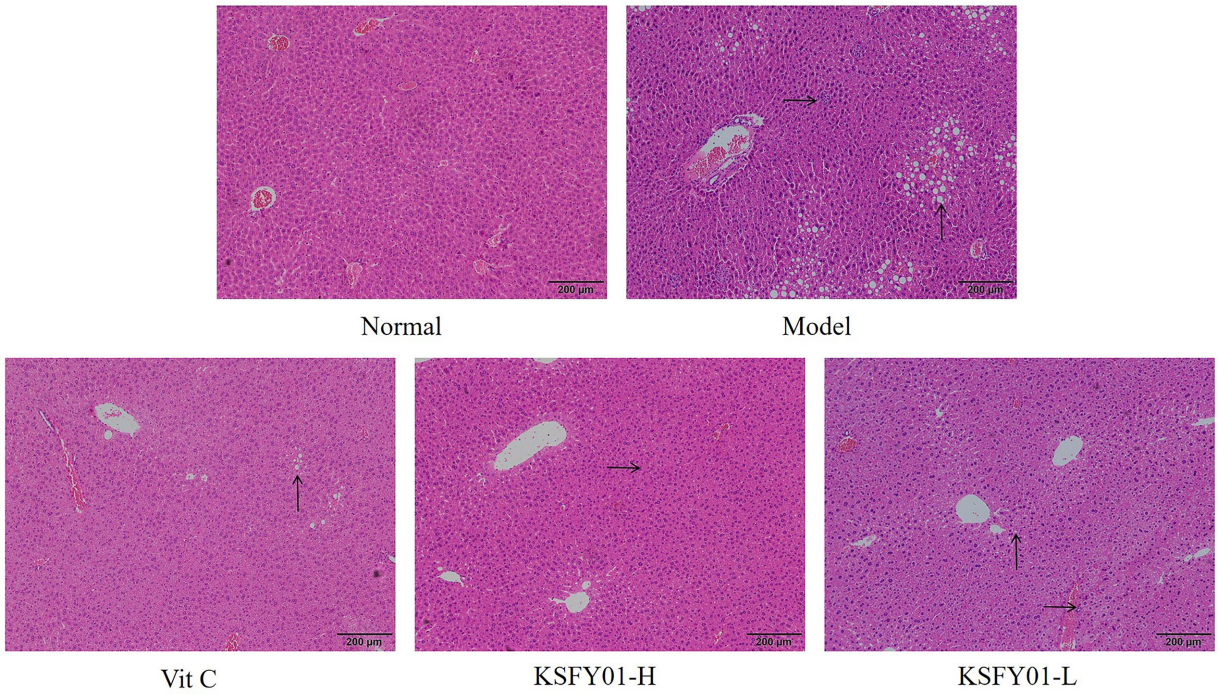


FIGURE 3
H&E pathological observation of liver tissue in aging mouse (magnification, ×100). Vit C, mice treated with vitamin C (200mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10}CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9}CFU/kg). → indicate adipocyte; ↑ indicate inflammatory cell in liver.

4. Discussion

With increasing age, the generation of endogenous free radicals increases in parallel, while homeostasis, stress resilience, and the function of the endogenous antioxidant system all gradually decline. With the abnormal and excessive accumulation of free radicals in the body, the balance between the oxidation and antioxidant systems in the body breaks down, thereby damaging organelles such as the mitochondria and cell membranes. These changes result in cellular dysfunction, in turn affecting the metabolism and accelerating the aging process (Qian et al., 2018). As people age, the conduction velocity of their motor and sensory nerves gradually decreases, and this decline in the function of the nervous system causes the weakening of motor ability, which is accompanied by the frequent occurrence of fatigue (Wu et al., 2022). Exercise can help clear free radicals and thereby allow normal cells to maintain the integrity of mitochondrial structure and function; simultaneously, being in good physical condition can facilitate the body to maintain exercise capacity (Vargas-Mendoza et al., 2021). Improvements in exercise capacity represent the most direct manifestation of bodily resistance to fatigue, and also serve as a key manifestation of resistance to aging (Zhong et al., 2022). Studies have confirmed that some LAB possess good antioxidant and anti-aging effects (Suo et al., 2018). This study also established a mouse aging model by the intraperitoneal injection of D-galactose. This was done to observe the effect of *L. plantarum* KSFY01 on endurance running in mice in an attempt to verify the intervention effect of *L. plantarum* KSFY01 on exercise capacity in the aging state. The experimental results also confirmed that *L. plantarum* KSFY01 could prolong the endurance running time of mice, enhance their exercise capacity, and relieve exercise-induced fatigue.

Long-term continuous exercise causes a substantial degree of bodily fatigue. In this study, we designed a high-intensity exercise treadmill experiment to induce exercise fatigue in mice. The depletion of hepatic glycogen and the accumulation of metabolic byproducts are each thought to contribute to bodily fatigue (Wang et al., 2015; Yi et al., 2021; Zhu et al., 2021). Physical exercise initiates with increases in aerobic muscle activity. If the exercise is intense, anaerobic metabolism will be employed, thereby resulting in the accumulation of LA. Increasing LA then reduces the pH value of both tissues and blood, consequently affecting the physiological and biochemical processes occurring in the body, and ultimately affecting bodily function (Peng et al., 2021). In this way, LA is an important indicator for measuring the level of fatigue. The normal function of LDH in cells is to catalyze the interconversion between pyruvate and lactate (Majid, 2022). BUN is a by-product of metabolic proteins that is produced by the body after excessive glycogen consumption, and it represents a sensitive parameter related to fatigue (Yang et al., 2022). The lower the physical endurance of an individual, the greater the increase in BUN levels during physical exertion (Lee et al., 2021). Therefore,

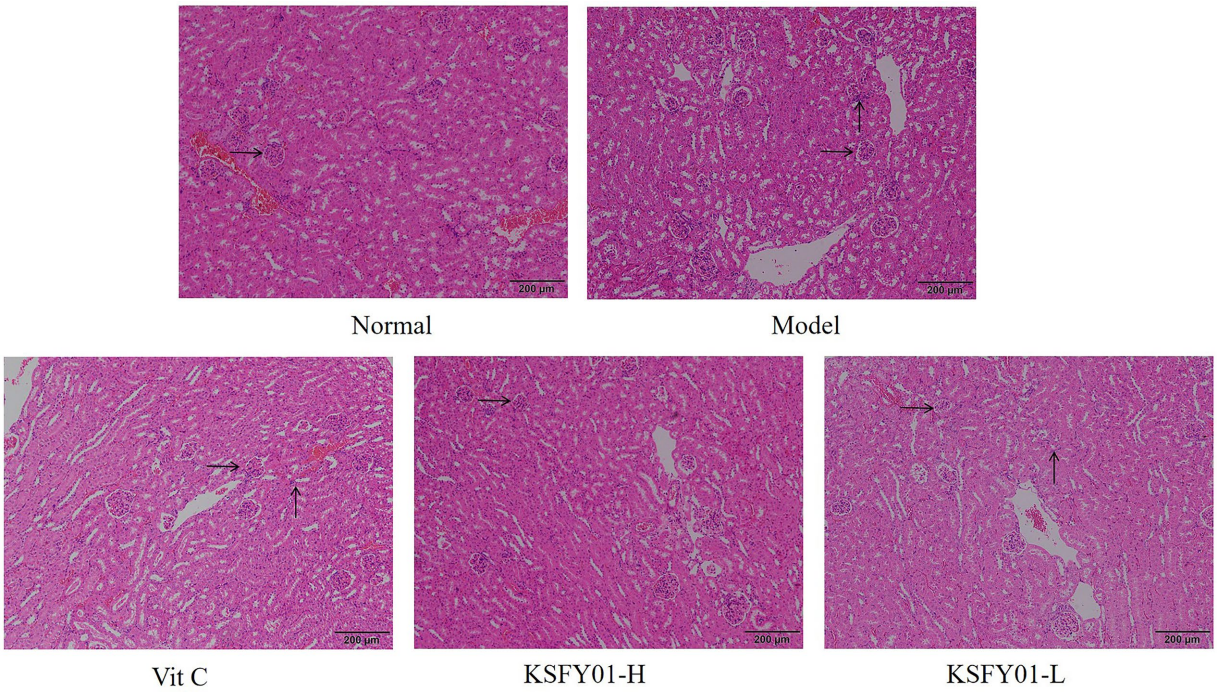


FIGURE 4
H&E pathological observation of kidney tissue in aging mouse (magnification ×100). Vit C, mice treated with vitamin C (200mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10}CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9}CFU/kg). → indicate glomerulus; ↑ indicate inflammatory cell in kidney.

serum levels of LA, LDH, and BUN can indicate both the speed and extent of fatigue that has developed within the body. In addition, the physical exhaustion of mice during long-term high-intensity exercise is related to changes in MG content. After a period of exercise, glycogen in the body is decomposed into lactic acid, and energy is then released to allow muscle activity. The greater the MG reserve, the better the endurance of the body, and the longer the exercise duration (Kim et al., 2020). In this study, compared with the model group, vitamin C and *L. plantarum* KSFY01 interventions each significantly attenuated the accumulation of these metabolites and regulated blood sugar levels. *L. plantarum* KSFY01 also reduced muscle damage by regulating LDH levels. This indicates that *L. plantarum* KSFY01 can relieve fatigue by effectively reducing the accumulation of metabolic byproducts, improving muscle damage, and enhancing energy storage capacity.

There is increasing evidence that high-intensity and acute exercise can each cause damage to hepatocytes by reducing blood flow to the liver and portal vein, which often leads to hepatocyte hypoxia, and ultimately, liver necrosis (Kinoshita et al., 2003). This injury is often accompanied by elevated levels of CK, AST, and ALT (Sangita et al., 2018). In this study, we also found that exercise fatigue caused a significant increase in the liver function indicators of serum CK, ALT, and AST, thereby indicating liver dysfunction. This outcome could be reversed by either the oral administration of vitamin C or *L. plantarum* KSFY01.

The liver is the central organ of oxidative stress reaction, which can significantly reflect the degree of oxidation of the body, and is also an organ easy to produce free radicals and lipid peroxides. The increase of free radicals caused by aging can be clearly shown in the pathological changes of liver tissue. In addition, with the aging of the body, the kidney gradually shrinks, and the secretion of prostaglandins decreases, leading to vasoconstriction and reduced blood flow. As aging continues, the vascular contraction frequency of liver and kidney decreases, and the blood supply of various organs decreases, which directly reduces the exercise ability (Aschner et al., 2021). In this study, pathological section observation also confirmed that aging led to liver and kidney tissue damage, *L. plantarum* KSFY01 can effectively reduce the decline and damage of liver and kidney tissues, and one of the important roles it plays may be to improve motor function.

During exercise, many free radicals (such as hydroxyl radicals and superoxide anion radicals) are generated, and this phenomenon is especially pronounced in the aged state (Mariusz and Sławomir, 2013). CAT, GSH, etc. are all important antioxidants and free radical scavengers in the human body. Their existence can maintain the normal oxidative stress homeostasis by removing excess free radicals. The excessive accumulation of ROS can cause oxidative stress and attack biological macromolecules, such as lipids, proteins, and nucleic acids, to form lipid peroxidation products, like MDA

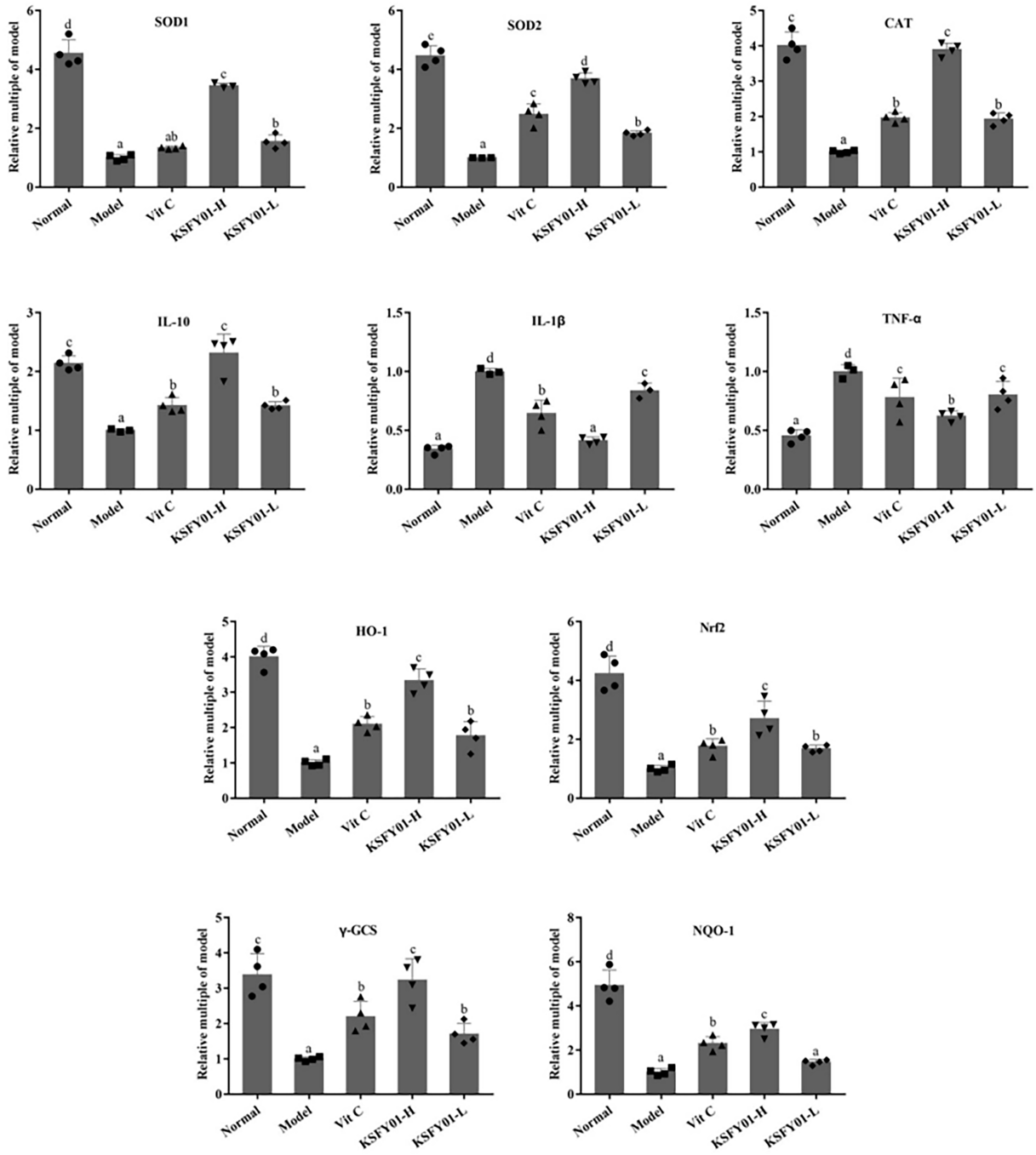


FIGURE 5
The SOD1, SOD2, CAT, IL-1β, IL-10, TNF-α, HO-1, Nrf2, γ-GCS, and NQO-1 mRNA expressions in mouse liver tissue. [a–e]Values in the same column with different letter superscripts indicate significant difference ($p<0.05$). Vit C, mice treated with vitamin C (200mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10}CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9}CFU/kg).

(Kheradmand et al., 2009; Li et al., 2022). MDA further damages the structure of the cell membrane, resulting in cell swelling and necrosis. After the intervention of either vitamin C or *L. plantarum* KSFY01, the content of MDA in the blood of mice was decreased, while the contents of CAT and GSH were increased. Therefore, our results suggest that *L. plantarum* KSFY01 has potent antioxidant activity and can be used to inhibit aging, whereby it exerts the effect of enhancing exercise endurance and performance.

In terms of inflammatory response, with the growth of age, the function of the immune system gradually declines, which induces some diseases that seriously affect tissues and organs, and

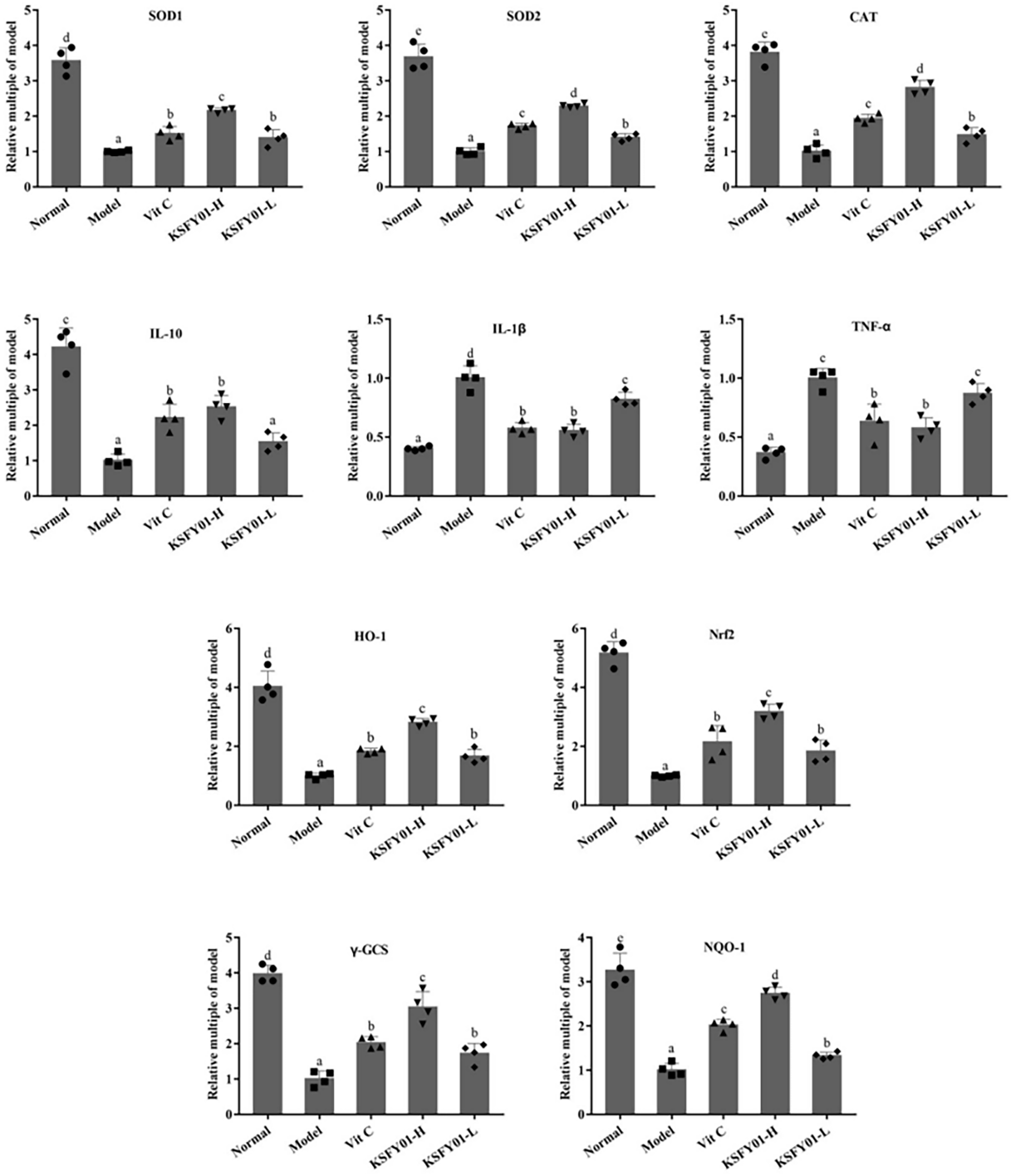


FIGURE 6
The SOD1, SOD2, CAT, IL-1β, IL-10, TNF-α, HO-1, Nrf2, γ-GCS, and NQO-1 mRNA expressions in mouse skeletal muscle tissue. [a–e]Values in the same column with different letter superscripts indicate significant difference (p<0.05). Vit C, mice treated with vitamin C (200mg/kg); KSFY01-H, mice treated with *L. plantarum* KSFY01 (1×10^{10}CFU/kg); KSFY01-L, mice treated with *L. plantarum* KSFY01 (1×10^{9}CFU/kg).

aggravates the aging of various systems of the body. TNF-α and IL-1β can activate T cells and enhance the immune response of the body. The results showed that in the model group, proinflammatory factor levels (TNF-α and IL-1β) were the highest, while anti-inflammatory factor levels (IL-10) were the lowest. In the normal group, a completely opposite trend was noted. After treatment with KSFY01, the expression of TNF-α and IL-1β was inhibited, while the expression of IL-10 was enhanced, indicating that the *L. plantarum* KSFY01 inhibits the production of inflammatory factors.

To elucidate the molecular mechanism by which *L. plantarum* KSFY01 affects fatigue and fatigue-related organ dysfunction in the aging state, we measured the expression of oxidative stress-related signaling genes in the liver and muscle tissue of mice. Many studies have shown that the Nrf2 signaling pathway serves as an extremely important endogenous defense system in the body (Sunil et al., 2021). Nrf2 is the most critical in the response to oxidative stress in cell. The protein primarily interacts with antioxidant response elements (AREs) to induce the expression of encoded antioxidant proteins and phase II detoxification enzymes (Johnson et al., 2008), thereby increasing cellular resistance against harmful stimuli within cells to play an important protective role. This confers anti-tumor, anti-stress, anti-apoptotic, anti-inflammatory, and neuroprotective effects (Vriend and Reiter, 2015; Zhang et al., 2022). Downstream antioxidant enzymes that are regulated by the Nrf2 signaling pathway include γ-GCS, SOD, CAT, and CSH-Px (Na and Surh, 2005), while the downstream phase II detoxification enzymes that are regulated by the Nrf2 signaling pathway include HO-1 and NQO1 (Keum, 2012). Oxidative stress generates a large amount of ROS to cause peroxidative damage, while the increase of ROS in mitochondria can lead to progressive lipid oxidation, generate more lipid peroxides, and induce tumor necrosis factor TNF-α (Wang W. et al., 2022; Wang L. et al., 2022). TNF-α not only damages the function of the mitochondrial respiratory chain and affects the electron transfer in the respiratory chain, but also opens the permeability transition pore of mitochondria, depletes cytochrome C in mitochondria, and then triggers the apoptosis and necrosis of liver cells (Wang W. et al., 2022; Wang L. et al., 2022). In this study, we used RT-PCR to analyze whether vitamin C and *L. plantarum* KSFY01 could affect the expression of these genes. The results showed that high-intensity exercise decreased the mRNA expression levels of Nrf2, NQO1, γ-GCS HO-1, SOD1, SOD2, CAT, and IL-10 in both liver and muscle tissue. Conversely, vitamin C and *L. plantarum* KSFY01 significantly increased the expression of these genes, and the intervention of *L. plantarum* KSFY01 was more significant. The intervention effect of *L. plantarum* KSFY01 was dose-dependent, and the intervention effect of high-dose *L. plantarum* KSFY01FDB was better than that of low-dose *L. plantarum* KSFY01. These findings suggest that the Nrf2 pathway is the underlying molecular mechanism by which *L. plantarum* KSFY01 exerts its antioxidant and anti-fatigue effects.

5. Conclusion

In this study, we established a mouse aging model to explore the antioxidant effect of *L. plantarum* KSFY01 and its effect on improving the ability of mice to run. The experimental results show that *L. plantarum* KSFY01 could alleviate exercise-induced fatigue and improve the exercise capacity of aging mice by improving their metabolite accumulation, glycogen storage, muscle and liver damage, and levels of oxidative stress. These results provide a reference for the future development of food-derived antioxidants for anti-fatigue effects, and for improving the motor function of the elderly. While these findings are promising, this study only examined the effect of LP-KFY04 in an animal model, so further experimentation is required to determine if a similar effect can be achieved in humans. In addition to fatigue, many diseases are related to oxidative stress, such as aging, diabetes and nonalcoholic fatty liver. In future research, other effects of *L. plantarum* KSFY01 can be further explored.

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 animal study was reviewed and approved by the Ethics Committee of Chongqing Collaborative Innovation Center for Functional Food.

Author contributions

QC performed the majority of the experiments and wrote the manuscript. CL, YZ, and SW contributed to the data analysis. FL designed and supervised the study and checked the final 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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Plantaricin BM-1 decreases viability of SW480 human colorectal cancer cells by inducing caspase-dependent apoptosis

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Plantaricin BM-1 is a class IIa bacteriocin produced by *Lactobacillus plantarum* BM-1 that has significant antimicrobial activity against food-borne bacteria. In this study, a cell proliferation assay and scanning electron microscopy were used to detect changes in the viability of SW480, Caco-2, and HCT-116 colorectal cancer cells treated with plantaricin BM-1. We found that plantaricin BM-1 significantly reduced the viability of all colorectal cancer cell lines tested, especially that of the SW480 cells. Scanning electron microscopy showed that plantaricin BM-1 treatment reduced the number of microvilli and slightly collapsed the morphology of SW480 cells. Fluorescence microscopy and flow cytometry demonstrated that plantaricin BM-1 induced apoptosis of SW480 cells in a concentration-dependent manner. Western blotting further showed that plantaricin BM-1-induced apoptosis of SW480 cells was mediated by the caspase pathway. Finally, transcriptomic analysis showed that 69 genes were differentially expressed after plantaricin BM-1 treatment ($p<0.05$), of which 65 were downregulated and four were upregulated. The Kyoto Encyclopedia of Genes and Genomes enrichment analysis showed that expression levels of genes involved in the TNF, NF-κB, and MAPK signaling pathways, as well as functional categories such as microRNAs in cancer and transcriptional misregulation in cancer, were affected in SW480 cells following the treatment with plantaricin BM-1. In conclusion, plantaricin BM-1 induced death in SW480 cells *via* the caspase-dependent apoptosis pathway. Our study provides important information for further development of plantaricin BM-1 for potential applications in anti-colorectal cancer.

KEYWORDS

apoptosis, bacteriocin, colorectal cancer, plantaricin BM-1, transcriptomics

1. Introduction

The colorectal cancer (CRC) is the third most common cancer worldwide and ranks second among the causes of global cancer mortality (Bray et al., 2018). Common colon cancer cell lines, such as Caco-2, SW480, HCT116, etc., are often used *in vitro* for preliminary screening of anticancer drugs and mechanism research. Besides, existing anticancer drugs kill cancer cells, but also have toxic side effects on normal cells (Schlichtig et al., 2019). Therefore, it is very meaningful to search for anticancer components with high efficacy and low toxicity from natural products. Generally, these natural products kill cancer cells by inhibiting cell proliferation and inducing apoptosis or necrosis (Cui et al., 2020). Apoptosis is a genetically controlled method of programmed cell death that does not induce an inflammatory response (Xu et al., 2019). Necrosis is usually associated with swelling and loss of membrane integrity as a result of physical injury that does not involve the active participation of the cell (Zhang et al., 2019). In contrast to necrosis, several morphological features, including chromatin condensation, cell contraction, nuclear fragmentation, and membrane permeability, commonly occur during apoptosis (Chen et al., 2018). The core executioners of apoptosis are the caspases family proteases. Among them, Caspase-3 is the ultimate executor of cell apoptosis (Yang et al., 2019). Cleaved caspase-3 disrupts anti-apoptotic proteins and releases pro-apoptotic C-terminal fragments (Asadi et al., 2022). Poly (ADP-ribose) polymerase (PARP-1) is the main substrate of caspase-3 and a key enzyme for detecting DNA damage. Cleaved caspase-3 triggers PARP-1 cleavage. The cleaved PARP-1 then accesses chromatin *via* endonucleases to induce apoptosis (Zhou et al., 2021).

Bacteriocins are short-chain peptides produced by bacterial ribosomes that are commonly used as antimicrobial agents in food and pharmaceuticals (Soltani et al., 2021). Bacteriocins are usually studied for their antibacterial effect, but they are also increasingly considered potential anticancer drugs (Baindara et al., 2018; Yaghoubi et al., 2020; Cesa-Luna et al., 2021). For example, colicins (A, E1, U, and E3; Arunmanee et al., 2021) and pediocin PA-1 (Villarante et al., 2011) were shown to inhibit HT29 CRC cell viability *in vitro*. In addition, antimicrobial peptides M2163 and M2386 induced apoptosis of SW480 CRC cells by enhancing cell membrane permeability (Tsai et al., 2015). Furthermore, the antimicrobial peptide KL15 obtained *via in silico* modification of the sequences of bacteriocins m2163 and m2386 damaged the cell membrane of SW480 cells by forming pores, thus inducing necrosis. Notably, KL15 was not cytotoxic to normal human mammary epithelial cells (Mejía-Caballero et al., 2021). In addition, nisin was shown to induce apoptosis in SW480 CRC cells through the intrinsic apoptotic pathway (Jaye et al., 2022). Microcin E492, a bacteriocin produced by the *Klebsiella pneumoniae* strain RYC492, showed antitumor activity against cancer cells and induced apoptosis by activating the caspase pathway at low concentrations and necrosis at high concentrations (Varas et al., 2020). However, the exact mechanism of action of bacteriocins against CRC cells has not yet been fully elucidated.

Plantaricin BM-1 is a novel class IIa bacteriocin produced by *Lactobacillus plantarum* BM-1, a probiotic isolated from a traditional natural fermented meat product. Our previous research showed that this bacteriocin has significant inhibitory activity against food-borne pathogenic bacteria, such as *L. monocytogenes*, *E. coli*, and *Salmonella* (Zhang et al., 2013), so it can be used to develop natural bio-preservatives. In the present study, we investigated whether plantaricin BM-1 has anticancer activity against SW480 CRC cells. We showed that plantaricin BM-1 induced apoptosis in SW480 CRC cells through the caspase-mediated pathway. Moreover, transcriptomic analysis was performed to provide detailed information on the potential molecular mechanisms of plantaricin BM-1 action against SW480 cells. This study provides an experimental basis for further development of plantaricin BM-1 as an anti-colorectal cancer compound.

2. Materials and methods

2.1. Preparation of plantaricin BM-1

A two-step method was used to purify plantaricin BM-1, as described in our previous study (Zhang et al., 2013). Briefly, *Lactobacillus plantarum* BM-1 was cultured in de Man, Rogosa, and Sharpe (MRS) broth at 37°C for 12 h and centrifuged at 4°C for 10 min at 10,000 rpm to collect the supernatant. Plantaricin BM-1 was purified *via* pH-mediated cell adsorption–desorption and cation-exchange chromatography, freeze-dried, and stored at −80°C.

2.2. Cell culture and culture conditions

SW480 (ATCC CCL-228), Caco-2 (ATCC HTB-37), and HCT-116 (ATCC CCL-247) cell lines were obtained from the Cell Resource Center of the Institute of Basic Medical Sciences (Chinese Academy of Medical Sciences, Beijing, China). The normal colonic epithelial cell line NCM460 (INCELL CVCL_0460) was obtained from iCell Bioscience, Inc. (Shanghai, China). SW480 and HCT-116 cells were cultured in Leibovitz's L-15 medium (Gibco, New York, NY, United States) and Iscove's Modified Dulbecco's medium (Gibco) containing 10% fetal bovine serum (Gibco), respectively. Caco-2 and NCM460 cells were grown in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum. Caco-2, HCT-116, and NCM460 cells were incubated in a humidified atmosphere of 95% air/5% CO_2, whereas SW480 cells were not exposed to additional CO_2. All cells were cultured in a CO_2 incubator (HF90; Shanghai Lishen Scientific Equipment Co. Ltd., Shanghai, China) at 37°C.

2.3. Cell viability assay

Cell Counting Kit-8 (CCK-8 kit; Dojindo, Kumamoto, Japan) was used to evaluate the effect of plantaricin BM-1 on cell viability. Briefly, the cells were seeded at a density of 1×10^4 cells/well in a 96-well plate 24 h before the addition of plantaricin BM-1. After overnight incubation, the medium was replaced by a fresh medium containing different concentrations of plantaricin BM-1 (181, 363, 726, 1,451, and 2,902 μg/ml, diluted with complete medium), and the cells were cultured. The medium was discarded after 1 h of reaction period. Then, 100 μl of the medium and 10 μl of the CCK-8 kit reagent were added to each well, and the plates were incubated at 37°C for 1 h. Cells treated with 100 μl of complete medium were used as the negative control. The final absorbance was measured at 450 nm using a microplate reader (ELx808; BioTek, Winooski, VT, United States). The following formula was used to assess viability: viability ratio (%) = [(absorbance of the experimental well − blank well absorbance)/(control well absorbance − blank well absorbance)] × 100%. Six biological replicates were used for each treatment. The IC_{50} value was estimated by viability rates and calculated using the logit of probit regression in SPSS 19.0.

2.4. Scanning electron microscopy

Approximately 2×10^5 SW480 cells were seeded on several 0.17-mm glass coverslips (Solarbio, Beijing, China) in a six-well plate (Corning, New York, NY, United States). After 24 h, the growth medium was replaced with the medium containing different concentrations of plantaricin BM-1 ($1/2 \times IC_{50}$, $1 \times IC_{50}$, or $2 \times IC_{50}$), and the cells were incubated for 1 h. Cells not exposed to plantaricin BM-1 were used as the negative control. Cells were washed with ice-cold phosphate-buffered saline (PBS; Solarbio) and fixed in 2.5% glutaraldehyde (Solarbio) for 2 h at room temperature. The cells were prepared for Scanning electron microscopy (SEM) analysis according to conventional methods. After the cells were dehydrated with 50, 70, 90, and 100% ethanol, they were frozen overnight. The samples were placed on a specimen holder and sputtered with 20-nm gold onto an ion coater. An ultra-high resolution scanning electron microscope (SU8010; Hitachi, Tokyo, Japan) was used to image the specimens.

2.5. Fluorescence microscopic analysis of apoptosis

SW480 cells (4×10^5 cells) were seeded in a 60-mm dish (Corning) and cultured for 24 h. After treatment with plantaricin BM-1 concentrations of 0, $1/2 \times IC_{50}$, $1 \times IC_{50}$, or $2 \times IC_{50}$ for 1 h, cells were directly incubated with 10 μg/ml Hoechst 33342 (Solarbio) at 37°C for 10 min to stain for the DNA. After staining, the cells were washed with ice-cold PBS and examined under a fluorescence microscope (XD; Sunny Optical Technology Co. Ltd., Hong Kong, China) at 100 and 200× magnifications to observe chromatin condensation.

2.6. Flow cytometry analysis of apoptosis

SW480 cells were seeded at a density of 1×10^5 cells/ml in six-well plate and treated with plantaricin BM-1 (0, $1/2 \times IC_{50}$, $1 \times IC_{50}$, or $2 \times IC_{50}$) for 1 h. The cells were then harvested *via* trypsinization (Solarbio). The harvested cells were washed twice with PBS. An annexin V-FITC/PI kit (4A Biotech, Suzhou, China) was used for double staining. Apoptotic cells were analyzed using flow cytometry (NovoCyte; ACEA Biosciences, San Diego, CA, United States). Based on the flow cytometry results, apoptotic cells were mainly composed of early (Annexin V-$FITC^+$/PI^-) and late (Annexin V-$FITC^+$/PI^+) apoptotic cells.

2.7. Western blotting analysis

To estimate the influence of plantaricin BM-1 on the cleavage of caspase-3 and PARP-1, SW480 cells were treated with the complete medium (negative control), different concentrations of plantaricin BM-1 ($1/2 \times IC_{50}$, $1 \times IC_{50}$, and $2 \times IC_{50}$), or with 5-fluorouracil (5-FU; 757.9 μg/ml; positive control), and incubated for 1 h. Then, the cells were lysed and proteins were extracted using a Protein Extraction Kit (GenePool, Beijing, China). The proteins were using an SDS-PAGE Gel Kit (GenePool), containing 10% (cleaved PARP-1) or 15% (cleaved caspase-3) separating gel and 5% stacking gel, and then transferred to a polyvinylidene fluoride (PVDF) membrane (GenePool) with a pore size of 0.22 μm for 0.5 h (cleaved PARP-1) or 2 h (cleaved caspase-3). After the transfer, the PVDF membrane was immersed in a milk-blocking buffer (GenePool)/bovine serum albumin-blocking buffer (GenePool) for 1 h to block non-specific binding. The membrane was incubated at 4°C overnight with anti-PARP (Bioss, Beijing, China, No. bs2138R), anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, United States, No. 9664), and anti-β-actin (Abcam, Cambridge, United Kingdom, No. ab6276) primary antibodies diluted with 1% BSA/5% milk. The membrane was washed with TBST (GenePool) three times and then incubated with a milk-blocking buffer containing either goat anti-rabbit IgG-HRP (Abcam, Cambridge, United Kingdom) or goat anti-mouse IgG-HRP (Abcam, Cambridge, United Kingdom) diluted at 1:5,000, and gently shaken for 50 min at room temperature. After washing with TBST four times, the PVDF membrane was immersed in an ECL Plus Western Blot Kit color-developing solution (GenePool) for 1 min and then exposed and developed in a dark room. Quantity One V.4.6.2 software was used to analyze gray values.

2.8. RNA-sequencing analysis and gene set enrichment analysis

RNA sequencing was performed on plantaricin BM-1-treated (D group) or complete medium-treated (C group) cell samples based on the BMKCloud platform.[1] And three biological replicates were used for D group (D1, D2, and D3) and C (C1, C2, and C3). In brief, SW480 cells (1×10^5 cells/ml) were treated with $1 \times IC_{50}$ concentration of plantaricin BM-1 for 1 h, trypsinized to collect cells, and rinsed three times with PBS. Total RNA was extracted using the TRIzol reagent at 4°C. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). RNA integrity was assessed using the RNA Nano 6000 Assay Kit on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, United States). RNA samples that met the conditions of $2.5 \geq OD_{260}$ nm/OD_{280} nm ≥ 1.7, $2.5 \geq OD_{260}$ nm/OD_{230} nm ≥ 0.5, and RNA integrity number ≥ 7.0 were used for subsequent experiments. Sequencing libraries were generated using the NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, United States) following the manufacturer's recommendations, and index codes were added to attribute annotations to each sample. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina platform. The expression levels of mRNAs identified by transcriptomic sequencing results were analyzed with reference to *Homo sapiens* GRCh38 genome. Genes with fold change ≥2 and false discovery rate < 0.01 were regarded as differentially expressed genes (DEGs). All the DEGs were used for annotation and enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases, and significant enrichment was considered at a value of $p < 0.05$.

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2.9. Statistical analysis

Data are expressed as the mean ± SD, and effects were considered significant if value of $p < 0.05$. SPSS 26.0 software was used for one-way ANOVA, following a *post hoc* analysis with the Duncan method. Histograms were constructed using Prism 9.1.1 (GraphPad Software Inc., San Diego, CA, United States).

3. Results

3.1. Plantaricin BM-1 inhibits the proliferation of CRC cells

To investigate the anti-proliferative effect of plantaricin BM-1, a CCK-8 kit was used to quantify the viability of HCT-116, Caco-2, SW480, and NCM460 cells. As shown in Figure 1, treatment with plantaricin BM-1 significantly reduced CRC cell viability in a dose-dependent manner at 1 h compared to that in negative control. In contrast, plantaricin BM-1 showed no cytotoxicity against the normal colonic epithelial NCM460 cell line. The IC_{50} values of plantaricin BM-1 were 1,578, 819.9, and 757.9 μg/ml in HCT-116, Caco-2, and SW480 cells, respectively. Among these, SW480 cells appeared to be the most sensitive to the inhibitory effect of plantaricin BM-1. Incubation of SW480 cells with 181, 363, 726, 1,451, and 2,902 μg/ml plantaricin BM-1 led to significant cytotoxic

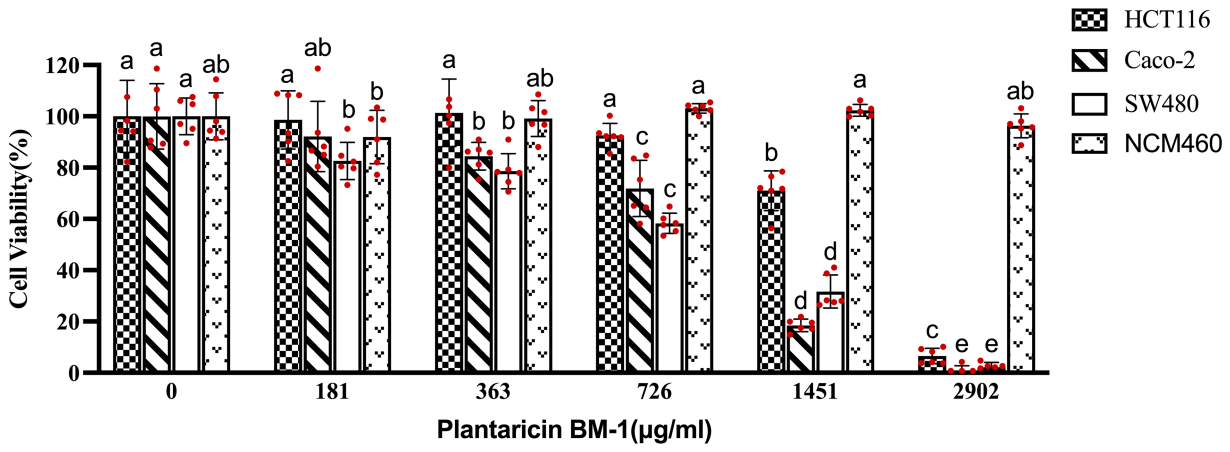

FIGURE 1
Effects of plantaricin BM-1 on cell viability in colorectal cancer (CRC) cells and normal intestinal epithelial NCM460 cells. The cells were treated with different concentrations of plantaricin BM-1 for 1 h and then, the cell survival rate (%) was determined by the CCK-8 kit. Each experiment was performed six times, and the results are presented in individual columns as the mean±SD; (a–e) Different letters in bars indicate that means are significantly different at $p<0.05$ according to one-way ANOVA in different plantaricin BM-1 concentration environments in the same cell line.

effects and decrease of cell viability to 82.6 ± 7.24, 78.6 ± 6.84, 58.29 ± 3.99, and 31.69 ± 6.45, and 2.14 ± 1.89%, respectively, of the values in negative control cells. Compared to its cytotoxic action in SW480 cells, the inhibitory effect of plantaricin BM-1 on HCT-116 cells was weak. Even when the concentration of plantaricin BM-1 was 1,451 μg/ml, the survival rate of HCT-116 cells remained high, at 71.01% of the negative control value. Except for the normal colonic epithelial NCM460 cells, none of the three other cell lines survived after treatment with 2,902 μg/ml plantaricin BM-1. The cell viability assay results indicated that plantaricin BM-1 was cytotoxic to CRC cells, especially SW480 cells, and showed no toxicity toward the normal colonic epithelial NCM460 cells. Therefore, SW480 cells were selected for subsequent experiments.

3.2. Scanning electron microscopy of cell morphology changes after plantaricin BM-1 treatment

Morphological changes in SW480 cells treated with different concentrations of plantaricin BM-1 for 1 h were examined by SEM. As shown in Figure 2A, in the negative control group without plantaricin BM-1 treatment, the cells were intact and surrounded by numerous microvilli. In contrast, the morphology of SW480 cells changed significantly after treatment with plantaricin BM-1 at $1/2 \times IC_{50}$ and $1 \times IC_{50}$: most of the microvilli vanished, and a few cells collapsed slightly (Figures 2B,C). In SW480 cells treated with $2 \times IC_{50}$ plantaricin BM-1, microvilli disappeared completely, and vacuolization and membrane perforation were observed (Figure 2D). These changes indicated that plantaricin BM-1 had a concentration-dependent cytotoxic action on SW480 cells, which was in agreement with the CCK-8 results.

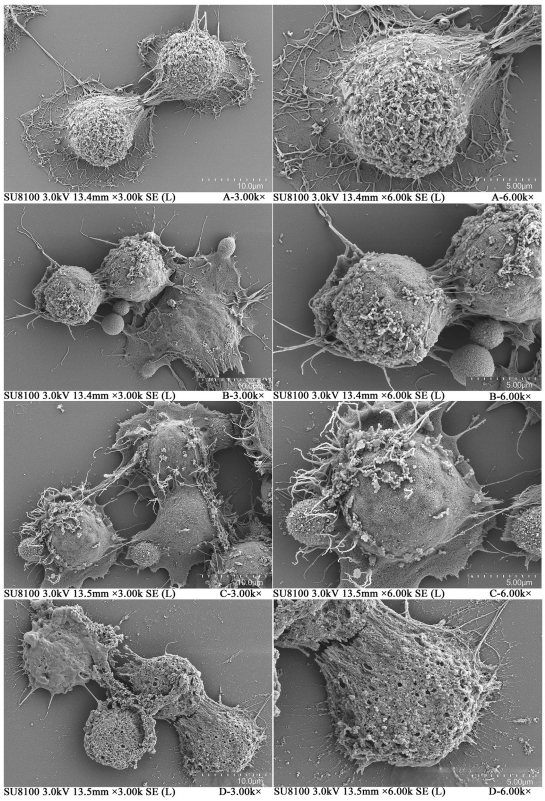

FIGURE 2
Scanning electron microscopy (SEM) of SW480 cells treated with plantaricin BM-1. Morphology of SW480 cells treated with different concentrations of plantaricin BM-1 for 1 h is illustrated as follows: **(A)** negative control, **(B)** $1/2 \times IC_{50}$, **(C)** $1 \times IC_{50}$, and **(D)** $2 \times IC_{50}$.

3.3. Plantaricin BM-1 induces apoptosis of SW480 cells

After treatment with $1/2 \times IC_{50}$, $1 \times IC_{50}$, and $2 \times IC_{50}$ of plantaricin BM-1 for 1 h, the morphology of SW480 cells was observed using Hoechst33342 staining under a fluorescence microscope. In the negative control group (Figure 3A), the cell bodies had a clear round nucleus and were intact, with weak fluorescence intensity. However, SW480 cells treated with

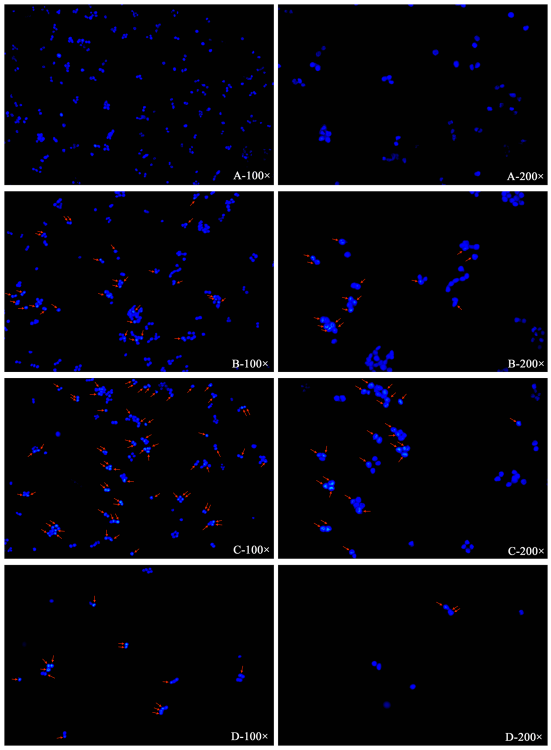

FIGURE 3
Induction of apoptosis in SW480 cells by plantaricin BM-1 revealed by fluorescence microscopy. SW480 cells were incubated for 1 h with various concentrations of plantaricin BM-1: **(A)** negative control, **(B)** $1/2 \times IC_{50}$ plantaricin BM-1, **(C)** $1 \times IC_{50}$ plantaricin BM-1, and **(D)** $2 \times IC_{50}$ plantaricin BM-1. Then, the cells were stained with Hoechst33342 and photographed under a fluorescence microscope at magnifications of 100 and 200×. The arrow shows typical apoptotic cell features, such as chromatin condensation, cell contraction, and nuclear cracking.

plantaricin BM-1 (Figures 3B–D) were brighter and had condensed chromatin and nuclear fragmentation, which are characteristic features of apoptosis. The number of apoptotic cells increased in a dose-dependent manner, and in the $1\times IC_{50}$ plantaricin BM-1 treatment group, it was significantly higher than that in the $1/2\times IC_{50}$ plantaricin BM-1 treatment group. In addition, the number of viable cells in the $2\times IC_{50}$ plantaricin BM-1 treatment group decreased significantly. This indicated that plantaricin BM-1 induced a concentration-dependent pro-apoptotic effect.

Apoptosis of SW480 cells treated with plantaricin BM-1 for 1 h was studied *via* flow cytometry with annexin V-FITC/PI double staining (Figure 4). After treatment with plantaricin BM-1 at $1/2\times IC_{50}$, $1\times IC_{50}$, and $2\times IC_{50}$, the apoptotic SW480 cells (including early and late apoptotic cells) comprised 4.33, 14.16, and 34.22%, respectively, of the total cells. This assay indicated that plantaricin BM-1 induced apoptosis of SW480 cells in a concentration-dependent manner, as determined by the increase in the signal detected in the first and fourth quadrants with an increase in the treatment dose (Figure 4). A total of 94.99% of the cells were still alive after treatment with $1/2\times IC_{50}$ of plantaricin BM-1. SW480 cells exposed to $1\times IC_{50}$ plantaricin BM-1 had a much larger cell population undergoing early apoptosis. A significant decline in cell viability and a drastic increase in apoptosis were observed in SW480 cells exposed to $2\times IC_{50}$ plantaricin BM-1.

Fluorescence microscopy and flow cytometry results indicated that the cytotoxicity of plantaricin BM-1 in SW480 cells was related to the promotion of apoptosis.

3.4. Plantaricin BM-1 activates caspase-3 and induces PARP-1 cleavage

We used western blot analysis to determine the levels of cleaved caspase-3 and cleaved PARP-1 in SW480 cells cultured with or without plantaricin BM-1. Cells treated with 757.9 μg/ml 5-FU were used as the positive control. Data were normalized by the level of β-actin expression used as the internal control. Western blot results showed that expression levels of cleaved caspase-3 and cleaved PARP-1 were directly proportional to the concentration of plantaricin BM-1 used to treat SW480 cells (Figure 5). These results indicated that plantaricin BM-1 induced caspase-dependent apoptosis in SW480 cells.

3.5. Transcriptomic analysis of the effects of plantaricin BM-1 treatment on gene expression in SW480 cells

In this study, we showed that plantaricin BM-1 inhibited the proliferation of SW480 cells by promoting caspase-induced apoptosis. To systematically elucidate the mechanism underlying the suppressive action of plantaricin BM-1 on SW480 CRC cells, we used RNA sequencing transcriptome analysis (RNA-Seq) to analyze three biological replicates in each group of samples. As shown in the volcano plot (Figure 6A), 69 differentially expressed genes were screened from 23,152

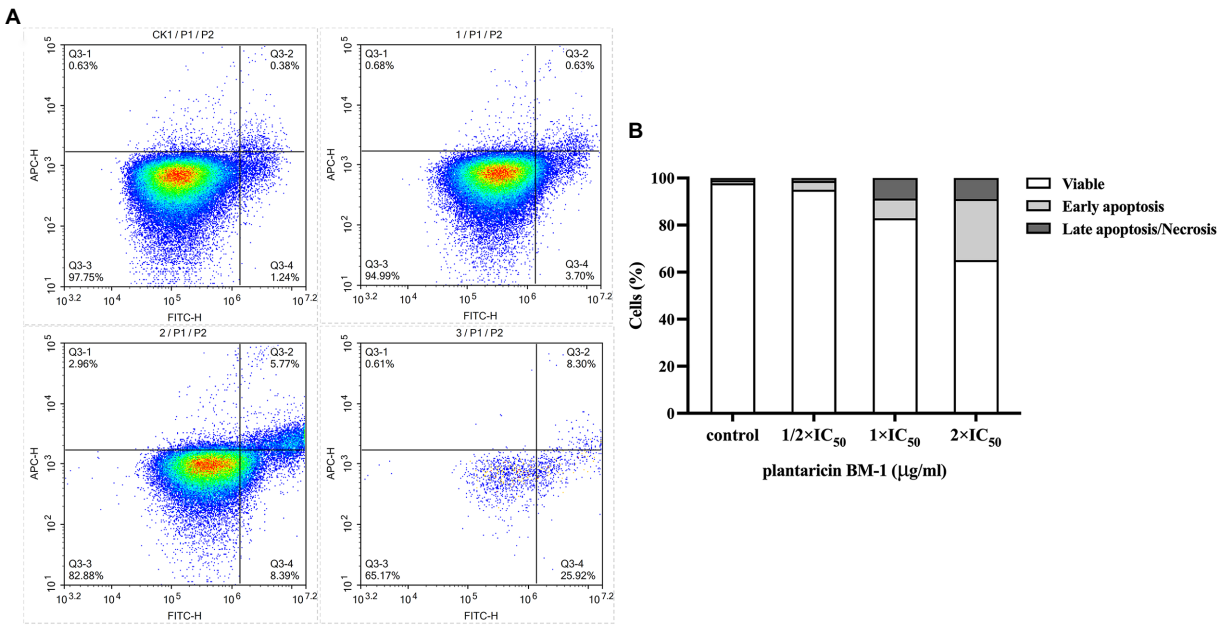

FIGURE 4
Quantitative analysis of cell apoptosis by flow cytometry with annexin V-FITC/PI double staining. **(A)** Flow cytometry was applied to analyze SW480 cell apoptosis after exposure to different concentrations of plantaricin BM-1 (CK1, control; 1, 1/2×IC_{50} plantaricin BM-1; 2, 1×IC_{50} plantaricin BM-1; and 3, 2×IC_{50} plantaricin BM-1) for 1 h. **(B)** Histograms show relative cell number in four different states: live, early apoptosis, late apoptosis, and necrosis.

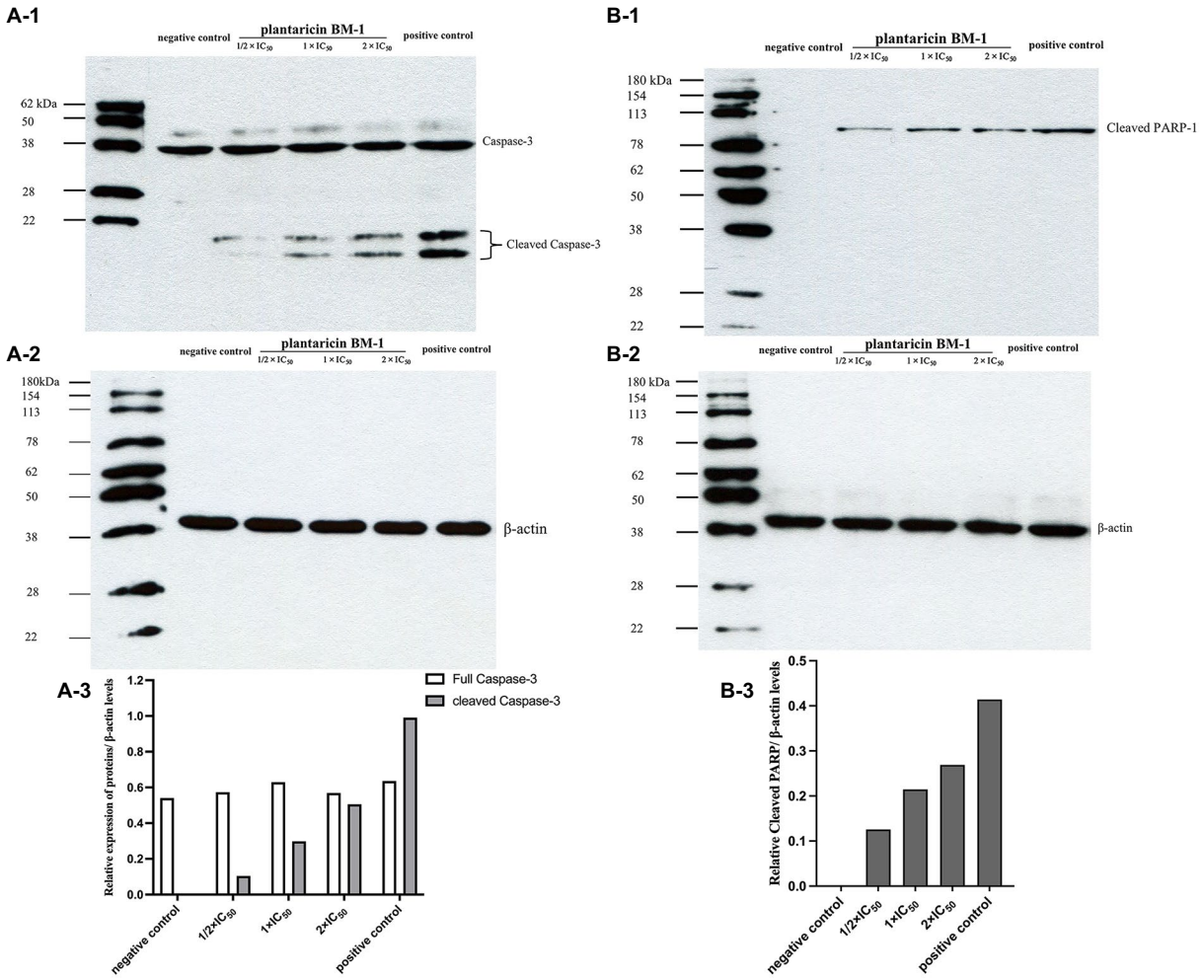

FIGURE 5
Western blot analysis of the levels of cleaved caspase-3 and cleaved PARP-1 after plantaricin BM-1 treatment. SW480 cells were treated with complete medium (negative control), plantaricin BM-1 (1/2×IC_{50}, 1×IC_{50}, or 2×IC_{50}), or 5-FU (positive control) for 1 h. **(A-1,A-3)** indicate the expression level of cleaved caspase-3. **(B-1,B-3)** indicate the expression level of cleaved PARP-1. Data were normalized to the level of β-actin protein expression, which was used as internal control (shown in **A-2** and **B-2**).

transcriptome-annotated genes, of which 65 genes were downregulated, and four genes were upregulated (fold change ≥ 2; false discovery rate < 0.01).

Gene Ontology enrichment analysis was used to further analyze DEGs with a focus on biological processes (BP), cellular components (CC), and molecular functions (MF). As shown in Figure 6B, the classification based on BP demonstrated that DEGs were mainly related to cellular processes (63.77%), single-organism processes (52.17%), metabolic processes (53.62%), biological regulation (55.07%), and response to stimulus (46.38%). From the perspective of the CC category, DEGs were mainly associated with the cell (65.22%), cell part (65.22%), organelle (52.17%), membrane (21.74%), and membrane part (13.04%). With regard to MF, DEGs were mainly related to binding (65.22%) and catalytic activity (20.29%).

Based on the KEGG pathway database for enrichment analysis, DEGs in SW480 CRC cells were significantly enriched in 34 KEGG pathways ($p < 0.05$). The top 20 enrichment pathways with the lowest value of *p*s are shown in Figure 6C. In addition, we investigated the relevant pathways that have been documented to regulate the growth and apoptosis of colon cancer cells (Supplementary Figure 1), including the TNF signaling pathway (ko04668), NF-κB signaling pathway (ko04064), MAPK signaling pathway (ko04010), microRNAs in cancer (ko05206), and transcriptional misregulation in cancer (ko05202). These pathways and DEGs are listed in Table 1. Twenty-seven DEGs were significantly downregulated in these signaling pathways in response to plantaricin BM-1 treatment.

To gain a systematic insight into the signaling networks involved in the induction of apoptosis and resulting growth inhibition in SW480 cells following exposure to plantaricin BM-1, we combined the results of differential expression analysis and the interaction pairs collected in the STRING database to construct a

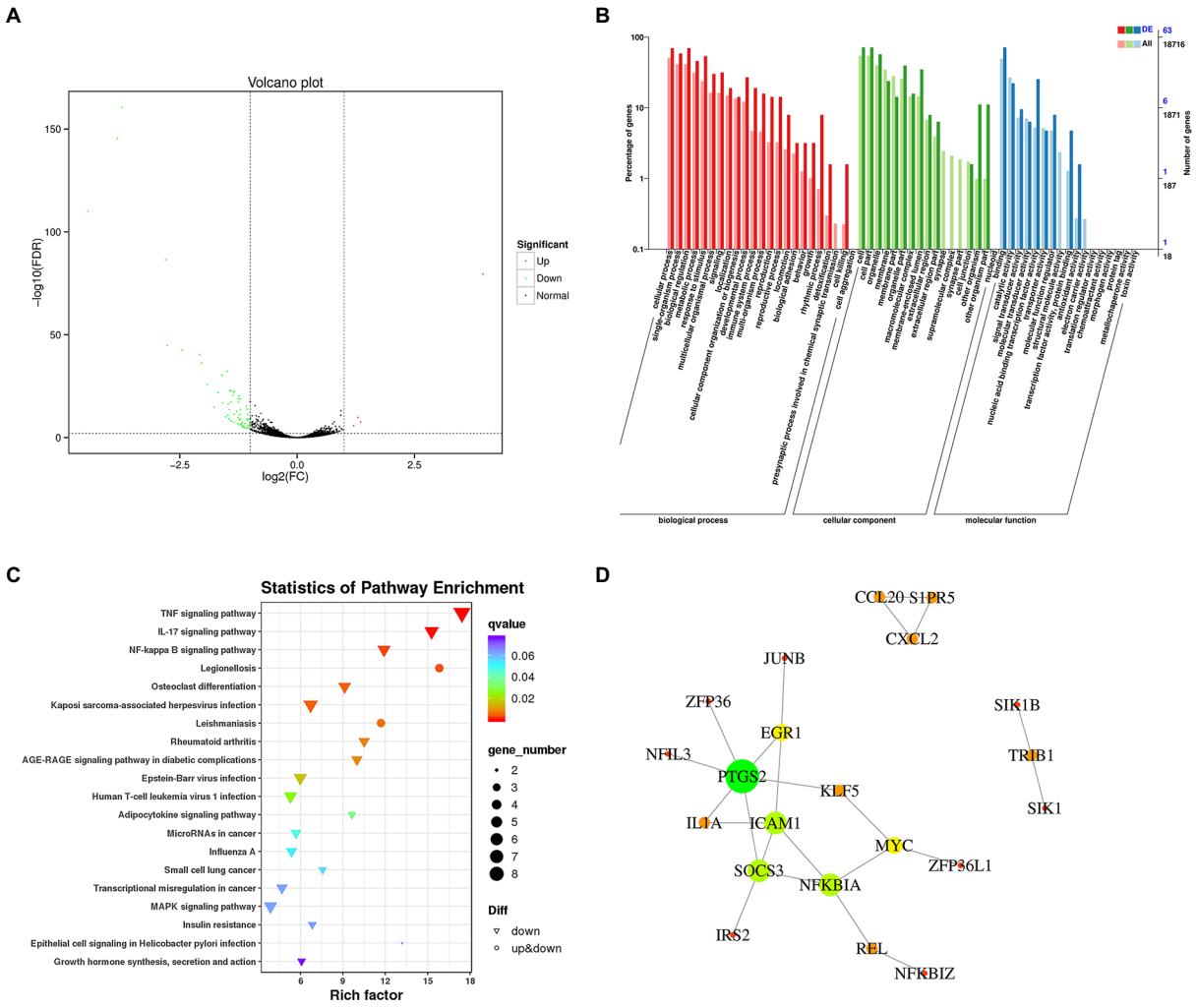

FIGURE 6
Transcriptome analysis of gene expression changes in SW480 cells after plantaricin BM-1 treatment. **(A)** Volcano plot. **(B)** GO enrichment analysis. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. **(D)** Protein–protein interaction network. The nodes and lines represent proteins and interactions, respectively. The node size is proportional to the number of edges connected to the node. The node color represents the clustering coefficient (red=high clustering coefficient; green=low clustering coefficient). A total of 21 nodes and 23 edges were identified.

protein interaction network of SW480 cell DEGs by using Cytoscape software. The protein–protein interaction network of DEGs is shown in Figure 6D. A total of 21 nodes and 23 edges were identified. Three highly connected clusters were obtained from the cluster analysis (Supplementary Table 1). These findings revealed complicated mechanisms underlying the effects of plantaricin BM-1 in SW480 cells.

4. Discussion

Plantaricin BM-1 is a class IIa bacteriocin that, similarly to other known class IIa bacteriocins, has antimicrobial activity against various Gram-positive and Gram-negative bacteria (Zhang et al., 2013). In this study, we found for the first time that plantaricin BM-1 inhibited SW480 CRC cell growth by reducing proliferation and inducing apoptosis.

Our results showed that plantaricin BM-1 specifically acted on cancer cells and was not toxic to normal cells. Moreover, the cytotoxicity of this bacteriocin in SW480 cells was stronger than that in the other two colon cancer cell lines, indicating that SW480 cells were particularly susceptible. In agreement with our present data, the antimicrobial peptides M2163 and M2386 were also significantly cytotoxic to SW480 and Caco-2 cells but not to normal cells. The MTT assay gives an IC_{50} of SW480 cells treated with either peptide m2163 or m2386 for 24 h was 40 μg/ml (Tsai et al., 2015). Compared with our results (Figure 1), plantaricin BM-1 could kill SW480 cells after 1 h treatment, although the IC_{50} was higher at 757.9 μg/ml. In addition, according to a previous report, nisin decreased the viability of SW480 cells in a

TABLE 1 Significantly enriched signaling pathways associated with CRC and the genes involved.

Pathway	Enrich_factor	Value of *p*	Down_genes	False discovery rate	Log2FC
TNF signaling pathway	17.43	1.26E−08	PTGS2	2.36E−08	−1.345696228
			CXCL2	2.82E−05	−1.013569651
			ICAM1	2.46E−05	−1.080316693
			NFKBIA	6.73E−05	−1.006230115
			CCL20	3.68E−05	−1.052786073
			TNFAIP3	9.22E−11	−1.522580166
			JUNB	1.82E−23	−1.435749373
			SOCS3	1.37E−09	−1.052087258
NF-kappa B signaling pathway	11.91	5.60E−05	PTGS2	2.36E−08	−1.345696228
			CXCL2	2.82E−05	−1.013569651
			ICAM1	2.46E−05	−1.080316693
			NFKBIA	6.73E−05	−1.006230115
			TNFAIP3	9.22E−11	−1.522580166
MAPK signaling pathway	3.87	8.74E−03	IL1A	5.97E−06	−1.154147302
			DUSP1	6.87E−33	−1.489214608
			NR4A1	2.55E−161	−3.729128244
			MYC	2.54E−06	−1.158861570
			DUSP5	1.15E−17	−1.589627729
MicroRNAs in cancer	5.68	5.12E−03	PTGS2	2.36E−08	−1.345696228
			MYC	2.54E−06	−1.15886157
			PIM-1	3.12E−21	−1.364514968
			IRS2	1.18E−05	−1.117769098
Transcriptional misregulation in cancer	4.68	1.01E−02	NR4A3	2.97E−43	−2.454790071
			MYC	2.54E−06	−1.158861570
			NFKBIZ	1.32E−45	−2.769516860
			REL	2.18E−09	−1.219135810

dose-dependent manner. Survival rates of 42.94, 41.77, 79.22, 130.6, 129, and 132.1% were detected after treatment with nisin at different concentrations (3,000, 2,500, 1,000, 750, 325, and 250 μg/ml, respectively) for 24 h (Jaye et al., 2022). Our results (Figure 1) showed that the IC_{50} value of plantaricin BM-1 in relation to its effect on SW480 cells was 757.9 μg/ml, indicating that the inhibitory effect of plantaricin BM-1 was better than that of nisin. Thus, plantaricin BM-1 specifically inhibited SW480 CRC cell proliferation at lower concentrations and over a shorter period of time.

To further investigate the mechanism underlying the cytotoxic action of plantaricin BM-1 on SW480 cells, SEM was used to observe changes in cell morphology and cell membrane surface after treatment. As was demonstrated by Mejía-Caballero et al. (2021), SW480 cells treated with the bacteriocin KL15 had pores in the membrane after treatment, and their microvilli disappeared, which is similar to the SEM images of plantaricin BM-1 after $2 \times IC_{50}$ treatment (Figure 2D). These results indicated that similar to KL15, $2 \times IC_{50}$ plantaricin BM-1 may also killed SW480 cells through necrosis. Similarly, the microvilli of cancer cells disappeared, and the membrane surface became smooth after treatment with the anticancer peptide LS10, as observed by SEM (Baindara et al., 2017), which was consistent with that after treatment with $1/2 \times IC_{50}$ and $1 \times IC_{50}$ plantaricin BM-1 (Figures 2B,C). This indicated that plantaricin BM-1 does not induce cell death through necrosis at lower concentrations, but only affected SW480 cell morphology, while the cell membrane remained intact.

Apoptosis is considered to be one of the most promising ways to inhibit cancers (Carneiro and El-Deiry, 2020). In this study, we focused on the suppression of SW480 cell treated with plantaricin BM-1 *via* induction of apoptosis. In previous studies, Hoechst33342 staining was used to observe the increase in fluorescence intensity of apoptotic cells, as well as aggregation and fragmentation of the nuclei (Goud et al., 2020; Wu et al., 2020; Huang et al., 2021; Alafnan et al., 2022). This

was similar to our results (Figure 3) using Hoechst33342 staining, which demonstrated that after receiving plantaricin BM-1 for 1 h, the initiation of the apoptosis process *via* enhanced chromatin condensation and the number of cells with condensed nuclei of high fluorescence intensity was increased dose-dependently. And the number of apoptotic cells increased in a dose-dependent manner. Compared with fluorescence microscopy, the flow cytometry analysis provides better qualitative and quantitative analyses of apoptotic cells (Pereira et al., 2021). The bacteriocin m2163 has been demonstrated to induce SW480 cell apoptosis by annexin V/PI staining and flow cytometry analysis after 24 h treatment (Tsai et al., 2015). Compared with m2163, plantaricin BM-1 could induce apoptosis in SW480 cells in a shorter time. Using this method, we showed that plantaricin BM-1 treatment of SW480 cells for 1 h also induced apoptosis. And the corresponding apoptosis rate at $1 \times IC_{50}$ concentration was 14.16% (Figure 4). This was mirrored by fluorescence microscopy results (Figure 3).

Caspase-3 protein is key regulator of the execution of apoptosis, which is responsible for the actual cleavages of intracellular proteins, such as PARP-1, resulting in apoptotic cell death (Yang et al., 2019; Zhou et al., 2021; Asadi et al., 2022). The observed cleavage of caspase-3 and cleavage of PARP-1 following plantaricin BM-1 treatment indicated that plantaricin BM-1 can induce caspase-dependent apoptosis in SW480 cells (Figure 5). This is similar to the mechanism of apoptosis induced by deoxypodophyllotoxin in SW480. Moreover, deoxypodophyllotoxin has also been confirmed to mediate the intrinsic mitochondrial pathway to induce CRC cell apoptosis by increasing the level of pro-apoptotic protein Bax and decreasing the level of anti-apoptotic protein Bcl-xL (Gamage et al., 2019), which was not involved in our study and can be carried out in subsequent studies. In addition, our findings suggested that the degree of caspase-3 cleavage after treatment with $1 \times IC_{50}$ plantaricin BM-1 was significantly lower than that after treatment with 5-FU at the same concentration. However, CCK-8 results showed that cell viability after plantaricin BM-1 treatment was significantly lower than that after 5-FU treatment (data not shown). This indicates that bacteriocin plantaricin BM-1 may induce SW480 cell death through pathways other than the caspase-dependent apoptosis pathway, which needs to be further studied.

We then applied transcriptomic technology and bioinformatics analysis to comprehensively reveal the molecular mechanism of plantaricin BM-1 effects on SW480 cells. Notably, our data suggested that plantaricin BM-1 triggered apoptosis and suppressed the viability of SW480 cells by regulating multiple signaling pathways. Changes in genes encoding the TNF signaling pathway components were the most significant. The TNF signaling pathway mediates apoptosis in SW480 cells. It has been reported that boric acid treatment activates the TNF signaling pathway, which, in turn, induces apoptosis in SW480 cells, and the qRT-PCR results showed that the expression levels of *TNF* and *TNFSF8* genes in the treatment group were significantly upregulated (Sevimli et al., 2022). However, the expression levels of these two genes were not significantly altered in our transcriptomic analysis. Our results showed that among the DEGs related to the TNF signaling pathway, *TNFAIP3* expression was most significantly downregulated in response to plantaricin BM-1 treatment. The TNFAIP3 protein disrupts the recruitment of death domain signaling molecules TRADD and RIP to receptor signaling complexes and protects cells from TNF-induced apoptosis (Martens and van Loo, 2020). In addition, the knockdown of *TNFAIP3* promoted TNF-induced apoptosis (Priem et al., 2019; Mulla and Venkatraman, 2022). As shown in previous studies, aspirin can induce apoptosis of CRC cells by degrading NFKBIA, leading to nuclear translocation of the NF-κB complex and activation of the NF-κB pathway (Prescott and Cook, 2018). Similarly, the expression of NFKBIA was significantly reduced in our study, and KEGG enrichment analysis revealed changes in the NF-κB signaling pathway. This indicates that plantaricin BM-1 may also induce apoptosis in SW480 cells by activating the NF-κB signaling pathway. The MAPK signaling pathway plays a regulatory role in cell survival, growth, and apoptosis (Yue and López, 2020). Asterosaponins induced apoptosis by inactivating the ERK1/2 MAPK pathway and downregulating *MYC* (Thao et al., 2014). Indeed, significant downregulation of *MYC* and alterations in MAPK signaling pathways were observed in our results. miRNAs play an important role in promoting or inhibiting cancer cell proliferation and apoptosis by regulating oncogenes or tumor suppressor genes (Zhi et al., 2018). Oncogene PIM-1 is usually overexpressed in colon cancer (Zhang et al., 2010). Knockdown or downregulation of PIM-1 was shown to inhibit tumor proliferation and induce mitochondria-mediated apoptosis by activating caspase-9 (Weirauch et al., 2013; Rathi et al., 2021). Our transcriptomic results also revealed a decrease in the *PIM1* gene expression. These results provide evidence that plantaricin BM-1 activates multiple pathways to induce apoptosis.

5. Conclusion

In this study, we demonstrated for the first time that plantaricin BM-1 shows no cytotoxicity against the normal colonic epithelial NCM460 cell line, but inhibits SW480 CRC cell proliferation. The IC_{50} was 757.9 μg/ml. Besides, plantaricin BM-1 induces apoptosis through the caspase-3 pathway *in vitro*. Furthermore, using transcriptomic and bioinformatic analyses, we explored possible mechanisms underlying the cytotoxic effects of this bacteriocin. We found that 65 genes were downregulated and four were upregulated. KEGG analysis showed that the dysregulation of the TNF, NF-kB, and MAPK signaling pathways, altered levels of cancer-related miRNAs, and changes in the expression of genes mediating transcriptional

misregulation in cancer were likely involved in the biological mechanism by which plantaricin BM-1 induced apoptosis in SW480 cells. This spectrum of biological activity shows that plantaricin BM-1 should be studied further as a promising anti-CRC compound.

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: NCBI, https://www.ncbi.nlm.nih.gov/sra/PRJNA910522.

Author contributions

YX and HW: conceptualization and methodology. HW and ZB: software. HW and JZ: validation. JJ, XP, and YH: resources. YX: data curation and writing—review and editing. HW: writing—original draft preparation. HZ and YX: supervision. 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/fmicb.2022.1103600/full#supplementary-material

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Improvement of halitosis by probiotic bacterium *Weissella cibaria* CMU: A randomized controlled trial

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Several *in vitro* and *in vivo* studies have evaluated the effect of probiotics on oral health; however, human clinical studies are still limited. Therefore, this study aimed to examine the effects of *Weissella cibaria* Chonnam Medical University (CMU)-containing tablets on halitosis. This randomized, double-blinded, placebo-controlled study included 100 adults with halitosis (age, 20–70years). The participants were randomly assigned to the test group (n=50) and control group (n=50). One tablet [1×10^8 colony forming units (CFU)/tablet] was to be taken each day over 8weeks. The concentrations of volatile sulfur compounds (VSCs), bad breath improvement scores, and oral colonization of *W. cibaria* were measured. Psychosocial indicators including depression, self-esteem, oral health-related quality of life, and subjective oral health status were evaluated. Most variables were assessed at baseline, 4, and 8weeks, and *W. cibaria* number and safety variables were assessed at baseline and 8weeks. Intergroup comparisons were carried out using Student's t-test, Chi-square test, or Fisher's exact test on per-protocol analysis. Intragroup differences before and after intake were analyzed using the linear mixed-effect model (LMM). Per-protocol analysis was carried out in the test group (n=45) and control group (n=46). Total VSC was significantly lower in the probiotics group than in the placebo group at baseline (week 0, p=0.046) and at 8weeks (p=0.017). The sum of hydrogen sulfide and methyl mercaptan did not differ significantly between the groups at baseline; however, it was significantly lower in the probiotics group than in the placebo group at week 8 (p=0.012). Bad breath improvement (BBI) scores were significantly reduced at week 8 (p=0.006) in the probiotics group. Statistically significant intergroup differences were observed for changes in the level of *W. cibaria* at week 8 (p<0.001). Psychological indicators significantly improved from baseline to week 8 in the probiotics group. No safety issues were observed in either group. The levels of *W. cibaria* was higher in patients with halitosis using *W. cibaria* CMU-containing tablets. The subjective degree of bad breath and psychological indicators were improved in patients with halitosis using *W. cibaria* CMU-containing tablets.

KEYWORDS

halitosis, probiotics, clinical study, safety, *Weissella cibaria*

1. Introduction

Halitosis refers to the bad oral breath during exhalation; it originates from the mouth, nasal cavity, upper respiratory tract, and upper digestive tract. It is generally a smell that makes others feel unpleasant (Scully and Felix, 2005). According to the recent international consensus (Seemann et al., 2014), halitosis is classified into genuine halitosis and pseudo-halitosis. Genuine halitosis is defined

as an overt malodor, with an intensity exceeding socially acceptable levels, whereas pseudo-halitosis refers to a case that is not detected as malodor by the clinician wherein the patient claims to be suffering from halitosis despite unable to provide reliable evidence (Murata et al., 2002). Genuine halitosis is subdivided into intra-oral halitosis and extra-oral halitosis according to the cause (Seemann et al., 2014). The causes of bad breath in the oral cavity are poor oral hygiene, periodontal disease, coated tongue, food impaction in the interdental sites, unsanitary dentures, and inappropriate prostheses (Ortiz and Filippi, 2021). Bad breath is usually caused by volatile sulfur compounds (VSCs), namely hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and dimethyl sulfide [$(CH_3)_2S$] (Tonzetich, 1977; Rosenberg, 1990). VSCs are mainly produced by the degradation of anaerobic bacteria in the oral cavity using proteins (L-cysteine, L-methionine containing sulfur) contained in gingival fissures, saliva, and food residues as substrates (Nakano et al., 2002a). *Treponema denticola*, *Prevotella intermedia*, *Prevotella loescheii*, *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* produces significant amounts of H_2S and CH_3SH in human serum (Persson et al., 1990).

Many recent studies have reported alternative ways to eliminate bad breath without altering the normal flora, including the use of oral probiotics (Petti et al., 2008) or interventions involving *Lactobacillus* (Keller et al., 2012; Teughels et al., 2013; Penala et al., 2016). Probiotics are believed to act through various mechanisms such as competitive inhibition of attachment and growth of pathogens, lowering of environmental pH levels, synthesis of antimicrobial substances, modulation of local and systemic immune responses, and direct antimicrobial effects (Fooks and Gibson, 2002). Specific strains of the genera *Lactobacillus, Streptococcus*, and *Weissella* are some of the most helpful probiotics used in the treatment or prevention of halitosis (Karbalaei et al., 2021). *Weissella cibaria* is a short-rod shaped gram-positive lactic acid bacterium (Bjorkroth et al., 2002). It is a dominant species in fermented foods, such as kimchi (Kwak et al., 2014). *W. cibaria* inhibits oral pathogens (Kang et al., 2006) and suppresses volatile VSCs (Lee et al., 2020). Kang et al. reported that *Fusobacterium nucleatum* could not produce VSCs in the presence of *W. cibaria* Chonnam Medical University (CMU) because the growth of *F. nucleatum* was inhibited by the hydrogen peroxide produced by *W. cibaria* (Kang et al., 2006).

Many *in vitro* and *in vivo* studies have evaluated the efficacy of probiotics in oral health; however, clinical studies on humans are still lacking. The objective of this study was to evaluate the impact of probiotic *W. cibaria* CMU-containing tablets on reducing the production of VSCs, bad breath improvement scores, and increasing *W. cibaria* oral colonization.

2. Materials and methods

2.1. Ethical considerations

This study was performed in accordance with the Declaration of Helsinki and Consolidated Standards of Reporting Trials (2010). Approval for the study was obtained from the Institutional Review Board of Seoul National University Dental Hospital (approval no. CRI19008) and registered in https://trialsearch.who.int (KCT0004291) on September 10, 2019. The participants were informed about the purpose and procedure of the study and that refusal to participate would not disadvantage them in any way. Written informed consent was obtained from all the participants prior to enrolment.

2.2. Study participants

According to a previous clinical research (Lee et al., 2020), the difference (mean ± standard deviation) in the improvement of bad breath between the test group and the control group was considered the primary outcome. The number of participants required for the independent t-test with significance level $\alpha = 0.05$, bilateral test, power = 0.8, was 80. The initial sample size was planned as 40 in each group, considering a dropout rate of 20%, and 100 participants were enrolled in the current study. Using a computer-generated random list, random allocation sequences were generated for the placebo (control) and probiotics (test) groups. The participants were assigned to the placebo and probiotics groups in a 1:1 manner by block randomization, and we ensured that the male-to-female ratio was similar in both groups as much as possible.

The participants for this study were recruited from the Department of Periodontology, Seoul National University Dental Hospital. Participants were included if they: were able to comply with the protocol, were aged 20–70 years with >20 natural teeth, had no tongue impediment, such as glossitis or tongue cancer, had no severe periodontal disease when periodontal treatment, antibiotics, or tooth extraction are required during oral examination as soon as possible, and had total VSC concentration of 1.5 ng/10 ml or higher. Recruited patients should maintain routine oral hygiene, but periodontal treatment, oral hygiene treatment, and using oral products other than the provided toothbrush and toothpaste were prohibited. Exclusion criteria were as follows: presence of systemic diseases such as digestive disease, kidney disease, Sjogren's syndrome, rheumatism, sinusitis or rhinitis, chronic gastritis, dry mouth, diabetes mellitus, uncontrolled hypertension (SBP 160 mmHg or DBP ≥ 100 mmHg), or allergies to lactose. Participants with compliance less than 80%, and individuals taking *Lactobacillus*-containing food, probiotic supplements, or medicine that could affect the outcomes within 1 week of their visit were dropped from the study. Those who received oral hygiene or periodontal treatment during the test period were also dropped out. Consumption of garlic, onions, green onions, and chives, which may affect bad breath, was prohibited the day before the visit. In addition, a smoking ban was provided from the start of the test to the end of the test. If the participants did not observe the above, the participants would be dropped out.

The assessments were conducted at Seoul National University Dental Hospital at baseline, 4, and 8 weeks. Participants and investigators were blinded to the intervention. In order to maintain double-blindness, envelopes containing tablets with a unique code of each assigned group were sealed and delivered to the research director. The allocations were not disclosed to the director until the end of the human application study except for cases of serious adverse events. Information related to the allocation of participants and distribution of tablets was managed by a third party who were not directly involved in the present study.

A total of 105 participants were screened, 5 participants who did not meet the inclusion criteria or refused to participate during the 2-week run-in period were excluded. Therefore, 100 were randomly assigned to the placebo group ($n = 50$) or the probiotics group ($n = 50$). Nine additional participants were excluded from the 8-week intervention

phase, and finally, 91 participants were included in the final analysis (Figure 1).

2.3. Study treatment

Each 800-mg probiotic tablet contained 1.0×10^8 colony forming units (CFU)/tablet of *W. cibaria* CMU (oraCMU®; OraPharm Inc., Seoul, Republic of Korea). This strain is used as a food supplement in Korea. Other ingredients included isomalt, sucralose, peppermint-flavored powder, maltodextrin, magnesium stearate. The placebo was a tablet with a similar taste, texture, and appearance, but without *W. cibaria* CMU. It was obtained from the same manufacturer, and contained isomalt, sucralose, peppermint flavor, maltodextrin, and magnesium stearate. The participants were instructed to chew on one tablet every night before bedtime, after brushing their teeth. Participants were not allowed to consume water and food after the treatment. The intervention period lasted for 8 weeks.

2.4. Study design and protocol

This was a randomized, double-blind, placebo-controlled trial. The probiotic tablet was administered to the test group and a placebo tablet of the same shape was administered to the control group. The participants were interviewed about their dietary habits and oral health problems at two visits (4 and 8 weeks) to assess the compliance, potential side effects, dietary lifestyle survey, vital signs, whether taking forbidden drugs or health functional food or not, and measurement of halitosis. Participants were instructed to bring the remaining test food or control food at visit 3 (4 weeks) and visit 4 (8 weeks) after ingestion, and the remaining amount of test food or control food was reconfirmed. Compliance was calculated as follows.

$$Compliance = \frac{Number\ of\ test\ foods\ (or\ control\ foods)\ actually\ consumed}{Number\ of\ test\ foods\ (or\ control\ foods)\ to\ be\ consumed} \times 100$$

In principle, from the day the test food or control food was distributed to the day before this visit, the remaining food was returned to the researcher. Compliance was considered if the consumption rate was 80% or higher. All interventions took place in a double-blind manner; the participants were identified only by their registration number, and the intervention providers did not know who was in the test or control group. The entire study process lasted from September 06, 2019 to March 2020.

2.5. Measurement of halitosis

Halitosis was measured before intake and 4 and 8 weeks after intake using the concentration of VSCs and bad breath improvement (BBI) scores. The levels of VSCs were measured by Oral Chroma (CHM-2; FIS, Inc., Hyogo, Japan). The participants were instructed to refrain from talking for 3 min before the measurements and close the mouth for 30 s with a gastight syringe in the mouth. Thereafter, the examiner aspirated 1 ml of mouth air from the participant and injected it into Oral Chroma to measure the VSC concentration. The VSC analysis included the total VSC, which is sum of H_2S, CH_3SH, and dimethyl sulfide [$(CH_3)_2S$], and sum of hydrogen sulfide and methyl mercaptan. The concentration of VSC was measured on the morning upon following the guidelines for the study participants, including the instructions to refrain from eating after they had brushed their teeth in the evening until the next morning. The VSC analyzes were conducted according to a previous study (Kang et al., 2006). BBI scores were determined by self-estimation of the oral odor on a scale of 1–5 (Rosenberg et al., 1995): Score 1 - overall, symptoms improved significantly (very good), score 2 - overall, symptoms improved (excellent), score 3 - there is no difference from before intake (unchanged), score 4 - overall, symptoms worsened (exacerbated), and score 5 - overall, symptoms worsened significantly (extremely worse).

2.6. Quantitative analysis *Weissella cibaria*

The amount of *W. cibaria* was examined by quantitative PCR (qPCR). For this process, the middle part of the tongue was rubbed five times (Lee et al., 2020) with a cotton swab (iClean Swab; Biofact, Daejeon, Korea). Then, bacterial genomic DNA was extracted from the swab using a genomic DNA extraction kit (Biofact), according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed in a total volume of 10 µl using a Gene Probe PCR kit (Qiagen, Hilden, Germany), which contained 2 µl of the template, 200 nM of the primers, and 100 nM of the probe. The qPCR conditions were as follows: denaturation at 95°C for 3 min, followed by 45 cycles at 95°C for 3 s and 58°C for 10 s. qPCR was performed using a Rotor Gene Q system (Qiagen). The sequences of the primers and dual-labeled probe used for *W. cibaria* were as follows: forward, 5-GTGAAAGCCCTCA GCTCAAC-3; reverse, 5-CTACGCATTTCACCGCTACA-3 and 5-FAM-TGGAAACTGGATGACTTGAGTGCA-BHQ-3′. The number of bacterial cells per sample was calculated from a standard curve constructed using the diluted genomic DNA from *W. cibaria*.

2.7. Assessment of psychosocial health

A questionnaire on the depression, self-esteem, oral health-related quality of life, and subjective oral health status was administered to the participants to evaluate the social and psychological health indicators according to a previous study (Lee et al., 2021). Depression was assessed using the Center for Epidemiologic Studies Depression Scale (Radloff, 1977; Chon et al., 2001), which consisted of 20 items. The 20 questions are rated on a four-point Likert scale; higher the total score, greater the depression. Self-esteem was evaluated using an instrument developed by Rosenberg, which consists of 10 items (Rosenberg, 1965). Each item was rated on a five-point Likert scale, with a higher total score indicating greater self-esteem. Oral-health-related quality of life was measured using the shortened version of the Oral Health Impact Profile (Slade and Spencer, 1994), which consists of 14 items. Each item was rated on a five-point Likert scale, with a higher total score indicatinggreater oral-health-related quality of life. Subjective oral-health status was evaluated using an instrument (Park, 2010), which consists of 10 items. Each item is rated on a five-point Likert scale, with a higher total score indicating poor oral-health status.

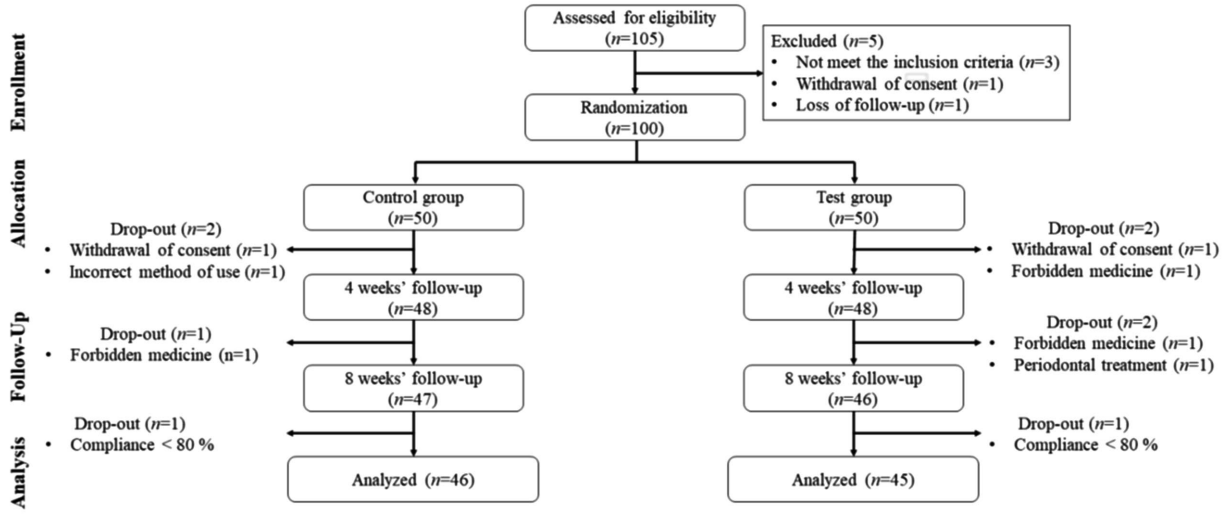

FIGURE 1
Flow chart of the study design.

2.8. Safety evaluation

The safety of this human clinical study was evaluated by monitoring the adverse events (AEs), vital signs, hematological findings (Hoffman and Monroe 3rd, 2001), indicators of liver function (Giovannini et al., 2008), kidney function, and electrolyte balance (Moreira et al., 2015) according to a previous study (Lee et al., 2020). AEs were monitored through interviews and self-report. In the event of an AE, based on the onset and disappearance of symptoms and signs as well as response actions were recorded, the event would be classified as either an AE or severe adverse event (SAE). In addition, blood samples were collected by nurses to determine the hematologic parameters and blood chemistry findings. All monitoring tests were performed at baseline and after 8 weeks.

2.9. Statistical analysis

A "per-protocol (PP)" analysis was performed on participants who completed the trial and whose compliance was ≥80%. Data with non-normal distribution were analyzed after being converted to ensure normal distribution. Intergroup comparisons of participant characteristics at baseline were carried out by Student's *t*-test for continuous variables and Chi-square test or Fisher's exact test for categorical variables. The compliance rates of the groups were compared using the Student's *t*-test. Intergroup comparisons of improvements in bad breath at 4 and 8 weeks were carried out using Student's *t*-test. Intergroup differences according to the intake period and intragroup differences before and after intake were analyzed using the linear mixed-effect model (LMM), with group, time, and interaction between group and time (group*week) included as random and fixed effects. Variables with significant differences between the groups (total VSC concentration and alcohol consumption) were corrected. The microbial index was analyzed by converting it to a $\log_{10}$ DNA copy value. For vital signs and hematological findings, differences between groups according to the intake period and differences within groups before and after intake were analyzed using the LMM. Adverse reactions were described in terms of the number of occurrences, type, symptom severity, and reaction to the test food by group and were compared between groups using the chi-square test or Fisher's exact test. Statistical analysis was performed using SAS version 9.4, and a two-sided test was performed, and results with a *value of p* ≤0.05 was considered significant.

3. Results

3.1. Study population

A total of 100 out of 105 screened patients were recruited and included in the study. Fifty patients each were allocated to either the control or test group. The test group (probiotics, $n = 45$) and control group (placebo, $n = 46$) underwent a per-protocol analysis. A detailed description on patient recruitment is presented in Table 1.

No significant differences were found in any of parameters except total VSC ($p = 0.046$) and alcohol consumption ($p = 0.043$) between the probiotics and placebo groups. Total compliance for 8 weeks was 96% or higher in both groups, with no significant difference.

3.2. Measurement of halitosis

Halitosis was measured using the total VSC, sum of H_2S and CH_3SH levels, and BBI score. The measurements of the total VSC and the sum of H_2S and CH_3SH are presented in Figure 2 and Table 2. Total VSC was significantly lower in the probiotics group compared to the placebo group at baseline (week 0, $p = 0.046$) and at 8 weeks ($p = 0.017$). As shown in Table 2, the sum of H_2S and CH_3SH did not differ significantly between the groups at baseline; however, it was significantly lower in the probiotics group than in the placebo group at week 8 ($p = 0.012$). Analysis of the group differences according to the intake period with LMM showed no significant differences in any of the indicators. The BBI scores are presented in Figure 3 and Supplementary Table S1. There was no significant difference between the groups in the mean BBI scores at week 4 and changes in BBI scores from week 0 to week 4, but there

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was a significant difference at week 8 ($p = 0.006$). The placebo group showed an increase in the BBI scores, and the probiotics group showed a decrease in the BBI scores.

TABLE 1 Baseline characteristics of the subject in the placebo and probiotic groups.

Variables	Placebo	Probiotic	Value of $p^{\dagger}$
Age (year)	51.3 ± 1.3	52.1 ± 1.2	0.655
Gender (male/female)	15/31	13/32	0.701
Body weight (kg)	63.2 ± 1.7	63.5 ± 1.7	0.898
Total volatile sulfur compound (ng/10 ml)	15.1 ± 3.1	8.2 ± 1.4	0.046
Alcohol drinker (yes/no)	21/25	12/33	0.060
Alcohol amount (abstention/less than 1 bottle/1–3 bottles/more than 4 bottles, per week)	25/17/4/0	33/7/3/2	0.043
Smoker (yes/no)	3/43	0/45	0.242
Smoking amount (cigarettes/day)	0.8 ± 0.5	0.0 ± 0.0	0.128
Compliance (week 4)	96.1 ± 0.8	96.8 ± 0.8	0.552
Compliance (week 8)	96.8 ± 0.8	97.7 ± 0.8	0.432

Values are mean ± SE (all such values). †Student's *t*-test for continuous variables and Chi-square or Fisher's exact test for categorical variables were used to compare the difference between the groups.

3.3. Quantitative analysis of *Weissella cibaria*

Quantitative values detected in the tongue at each time point were analyzed to confirm the oral colonization of *W. cibaria*. Quantitative values were analyzed by conversion to $\log_{10}$ DNA copy values (Figure 4). Table 3 shows the results of the comparative analysis and statistical significance between the test and placebo groups. No statistically significant differences in the levels of *W. cibaria* were found between the groups at week 0 ($p = 0.619$). However, at week 8, the levels of *W. cibaria* were significantly higher in the probiotics group than in the placebo group ($p < 0.001$). There was a significant difference between the groups in terms of the changes in the proportion *W. cibaria* from baseline to 8 weeks ($p < 0.001$).

3.4. Evaluation of psychosocial health

As shown in Supplementary Table S2, depression ($p = 0.049$), oral health-related quality of life ($p = 0.001$), and subjective oral health status ($p = 0.007$) significantly improved from baseline to week 8 in the probiotics group. However, there were no significant differences between the groups at each visit (Student's *t*-test) and according to the intake period (LMM) for all indicators.

3.5. Safety evaluation

Vital signs, hematological findings, blood chemistry results, and all AEs were monitored to evaluate the safety of this clinical study (Supplementary Tables S3–S6). During the study period, some of these safety variables showed significant intragroup differences; however, there were no clinically significant changes in any of the indicators. In addition, no SAEs occurred, the symptoms of the AEs were mild, and no reaction to the test food was reported (Supplementary Table S6).

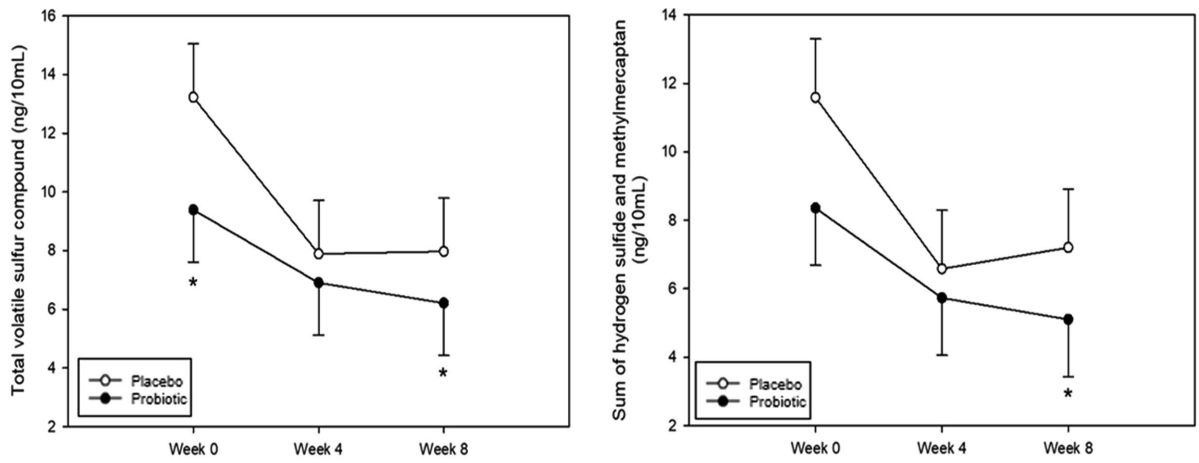

FIGURE 2
Analysis of volatile sulfur compounds. **(A)** Total volatile sulfur compound (VSC). Total VSC was significantly lower in the probiotics group than in the placebo group at baseline (week 0, p=0.046) and at week 8 (p=0.017). **(B)** Sum of hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH). The sum of H_2S and CH_3SH levels was significantly lower in the probiotic group than in the placebo group at week 8 (p=0.012). Each line represents the least squares mean (LSmean)±standard error (SE). *Value of $p \leq 0.05$, Student's *t*-test.

TABLE 2 Total volatile sulfur compound and sum of H_2S and CH_3SH at 0(baseline), 4, and 8weeks (unit: ng/10ml).

Variables	Placebo	Probiotic	Value of $p^{\dagger}$	Value of $p^{\ddagger}$
Total VSCs				
Week 0	13.23 ± 1.82	9.39 ± 1.78	0.046	
Week 4	7.89 ± 1.82	6.90 ± 1.78	0.130	
Week 8	7.97 ± 1.82	6.21 ± 1.78	0.017	0.642
value of $p^{\S}$	0.018	0.153		
Sum of hydrogen sulfide and methyl mercaptan				
Week 0	11.59 ± 1.71	8.36 ± 1.68	0.057	
Week 4	6.58 ± 1.71	5.73 ± 1.68	0.156	
Week 8	7.20 ± 1.71	5.10 ± 1.68	0.012	0.703
value of $p^{\S}$	0.029	0.108		

Values are LSmean ± SE (all such values). †Student's *t*-test was used to compare differences between the groups. ‡Linear mixed-effect model adjusted with total volatile sulfur compound, alcohol drinker and alcohol amount at baseline was used to analyze the effects of group* week. §Linear mixed-effect model adjusted with total volatile sulfur compound, alcohol drinker and alcohol amount at baseline was used to analyze the difference within each group.

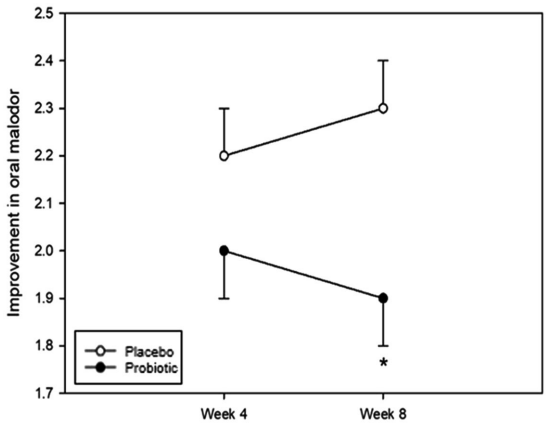

FIGURE 3
Analysis of bad breath improvement (BBI) scores. The BBI score was not significantly different between the groups at week 4; however, there was a significant difference at week 8 (p=0.006). Each line represents the LSmean ± SE. *Value of $p \leq 0.05$, Student's *t*-test.

There were no significant differences between the groups in terms of the occurrence, type, symptom severity, or reaction to the test food.

4. Discussion

This study aimed to evaluate the effect of the intake of an oral probiotic containing of *W. cibaria* on patients with halitosis. The results of this clinical trial are meaningful since they supported the effect of *W. cibaria* on the improvement of halitosis.

The effects of probiotic agents on halitosis have been demonstrated using *Lactobacillus* strains in several probiotics studies (Keller et al., 2012; Suzuki et al., 2014; Penala et al., 2016). Lactic acid-producing bacteria, such as *Lactobacillus acidophilus* and *Lactobacillus casei*, have usually been chosen because of their inhibitory effect on anaerobic bacterial proliferation *via* the production of strong acids. However, this strong acid can

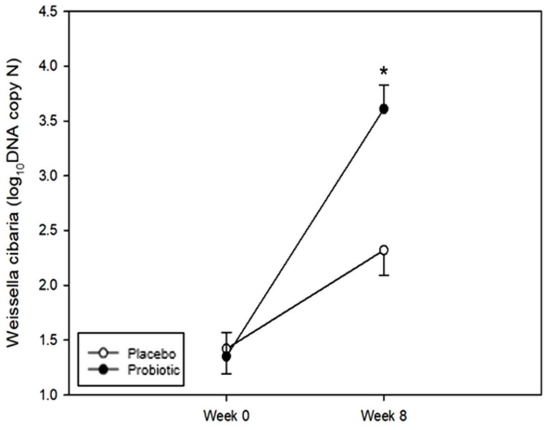

FIGURE 4
Changes in *Weissella cibaria*. The levels of *W. cibaria* were not significantly different between the groups at week 0 (p=0.619). The levels of *W. cibaria* were significantly higher in the probiotic group than in the placebo group at week 8 (p<0.001). Each line represents the LSmean ± SE. *Value of $p \leq 0.05$, Student's *t*-test.

TABLE 3 Number of *Weissella cibaria* measured at 0 and 8weeks (unit: log_{10}DNA copy N).

Week	Placebo	Probiotic	Value of $p^{\dagger}$	Value of $p^{\ddagger}$
Week 0	1.42 ± 0.23	1.35 ± 0.22	0.619	
Week 8	2.32 ± 0.23	3.61 ± 0.22	<0.001	<0.001
Value of $p^{\S}$	<0.001	<0.001		

Values are LSmean ± SE (all such values). †Student's *t*-test was used to compare differences between the groups. ‡Linear mixed-effect model adjusted with total volatile sulfur compound, alcohol drinker and alcohol amount at baseline was used to analyze the effects of group*week. §Linear mixed-effect model adjusted with total volatile sulfur compound, alcohol drinker and alcohol amount at baseline was used to analyze the difference within each group.

be neutralized by the buffering function of saliva in the oral cavity of a healthy person and has the potential to induce dental caries (Babaahmady et al., 1998). In contrast, *W. cibaria* can prevent dental caries because it inhibits biofilm formation by *Streptococcus mutans* (Jang et al., 2016) and has a higher ecological pH than Lactobacilli strains (Kang et al., 2006). In light of these results, a randomized controlled clinical trial on the effect of *W. cibaria* CMU-containing probiotics on halitosis was conducted in this study. The total VSCs and the sum of H_2S and CH_3SH emitted from the mouth were measured. Usually, the concentration of VSCs is used as an indicator of halitosis severity (Rosenberg et al., 1991). The most common VSC is H_2S from the back of the tongue (Washio et al., 2005), methyl mercaptans in the case of periodontal disease (Nakano et al., 2002b), and dimethyl sulfide, which originates extra-orally in the gut (Tangerman and Winkel, 2010). In our study, total VSC and sum of H_2S and CH_3SH showed similar patterns. Therefore, even if only one of the two aspects is measured, halitosis would be sufficiently evaluated in a clinical study. *W. cibaria* CMU-containing tablets significantly reduced the total VSC and the sum of H_2S and CH_3SH levels after week 8, and the number of *W. cibaria* in the mouth increased with a corresponding reduction in the severity of halitosis. These results are similar to those of previous studies where *W. cibaria* led to a reduction in the levels of H_2S and CH_3SH (Kang et al., 2006; Jang et al., 2016; Do et al., 2019). The results of our study also corroborate the findings from previous studies which showed that H_2S and CH_3SH concentrations decreased when participants gargled using mouthwash containing

W. cibaria (Kang et al., 2006). Despite the differences in food type and treatment duration, both results showed an improvement in halitosis.

The decrease in total VSC and the sum of H_2S and CH_3SH over 8 weeks could be explained by the increase in the proportion of *W. cibaria*. Some gram-negative bacteria including *F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *T. denticola* have been found to cause bad breath (Salako and Philip, 2011; Ouhara et al., 2015). These bacteria produce sulfur-containing compounds, such as H_2S, by decomposing cysteine and methionine (Persson et al., 1990). The antibacterial effect of *W. cibaria* strains against representative oral bacteria, including *Aggregatibacter actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *Tannerella forsythia*, and *T. denticola*, has been reported in a previous study (Lim et al., 2018; Do et al., 2019). Among them, *F. nucleatum* serves as a bridge organism for other bacteria to engage in cohesion and coaggregation and can help them inhabit the oral cavity (Kolenbrander, 2000); *F. nucleatum* survives in the oral cavity because saliva cannot easily remove it (Jang et al., 2016). However, the proportion of *F. nucleatum* was found to decrease upon exposure to *W. cibaria* strains (Kang et al., 2006), and *W. cibaria* strains have been reported to strongly coaggregate with *F. nucleatum* and efficiently adhere to epithelial cells (Kang et al., 2005). In addition, *W. cibaria* produces higher levels of hydrogen peroxide, a representative antibacterial substance, than other *lactobacilli* (Jang et al., 2016). Hydrogen peroxide has been reported to alter the bacterial community in the oral cavity and inhibit the growth of *F. nucleatum* (Kang et al., 2006). The proportion of *F. nucleatum* could be significantly reduced in the oral cavity owing to the co-aggregation with *W. cibaria*, eliminating pathogenic bacteria thereby preventing VSC production (Kang et al., 2020).

Halitosis can be measured using an organoleptic method or gas chromatography analysis (Murata et al., 2002). In this study, the organoleptic method was not used to determined halitosis. The organoleptic method is practical and commonly used to measure bad breath; however, it has the limitation of less objectivity and low reproducibility since it is performed by directly sniffing the patient's breath (Oho et al., 2001). Halitosis was measured by using the critical discrimination value as determined using gas chromatography based on a previous study that demonstrated the measurement of halitosis determined using gas chromatography to be correlated with the results of the organoleptic measurement (Murata et al., 2002).

The BBI scores had improved in the probiotics group than in the control group, similar to the results of another study (Lee et al., 2020). Bad breath is one of the common discomforts affecting many adults in modern society. Self-perceived bad breath is a factor that hinders the quality of life, owing to psychological discomfort, poor social ability, and social isolation (Bornstein et al., 2009; Kato et al., 2019). In the present study, it was shown that depression, oral health-related quality of life, and subjective oral health status had significantly improved in the probiotics group after 8 weeks; however, careful analysis is needed because we used subjective measures. For the safety evaluation of *W. cibaria* CMU-containing tablets, vital signs, hematology parameters, blood chemistry findings, and AEs were monitored. No significant problems were observed in the blood counts, liver function indicators, and renal function indicators, and no AEs were reported, suggesting that *W. cibaria* CMU-containing tablets could be safe for clinical application.

The purpose of this study was to evaluate whether *W. cibaria* CMU was settled in the test group, in which *W. cibaria* CMU-containing tablets were provided to the patients. The number of *W. cibaria* increased both in the test group and the control group in this study. However, a statistically significant difference between the two group, specifically increasing in the test group, was confirmed. For the measurement of *W. cibaria*, qPCR was quantified using *W. cibaria*-specific primer; however, whole genome sequencing was impossible. An increasing trend of *W. cibaria* in the control group may be explained by the fact that the patients who originally had *W. cibaria* showed it's detected.

However, this study has a few limitations. First, the changes in the proportions of halitosis-inducing bacteria were not assessed. *W. cibaria* CMU has been known to inhibit the growth of VSC-producing bacteria by producing high amounts of hydrogen peroxide (Lim et al., 2018; Do et al., 2019). Many other studies based on *W. cibaria* CMU showed VSC reduction; however, this study did not show a statistically significant reduction in VSC in the probiotic group. Further studies are warranted to determine whether *W. cibaria* CMU significantly inhibits VSC-producing bacteria by assessing the amount of the bacteria. Second, despite randomization, it was difficult to find statistically significant differences between the groups because the concentrations of VSCs in the control and test groups were slightly different at baseline. Since total VSC and the sum of hydrogen sulfide and methylmercaptan were significantly higher in the control group from baseline, there seems to be a trend toward improvement in the control group over time. Further studies should be conducted with patients with similar baseline data to confirm the extensive clinical effects of *W. cibaria* on halitosis and mode of action of *W. cibaria* using next-generation sequencing technology.

The levels of *W. cibaria*, which, is known to led to a reduction in the levels of VSCs, is higher in patients with halitosis using *W. cibaria* CMU-containing tablets. The subjective degree of bad breath and psychological indicators were improved in patients with halitosis using *W. cibaria* CMU-containing tablets. *W. cibaria* CMU-containing tablets may be considered an adjunctive treatment for halitosis.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of Seoul National University Dental Hospital (approval no. CRI19008). The patients/participants provided their written informed consent to participate in this study.

Author contributions

H-sH and HY performed patient check-up, recorded clinical parameters, collected the samples, and analyzed the data. H-sH wrote the manuscript. Y-DC and SK supervised the research and revised the manuscript and corresponded. 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/fmicb.2023.1108762/full#supplementary-material

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Comprehensive analysis of metabolites produced by co-cultivation of *Bifidobacterium breve* MCC1274 with human iPS-derived intestinal epithelial cells

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Examining how host cells affect metabolic behaviors of probiotics is pivotal to better understand the mechanisms underlying the probiotic efficacy *in vivo*. However, studies to elucidate the interaction between probiotics and host cells, such as intestinal epithelial cells, remain limited. Therefore, in this study, we performed a comprehensive metabolome analysis of a co-culture containing *Bifidobacterium breve* MCC1274 and induced pluripotent stem cells (iPS)-derived small intestinal-like cells. In the co-culture, we observed a significant increase in several amino acid metabolites, including indole-3-lactic acid (ILA) and phenyllactic acid (PLA). In accordance with the metabolic shift, the expression of genes involved in ILA synthesis, such as transaminase and tryptophan synthesis-related genes, was also elevated in *B. breve* MCC1274 cells. ILA production was enhanced in the presence of purines, which were possibly produced by intestinal epithelial cells (IECs). These findings suggest a synergistic action of probiotics and IECs, which may represent a molecular basis of host-probiotic interaction *in vivo*.

KEYWORDS

***Bifidobacterium*, intestinal epithelial cells, metabolite, co-culture, host–microbe interaction, indole-3-lactic acid**

1. Introduction

Bifidobacteria are one of the major bacterial genera constituting the human gut microbiota, and several strains have been industrially used as probiotics (Turroni et al., 2011; Alessandri et al., 2021). Bifidobacteria produce various beneficial metabolites such as acetic and lactic acids by utilizing hexose sugars (Suzuki et al., 2010). These metabolites contribute to maintaining intestinal barrier function and enhancing immune function (Fukuda et al., 2011; Lee et al., 2018; Morita et al., 2019). Some bifidobacterial species possess a metabolic pathway for synthesizing aromatic lactic acids (ALAs) from dietary aromatic amino acids (Sakurai et al., 2019; Laursen

et al., 2021; Sakurai et al., 2021). An example of an aromatic lactic acid is indole-3-lactic acid (ILA), which exerts anti-inflammatory activity *via* the aryl hydrocarbon receptor (AhR) (Ehrlich et al., 2020; Meng et al., 2020). In addition to the above metabolites, certain bifidobacteria produce vitamins and conjugated linoleic acid (Coakley et al., 2003; Sugahara et al., 2015; Mei et al., 2022), which can act as signaling molecules and affect host physiology.

Since probiotics consumed orally exert its effects only transiently in the colon, it remains challenging to investigate the *in vivo* metabolic behavior of bifidobacteria when taken as probiotics due to the complexity of the gut environment, including the interaction of probiotics with host-gut microbes. Thus, an *in vitro* co-culture system capable of mimicking the gut environment can help to determine the metabolites produced by gut microbes in the human intestinal tract. Recently, several culture systems have been developed to address the difference in oxygen demand between the host and bacteria using a static two-layer culture system and microfluidic devices (Shah et al., 2016; Jalili-Firoozinezhad et al., 2019; Shin et al., 2019; Kwon et al., 2022). In addition to these culture techniques, attempts have been made to combine intestinal organoids to create an environment similar to that of the gastrointestinal tract (Sasaki et al., 2020; Gazzaniga et al., 2021; Puschhof et al., 2021; Zhang et al., 2021). Sasaki et al. reported a successful co-culture of colonic organoid-derived monolayers with some anaerobic bacteria, including *Bifidobacterium* species. While these new technologies are still in their infancy, they could advance research on host–bifidobacterial interactions.

Orally supplemented probiotics pass through the small intestine where they interact with intestinal epithelial cells (IECs) and affect the small intestine environment. Some probiotic bifidobacteria were detected in terminal ileum with colony-forming units (Pochart et al., 1992; Takada et al., 2020). Microbes in the small intestine play an important role in host physiology, including metabolism, immunity, and gastrointestinal motility (Saffouri et al., 2019; Kastl et al., 2020; Vonaesch et al., 2022). These reports highlight the importance of understanding the metabolic behavior of probiotics in the small intestine.

In this study, we used a two-chamber co-culture device to evaluate the interaction between *B. breve* MCC1274, which has been reported to improve cognitive function in patients with suspected mild cognitive impairment (Xiao et al., 2020; Asaoka et al., 2022), and induced pluripotent stem cells (iPS)-derived small intestinal-like cells to construct a small intestinal tract-like environment *in vitro*. The aim of this study was to determine how a co-culture of human IECs with the probiotic *B. breve* MCC1274 alters their metabolic behavior.

Abbreviations: AAAs, aromatic amino acids; ALAs, aromatic lactic acids; AhR, Aryl hydrocarbon receptor; CE-FTMS, capillary electrophoresis Fourier-transform mass spectrometry; CFU, colony-forming units; DDBJ, DNA Data Bank of Japan; HCA3, hydroxycarboxylic acid receptor 3; DEG, differential gene expression; FC, fold change; HpyA, phenylpyruvic acid; IECs, intestinal epithelial cells; ILA, Indole-3-lactic acid; iPS, induced pluripotent stem cells; IpyA, indole pyruvic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PBS, phosphate-buffered saline; PCA, principal component analysis; PLA, phenyllactic acid; PpyA, phenylpyruvic acid; RNA-seq, RNA sequencing; TOS, Transgalactosylated oligosaccharide; 4-OH PLA, 4-hydroxyphenyllactic acid.

2. Materials and methods

2.1. Bacterial culture

B. breve MCC1274 was obtained from the Morinaga Culture Collection (Morinaga Milk Industry, Tokyo, Japan). This strain was cultured in De Man, Rogosa, and Sharpe broth (BD Biosciences, Franklin Lakes, NJ, United States) supplemented with 0.05% (w/v) L-cysteine hydrochloride (Kanto Chemical, Tokyo, Japan) for 16 h at 37°C under anaerobic conditions using an Anaero Pack (Mitsubishi Gas Chemical, Tokyo, Japan). The culture medium was centrifuged (1,000 × *g*, 5 min, 4°C), and the pellet was washed with PBS (FUJIFILM Wako Pure Chemical, Osaka, Japan) and suspended to approximately 1.0×10^9 CFU/ml in PBS. The number of colony-forming units (CFU) on transgalactosylated oligosaccharide (TOS) propionate agar (Yakult Pharmaceutical Industry, Tokyo, Japan) was used to calculate the number of *B. breve* MCC1274 cells.

2.2. Monolayer culture of human small intestinal-like epithelial cells

Monolayers of human iPS-derived small intestinal epithelial-like cells were generated from FUJIFILM human iPS cell-derived Small Intestinal Epithelial-like Cells (F-hiSIECs) (FUJIFILM Wako Pure Chemical, Osaka, Japan) according to the manufacturer instructions with minor modifications. Briefly, Transwell culture inserts (12-well insert, 0.4-μm pore polyester membrane; Greiner bio-one, Kremsmunster, Austria) were coated with Matrigel (Corning, Corning, NY, United States) diluted 1:30 in DMEM/F12 medium (Gibco, Thermo-Fisher Scientific, Waltham, MA, United States) at least 1 day before F-hiSIEC seeding. Next, thawed F-hiSIECs were suspended to 1×10^6 cells/mL in seeding medium, and 340 μL of the cell suspension was seeded on each Transwell insert. The F-hiSIEC culture medium was changed every 2–3 days, and in the last medium change before using the co-culture device, antibiotics were omitted from the F-hiSIEC culture medium. Transepithelial electrical resistance (TER) values, measured using the Millicell-ERS system (Merck Millipore, Burlington, MA, United States), were used to evaluate monolayer maturation.

2.3. Co-culture of bacteria and IECs

To co-culture IECs and *B. breve* MCC1274, we used a two-chamber co-culture device (Shimadzu, Kyoto, Japan) (Supplementary Figure S1). The device could culture anaerobic bacteria on the apical side of IECs while maintaining aerobic conditions on the basolateral side by sealing the culture cup with the Transwell culture insert. IECs monolayers on Transwell inserts were placed on the device, and F-hiSIEC culture medium without antibiotics was filled up at the basolateral side of the device. The device was then transferred into a TYPE-B, flexible vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). On the apical side, the medium was replaced with Yeast Casitone (YC) medium and left in the anaerobic chamber overnight. The composition of the YC medium is described in the Supplementary Table S1. The device was maintained at 37°C for 24 h before starting co-culture. Next, 1% (v/v)

of a pre-conditioned *B. breve* MCC1274 suspension was inoculated into the apical medium. As the control group, the same volume of bacterial suspensions was added to conical tubes containing an equal volume of YC medium on the apical side of the device but no IECs. After incubating for 24 h at 37°C, the apical medium of the device and the culture medium of the conical tube were collected to calculate the number of *B. breve* MCC1274 by counting the number of CFU on TOS propionate agar (Yakult Pharmaceutical Industry). Then, the culture medium was centrifuged (10,000 × g, 5 min, 4°C) and the supernatant was stored at −20°C until use.

2.4. Culturing *Bifidobacterium breve* MCC1274 with purines

B. breve MCC1274 was cultured in YC medium supplemented with 200 μM each of adenosine, inosine, hypoxanthine, and xanthine (FUJIFILM Wako Pure Chemical) for 24 h under anaerobic conditions. Then, the culture medium was centrifuged (10,000 × *g*, 5 min, 4°C), and the supernatant was stored at −20°C until analysis.

2.5. Metabolome analysis

A comprehensive hydrophilic metabolite analysis was conducted using the ω-Scan package (Human Metabolome Technologies (HMT), Yamagata, Japan). Briefly, culture supernatant samples were diluted with 10 μM internal standard sample, and centrifuged (9,100 × *g*, 4°C, 60 min) using a centrifugal filter (5-kDa Ultra-free MC-PLHCC, HMT). Next, capillary electrophoresis Fourier-transform mass spectrometry (CE-FTMS) was used for metabolomic analysis of the samples. Peaks with a signal-to-noise ratio (S/N) of three or higher were extracted using the MasterHands software (v 2.19.0.2 Keio University, Yamagata, Japan). Peak information, such as mass-to-charge ratio (m/z), peak area, and migration time (MT), was also obtained using the same software. The extracted peaks were annotated based on the m/z values and MT, using the HMT metabolome database. Three samples from one experiment were analyzed in this analysis. Metabolites with initials XC or XA, which were detected in multiple biological sample types in the HMT assay results, were annotated by the HMT Known-Unknown peaks library. The relative peak area were shown in Supplementary Table S2.

2.6. Quantification of metabolite concentrations using LC–MS/MS

The concentrations of the aromatic amino acids and aromatic pyruvic acids in the medium were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Vanquish HPLC connected with the TSQ-FORTIS (Thermo Fisher Scientific, Waltham, MA, United States). For deproteinization, culture medium supernatant was mixed with methanol at a ratio of 1:9 and centrifuged (10,000 × g, 5 min, 4°C) to remove aggregation. The supernatants were evaporated (miVac Quattro LV, Genevac Ltd., Ipswich, United Kingdom) and resuspended in Mobile phase A with 100 ng/ml 1-methyl-2-oxidole as the internal standard. As an elution method, method 1 described below was used in the experiments for comparing co-culture and monoculture supernatant, while method 2 for evaluating the effect of purine supplementation. The conditions used to analyze in each elution method are described in Supplementary Tables S3, S4.

2.6.1. Method 1

LC–MS/MS quantification was measured using an XBridge® C18 column (4.6 mm × 150 mm, 5 μm) (Waters Corporation, Milford, MA, United States) and the protocols mobile phase A (1 g/L ammonium acetate) and mobile phase B (methanol), were applied at a flow rate of 0.2 mL/min. Gradient elution was performed by changing the percentage of mobile phase B in the following steps: (i) maintenance of 2% for 2 min, (ii) increase from 2 to 35% until 5 min, (iii) increase from 35 to 75% until 21 min, (iv) increase from 75 to 77% until 25 min, (v) increase from 77 to 99% until 30 min, (vi) maintenance of 99% until 38 min, (vii) decrease from 99 to 2% until 40 min, and (viii) maintenance of 2% until 55 min. Six samples from two independent experiments were analyzed in this assay.

2.6.2. Method 2

LC–MS/MS quantification was measured using an XBridge® C8 column (4.6 mm × 150 mm, 3.5 μm) (Waters Corporation, Milford, MA, United States) and the protocols mobile phase A (0.5 g/l ammonium formate) and mobile phase B (methanol) were applied at a flow rate of 0.2 ml/min. Gradient elution was performed by changing the percentage of mobile phase B in the following steps: (i) maintenance of 2% for 2 min, (ii) increase from 2 to 65% until 40 min, (iii) increase from 65 to 99% until 45 min, (iv) maintenance of 99% until 55 min, (vii) decrease from 99 to 2% until 60 min, and (viii) maintenance of 2% until 75 min. Three samples from one experiment were analyzed in this assay.

2.7. RNA sequencing

The bacterial cells were treated with RNAProtect Bacteria Reagent (Qiagen, Hilden, Germany) and stored at −80°C. The pellets were suspended in TE Buffer (pH 8.0) containing 30 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, United States), 5,000 U/ml mutanolysin (Sigma-Aldrich), and 20 mg/ml protease K (Qiagen) and incubated for 1 h at room temperature (20–25°C). Total RNA was extracted using the RNeasy Mini Kit (Qiagen) with an RNase-Free DNase Set (Qiagen). Library preparation was performed using Illumina Stranded Total RNA Prep with Ribo-Zero Plus and IDT® for Illumina® RNA UD Indexes Set A-B Ligation (Illumina, San Diego, CA, United States). The concentration and quality of the extracted RNA and adapter-tagged sequence library were calculated using an Agilent RNA 6000 Nano and Agilent High Sensitivity DNA Kits (Agilent Technologies, Santa Clara, CA, USA), respectively. Sequences were obtained using the NextSeq 1,000 system with the Illumina NextSeq 1000/2000 P2 Reagent kit (100 cycles) (Illumina). Trimming and mapping were conducted using CLC workbench (v 8, Qiagen) software. Normalization from total read counts, and the identification of differentially expressed genes (DEGs) were performed *via* iterative differential expression analysis (DESeq2) using TCC-GUI software (Su et al., 2019). The KEGG (Kyoto Encyclopedia of Genes and Genomes) Ortholog (KO) number and functional annotation of DEGs were assigned using GhostKOALA (v 2.2, Kanehisa et al., 2016).

Functional classification and reconstruction of metabolic pathways of annotated DEGs were performed using KEGG Mapper (v 5.0). Five samples monocultured in tubes and six co-cultured samples from two independent experiments were analyzed.

2.8. Statistical analysis

To calculate the significance of *B. breve* MCC1274 cell count, the relative abundance of each metabolite in the metabolome analysis, and the concentrations of ALAs and aromatic pyruvic acids, two-sided Welch's *t*-test was used. The comparison of the effects of purines for ALAs synthesis was assessed using Dunnett's test. For metabolome analysis, principal component analysis (PCA) and heatmap analyses were performed with the scaled value of the annotated peak intensity. The value of no detection (N.D.) in the metabolomics analysis data was regarded asset at eps (2^{-52}). Heatmaps described the significantly different metabolites (q-value <0.1), as calculated with the Kruskal–Wallis test and Benjamini–Hochberg post-hoc test. Vertical and horizontal hierarchical clustering was calculated using Spearman's correlation distance and Ward's clustering method. For RNA sequencing (RNA-seq) analysis, genes with a q-value <0.05 and a fold change (FC) of >2.0 were defined as DEGs. Statistical analyses were performed using R (version 4.1.3) with the FactoMineR package (version 2.4) and factoextra package (version 1.0.7) for PCA analysis, the ComplexHeatmap package (version 2.10.0) for heatmap analysis, and the Enhancedvolcano package (version 1.14.0) for visualizing DEGs of RNA-seq data.

3. Results

3.1. Evaluation of metabolic properties of the basolateral and apical media in the co-culture device

Initially, we confirmed the functionality of the device by evaluating the metabolites in the apical (YC medium) and basolateral sides (F-hiSIEC culture medium) of IECs without co-cultivation with bacteria. Based on total peak-annotated metabolites, we confirmed that distinctly different clusters were formed in the medium of the basolateral and apical sides, as can be seen in PCA (Supplementary Figure S2A) and heatmap analysis (Supplementary Figure S2B). These data indicate that there were less leaks of metabolites from one side to the other, suggesting that IECs were not in a leaky state and this device is suitable for subsequent experiments.

3.2. Differences in the metabolic profiles of IEC supernatants in the presence or absence of *Bifidobacterium breve* MCC1274

To explore the metabolite changes that were caused from the co-cultivation, we compared the metabolites in the supernatants of the probiotic monoculture with those in the apical side of the co-culture device, in the presence or absence of *B. breve* MCC1274. A total of 417 metabolites were detected, of which 237 were significantly different between the groups according to the Kruskal–Wallis test (q-value <0.1). The metabolite profile was divided into four distinct groups by PCA (Figure 1A). The groups were separated on the PC 1 axis by the presence of IECs, and on the PC 2 axis by the presence of *B. breve* MCC1274. The number of viable *B. breve* cells was almost the same in both the co-culture and the monoculture (Figure 1B).

To explore the different metabolites in each group, cluster classification based on the relative abundance of each metabolite was performed (Figure 1C; Supplementary Table S2). The metabolites were divided into six clusters as follows:

A. Cluster I, the metabolites with higher abundance in the culture medium of IECs.
B. Cluster II, the metabolites with higher abundance in the applied medium, but lower in the presence of IECs or *B. breve* MCC1274.
C. Cluster III, the metabolites with higher abundance in the culture medium of IECs, but lower in the presence of *B. breve* MCC1274.
D. Cluster IV, which is subdivided into two clusters IV-1 and IV-2, the metabolites with higher abundance in the applied medium without IECs.
E. Cluster V, the metabolites with higher abundance in the culture medium of *B. breve* MCC1274 with IECs.
F. Cluster VI, the metabolites with higher abundance in the culture medium of *B. breve* MCC1274 without IECs.

Cluster V represented the increase in the abundance of metabolites produced by *B. breve* MCC1274 when co-cultured with IECs, compared with that in the monoculture alone. We found that three ALAs, namely, PLA, 4-hydroxyphenyllactic acid (4-OH-PLA), and ILA; as well as the structurally similar metabolites imidazole lactic acid, and 2-Hydroxy-4-methylvaleric acid, fell into Cluster V (Figures 1C, 2A). Some of these ALAs were not annotated as a single substance; for example, a single peak was annotated as both Indole-3-lactic acid and 5-Methoxyindoleacetic acid. In contrast, no significant increase was observed in the amino acids phenylalanine, tyrosine, and tryptophan as substrates for the corresponding metabolite (Figure 2B). These results suggest that the increase in ALAs in the co-culture conditions was attributed to metabolic changes in either *B. breve* MCC1274 or IECs, and not due to a substrate increase in the co-culture environment.

3.3. Quantification of ALAs and aromatic pyruvic acids

Since some metabolites shown in Figure 2A were not annotated as a single substance using CE-FTMS, we performed LC–MS/MS to quantify ALAs and the corresponding aromatic pyruvic acids (Figure 3). We confirmed that PLA and ILA concentrations were significantly increased ($p < 0.01$), while 4-OH-PLA concentration showed a tendency to increase ($p = 0.09$) in the co-culture conditions compared with the concentrations in the monoculture of *B. breve* MCC1274. The concentration of the precursor metabolite phenylpyruvic acid (PpyA) significantly increased in the co-culture group ($p < 0.01$), while 4-hydroxyphenylpyruvic acid (HpyA) was only detected in the co-culture group. Contrastingly, the level of indole pyruvic acid (IpyA) was under detection limit (data not shown).

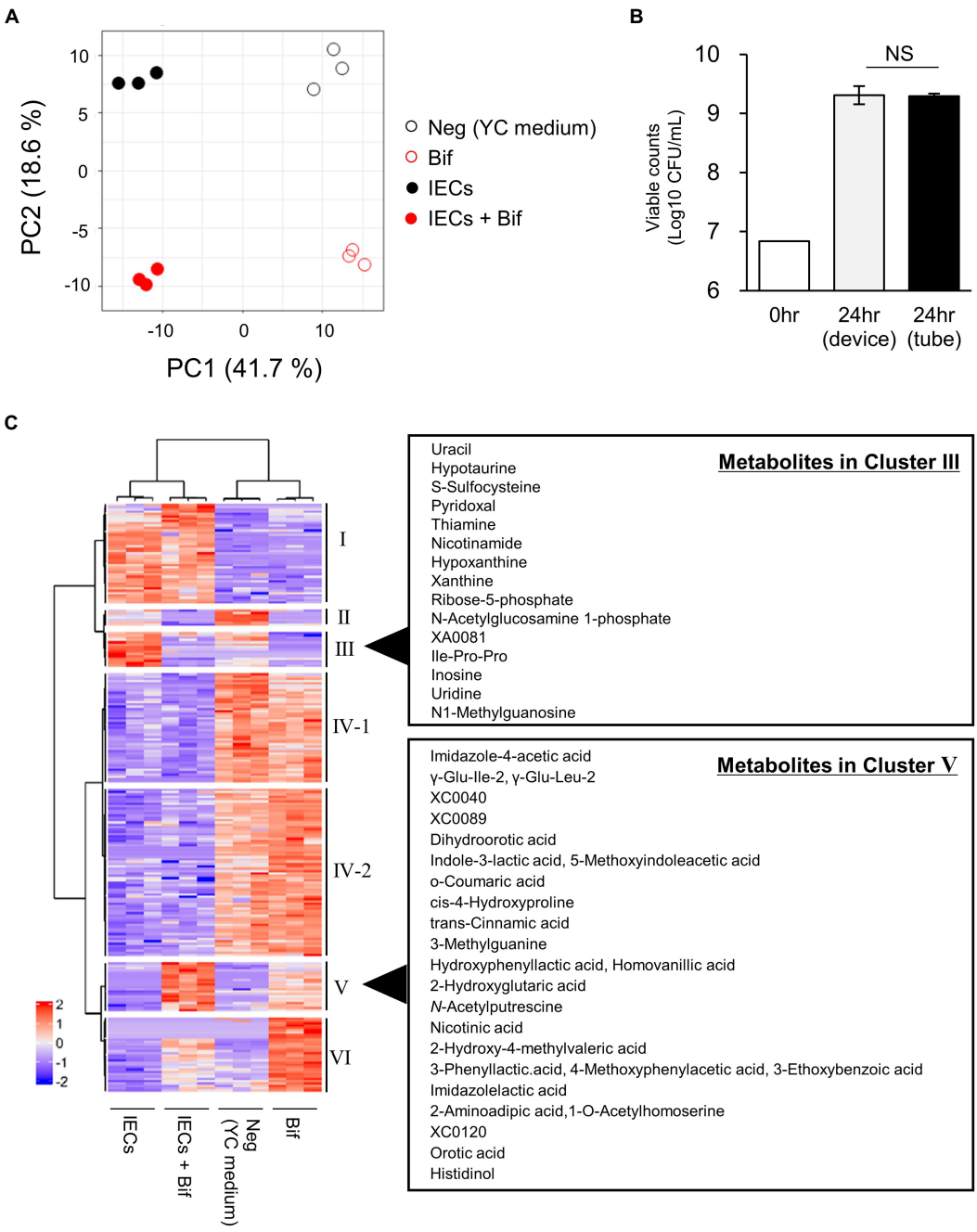

FIGURE 1
Comparison of the metabolites in the culture supernatants of *Bifidobacterium breve* MCC1274 with or without IECs. **(A)** PCA plot based on the total metabolites detected in the apical side of the device. Neg, YC medium; Bif, supernatant of *B. breve* MCC1274 monocultured for 24h in tube. IECs, supernatant of IECs monocultured in the apical side of the co-culture device cultured for 48h; IECs + Bif, supernatant of IECs co-cultured with *B. breve* MCC1274 in the apical side of the co-culture device cultured for 24h. **(B)** Viable cell count of *B. breve* MCC1274 before (0h) and after (24h) cultivation in the tube as a monoculture, and in the device as a co-culture with IECs. The data represent the average of two samples (0h) and six samples (24h) from each group, from two independent experiments (Average±standard deviation). Standard deviation were not described in before cultivation samples. NS, not significant (p=0.83, Welch's t-test). **(C)** A heatmap of the scaled intensity value of each metabolite with a significant difference (q-value <0.1) in the Kruskal–Wallis and Benjamini–Hochberg tests.

3.4. *Bifidobacterium breve* MCC1274 gene expression

To further clarify the mechanism behind the increase in ALA concentrations, we compared the gene expression profile of *B. breve* MCC1274 in the monoculture with that in the co-culture with IECs. A total of 446 genes were identified as DEGs (Figure 4A). We further classified the annotated DEGs, 173 upregulated and 77 downregulated genes, using the KEGG functional annotation (Figure 4B). The upregulated DEGs were mainly involved in translation and amino acid metabolism pathways. Moreover, the top ten upregulated genes,

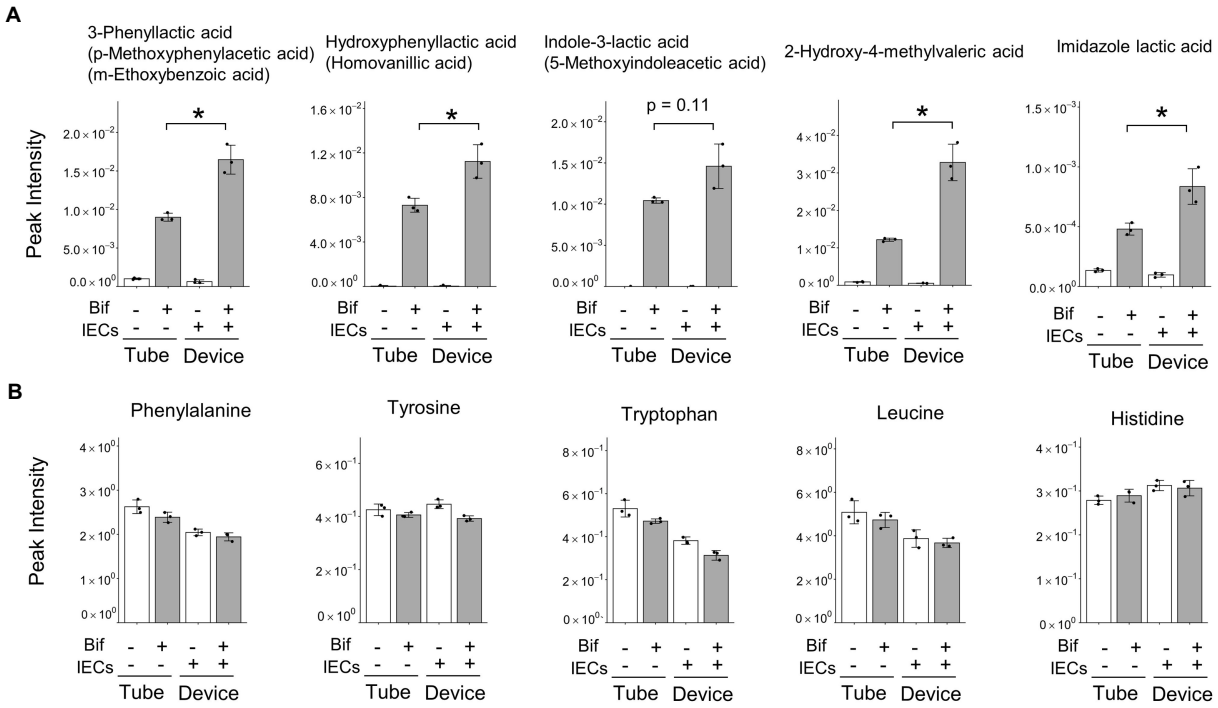

FIGURE 2
Peak intensity of amino acid-derived metabolites and their precursor amino acids. **(A)** Amino acid-derived metabolites, alternatives are shown in parentheses when peaks were annotated with more than one metabolite. **(B)** Precursor amino acids corresponding to the five metabolites shown in Figure 3. **(A)** Data are expressed as the mean±SD (n=3),* p<0.05, ** p<0.01 (Welch's t-test).

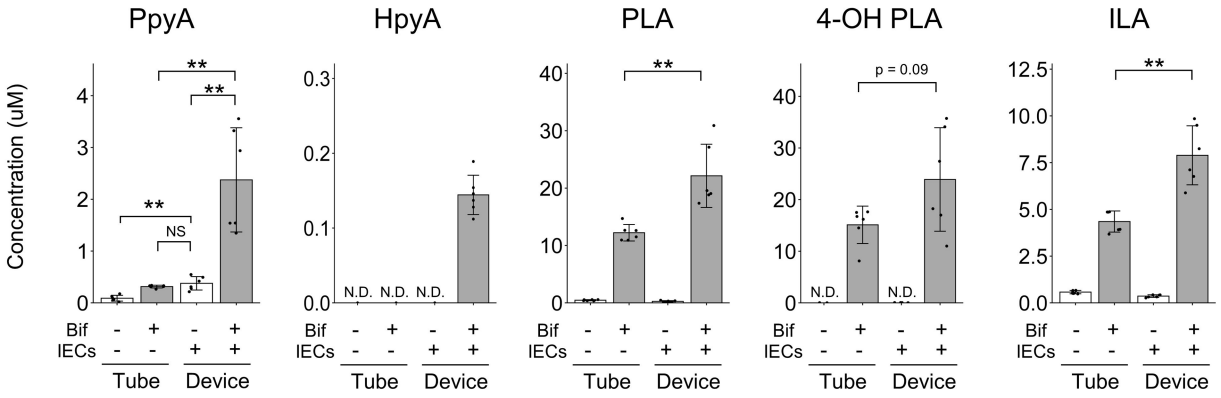

FIGURE 3
Quantitative analysis of aromatic lactic acids and their precursor metabolites. The concentrations of aromatic lactic acid-related metabolites. The data represent the average of six samples from each group, from two independent experiments. *p<0.05, **p<0.01 (Welch's t-test) N.D., not detected.

ranked by q-value, included genes involved in amino acid biosynthesis (*trpA, trpBC, livC2*, and *tpiA*) (Table 1), suggesting that amino acid metabolism by *B. breve* MCC1274 increased in the co-culture with IECs.

Next, we focused on ALA synthesis-related genes. In the co-culture, the gene expression of the aminotransferase gene (*aat*) was significantly increased (2.06-fold, p < 0.01, q < 0.01), while that of the lactate dehydrogenase type 4 gene (ldh4) tended to be increased (1.54 fold, p = 0.06, q = 0.11) when compared with the expression of those genes in a monoculture (Figure 4D). Other transcriptional changes related to ALA synthesis in the co-culture included the enhanced expression of genes involved in tryptophan and NADH synthesis (Figures 4E,F).

3.5. *Bifidobacterium breve* MCC1274 purine metabolism

To identify metabolites that enhance the production of ILA in the co-culture conditions, the metabolites with an increased abundance in cluster III of Figure 1C were considered for further analysis. The increase in their abundance in the IECs group and

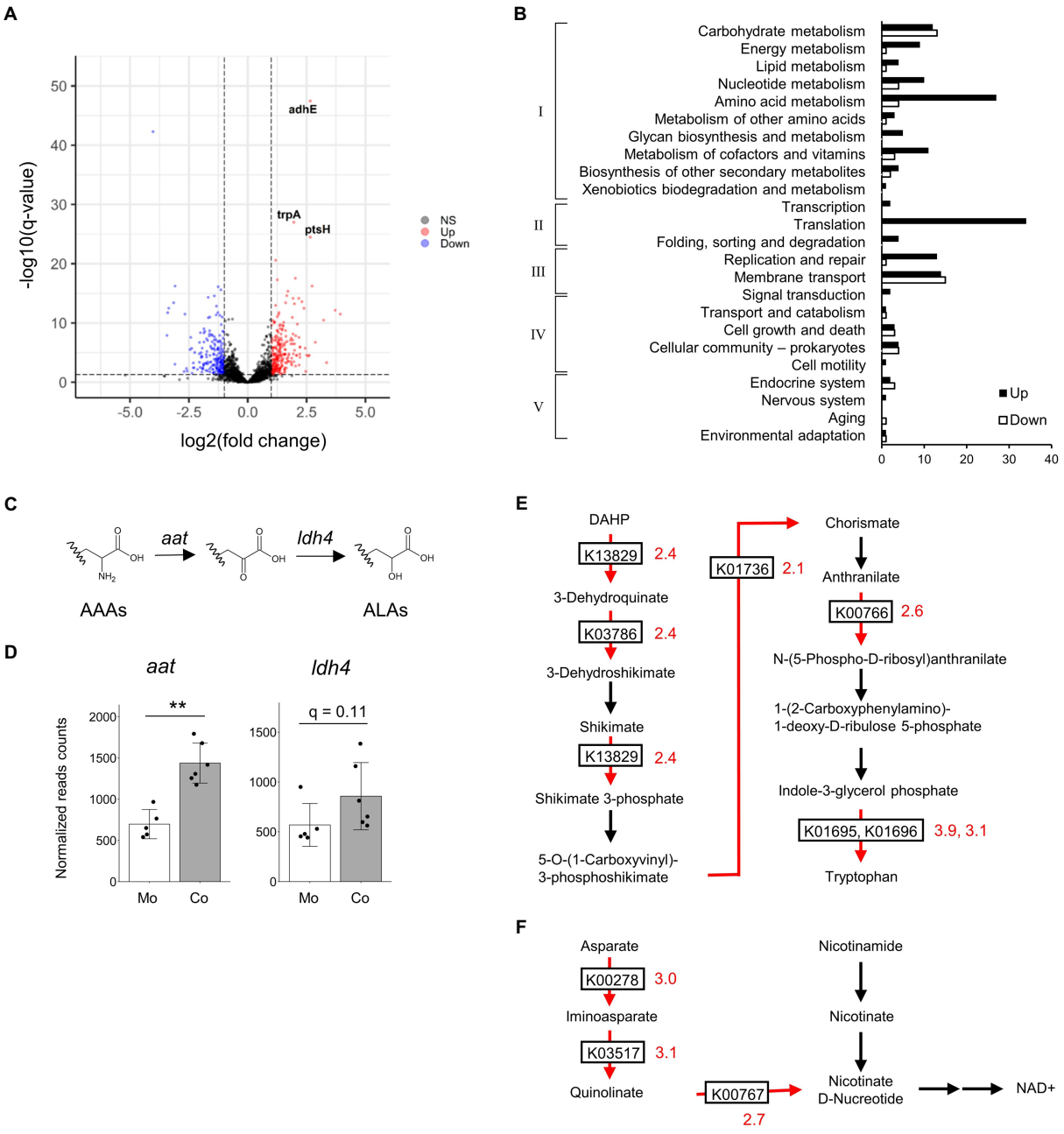

FIGURE 4
Comparison of gene expression profiles of *B. breve* MCC1274 in monoculture and in co-culture with IECs. **(A)** Volcano plot of total gene expression. Vertical and horizontal dotted lines indicate the fold-change value |FC|=2 and a *q*-value of 0.05, respectively. Upregulated DEGs in the co-culture conditions are labeled in red, while downregulated DEGs are labeled in blue. RNA-seq was performed on five to six samples from two independent experiments. **(B)** KEGG Orthology (KO) functional classification of annotated DEGs. The horizontal axis represents the number of genes. Categories I–V represent the following mapped KEGG pathway categories: I, Metabolism; II, Genetic Information Processing; III, Environmental Information Processing; IV, Cellular Processes; V, Organismal Systems. **(C)** Aromatic amino acid synthesis pathway. **(D)** The normalized read counts from RNA-seq analysis. Mo, *B. breve* MCC1274 monocultured in the tube; Co, *B. breve* MCC1274 co-cultured with IECs. ***q*<0.01 (Deseq2). **(E,F)** Tryptophan synthesis pathway components: **(E)** NADH synthesis **(F)** The red arrow indicates the enzymes whose gene expression was upregulated in co-culture (fold change >2, *q*-value <0.05). Black arrows indicate gene expression that was not significantly changed. The numbers next to the boxes describing KEGG Orthology indicate fold change. AAAs, aromatic amino acids; ALAs, aromatic lactic acids; APAs, aromatic pyruvic acids; DHAP, 2-Dehydro-3-deoxy-D-arabino-heptonate 7-phosphate.

decrease in the co-culture group, suggesting that they were produced by IECs and utilized by *B. breve* MCC1274. Most of these were nucleosides and nucleobases (Figure 1C). On the one hand, in the presence of IECs, the difference in adenosine levels in the medium on the apical side indicated its conversion to uric acid and its intermediate metabolites, such as inosine, hypoxanthine, and xanthine (Figures 5A,B). Corresponding to the conversion, in co-culture conditions, instead of adenosine, *B. breve* MCC1274 consumed inosine, hypoxanthine, and xanthine more. In fact, *in vitro* assays without IECs showed that supplementation with

TABLE 1 List of top 10 up- and down-regulated genes of *B. breve* MCC1274 in the co-culture with IECs compared with the mono-culture.

Rank	KO	Annotation	FC	*q*-value
Up-regulated gene				
1	K04072	adhE; acetaldehyde dehydrogenase / alcohol dehydrogenase	6.26	3.0.E-48
2	K01695	trpA; tryptophan synthase alpha chain	3.86	7.8.E-27
3	K02784	ptsH; phosphocarrier protein HPr	6.29	3.0.E-25
4	K01000	mraY; phospho-N-acetylmuramoyl-pentapeptide-transferase	2.27	1.2.E-20
5	K00053	ilvC; ketol-acid reductoisomerase	4.04	5.7.E-18
6	K01925	murD; UDP-N-acetylmuramoylalanine--D-glutamate ligase	2.38	1.2.E-17
7	K03502	umuC; DNA polymerase V	6.61	4.6.E-17
8	K03551	ruvB; holliday junction DNA helicase RuvB	3.24	3.5.E-16
9	K01696	trpB; tryptophan synthase beta chain	3.13	2.2.E-15
10	K01803	TPI, tpiA; triosephosphate isomerase (TIM)	4.48	5.9.E-15
Down-regulated gene				
1	–	Hypothetical protein	0.06	2.3.E-43
2	–	Hypothetical protein	0.12	4.2.E-17
3	–	Hypothetical protein	0.42	1.2.E-16
4	–	Hypothetical protein	0.45	6.8.E-16
5	K03484	scrR; LacI family transcriptional regulator, sucrose operon repressor	0.37	3.4.E-15
6	K01768	E4.6.1.1; adenylate cyclase	0.31	6.1.E-15
7	–	Hypothetical protein	0.11	2.8.E-14
8	–	Hypothetical protein	0.40	8.7.E-14
9	–	Hypothetical protein	0.10	2.4.E-13
10	K05349	bglX; beta-glucosidase	0.09	1.6.E-12

FC, Fold change, KO, KEGG Orthology.

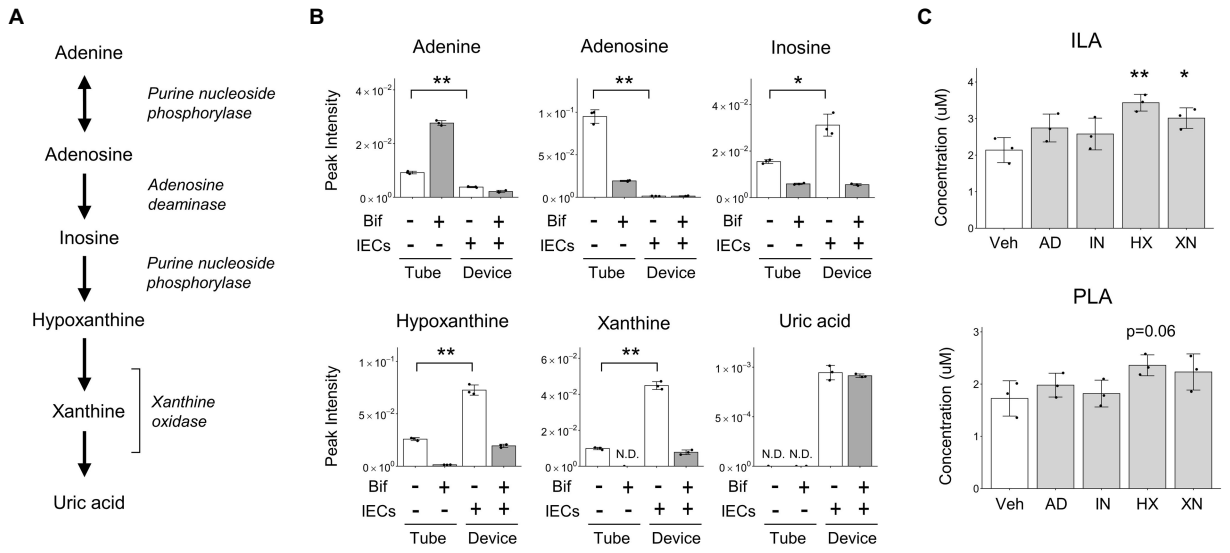

FIGURE 5
B. breve MCC1274 purine metabolism. **(A)** The purine metabolism pathway from adenine to uric acid. **(B)** Peak intensity of purine metabolites detected using CE-FTMS, *$p<0.05$, **$p<0.01$ (Welch's *t*-test). **(C)** Aromatic lactic acid concentrations when cultured in the YC medium with purines. *$p<0.05$, **$p<0.01$ (Dunnett's test). Data are expressed as the mean±SD ($n=3$). AD, Adenosine; HX, Hypoxanthine; IN, Inosine; Veh, vehicle; XN, Xanthine.

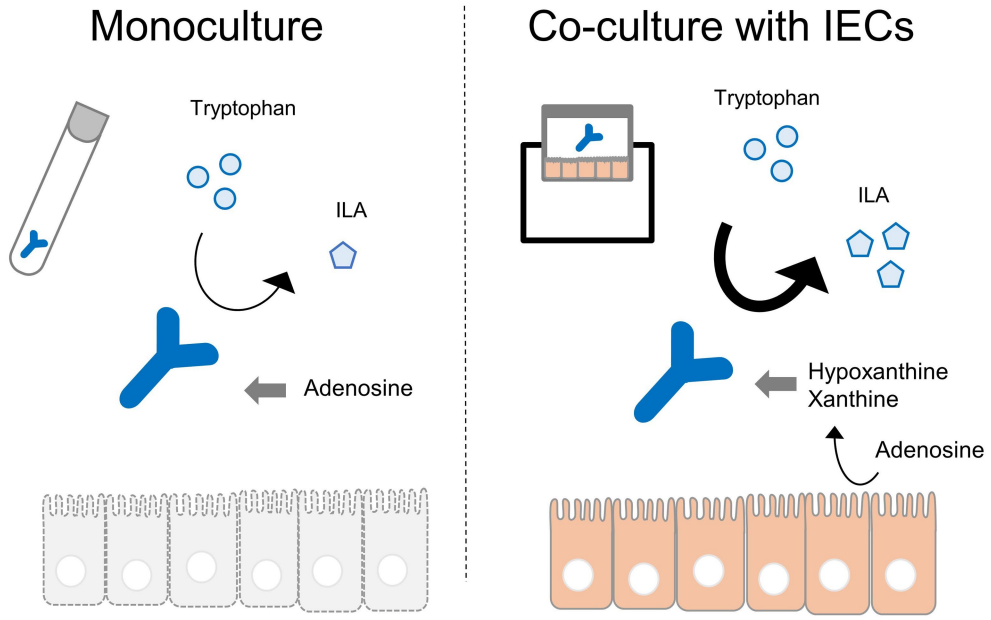

FIGURE 6
Graphical abstract.

xanthine and hypoxanthine significantly increased *B. breve* MCC1274 ILA production, whereas adenosine supplementation did not cause a significant increase (Figure 5C). These data indicate that the hypoxanthine and xanthine derived from IECs could enhance ILA production by *B. breve* MCC1274 (Figure 6).

4. Discussion

In the intestines, IECs interact with microorganisms on the luminal side. However, our knowledge regarding the metabolic interactions between the two is limited due to difficulty in mimicking the gut environment, such as oxygen requirements. To overcome this problem, a Transwell-based static two-layer co-culture system was used in this study. In addition to the two-chamber device, we used F-hiSIEC; iPS-derived small intestinal-like cells, which reflect the small intestine in terms of metabolic activity than well-known intestinal cell lines, such as Caco-2 cell (Onozato et al., 2018). While we did not measure oxygen concentrations in the apical and basolateral sides, with respect to the barrier function, the metabolite compositions were clearly different between the apical and basolateral sides (Supplementary Figures S2A,B). This suggests that the intestinal barrier function was maintained even when the co-culture devise was put under the anaerobic conditions for 48 h.

B. breve MCC1274 produces ALAs and their corresponding precursors *in vitro* (Sakurai et al., 2021). To the best of our knowledge, this is the first report of an enhanced production of these metabolites in a co-culture with IECs. Our findings suggest that co-culturing *B. breve* MCC1274 with IECs has a synergistic effect, where the production of ALAs, such as PLA and ILA, is significantly enhanced (Figure 3). PLA and ILA exert immunomodulatory effectors *via* the AhR and hydroxycarboxylic acid receptor 3 (HCA3) (Peters et al., 2019; Sakurai et al., 2019; Meng et al., 2020; Laursen et al., 2021; Sakurai et al., 2021). D-PLA induces pertussis toxin-sensitive migration in human monocytes in an HCA3-dependent manner (Peters et al., 2019). ILA has been reported to attenuate IL-8 production stimulated by interleukin-1β in primary enterocyte derived from necrotizing enterocolitis (NEC) patients and immature human intestinal organoid (Meng et al., 2020). In clinical studies, Laursen et al. reported that ILA reduced IL12p70 production from monocyte stimulated with lipopolysaccharide (Laursen et al., 2021). Another study suggested that ILA up-regulated the gene expression of galectin-1, which suppresses T-cell activation in Th2 and Th17 cells (Henrick et al., 2021). These reports suggest an anti-inflammatory effect of ILA. PLA also has antimicrobial properties (Ning et al., 2017; Sorrentino et al., 2018), and, along with 4-OH PLA, exerts antioxidant activity *via* suppressing ROS production in mitochondria and neutrophils (Beloborodova et al., 2012). Similarly, 2-Hydroxy-4-methylvaleric acid, the concentration of which was also enhanced in the co-culture (Figure 2A), has also been reported as a possible HCA3 ligand with antimicrobial activities against harmful bacteria (Sakurai et al., 2021; Pahalagedara et al., 2022).

In RNA-seq analysis, the upregulated genes were mainly involved in translation and amino acid metabolism pathways, especially amino acid biosynthesis. This suggests that amino acid metabolism by *B. breve* MCC1274 stimulated in the co-culture (Figure 4B). Moreover, guided by metabolomics analysis, of particular interest in this study were the ALA synthesis-related genes. *Bifidobacterium* species that colonize the intestines of breastfed infants have, in addition to human milk oligosaccharide utilization genes, a gene cluster composed of *aat* and *ldh4* (Sakanaka et al., 2019). The former gene presumably encodes aromatic amino acid transaminase that converts aromatic amino acids to aromatic pyruvic acids, while the latter gene encodes lactate dehydrogenase that converts aromatic pyruvic acids into their respective ALAs

(Figure 4C; Laursen et al., 2021). In the co-culture, the gene expression of *aat* was significantly increased and that of *ldh4* showed a tendency to increase. These results imply that under the co-culture conditions, the expression of both lactate dehydrogenase and transaminase involved in ALAs production was upregulated, which probably contribute to the conversion of aromatic amino acids into aromatic lactic acids *via* aromatic pyruvic acids. In addition, in the co-culture, there was an enhancement in genes involved in tryptophan and NADH synthesis. Tryptophan is the substrate for ILA synthesis. NADH acts as a cofactor for the dehydrogenase reaction. The data from this study imply that in the co-culture, not only the gene expression directly related to ALA synthesis is enhanced, but so it substrate and cofactor synthesis, which ultimately increased PLA and ILA concentrations.

The relative abundance of purines in the medium was altered in the presence of IECs. Our results suggest that the increase in ILA in the co-culture with IECs was partly due to purine metabolites derived from IECs. Since the enzymes involved in purine metabolism, converting adenosine to uric acid, were highly expressed in the human small intestine (Uhlen et al., 2010) (Human Protein Atlas proteinatlas.org), food-derived adenosine could also convert to inosine, hypoxanthine, and xanthine *in vivo* by IECs.

This indicates that substrates that affect ILA production by *B. breve* MCC1274 are assumed to be more diversified *in vivo*, similar to the results observed in the *in vitro* co-culture system. Some lactic acid bacteria have been reported to utilize extracellular purines for growth (Kilstrup et al., 2005; Martinussen et al., 2010; Yamada et al., 2017). Also, purine nucleoside starvation of *Lactococcus lactis* subsp. *cremoris* could affect its protein expression (Kilstrup et al., 2005). These reports suggest that alteration of purine nucleoside concentrations in the medium could affect the growth and amino acid metabolism of *B. breve* MCC1274, ultimately affecting their ability to synthesize ILA. However, supplementation with xanthine and hypoxanthine had a limited effect on PLA synthesis (Figure 5C). The mechanism underlying the enhancement of ILA production in the *B. breve* MCC1274 *via* supplementation of hypoxanthine and xanthine is yet to be elucidated.

Another possible mechanism related to the enhancement of ALAs production in the co-culture is that IECs and microbes collaborate to synthesize ALAs substrates. Humans possess a gene encoding transaminase for metabolizing phenylalanine and tyrosine to PpyA and HpyA, respectively. In this study, IECs cultured alone produced the same amount of PpyA as *B. breve* MCC1274 cultured alone (Figure 3). Conversely, in co-culture, a significant enhancement in PLA concentration was observed. Thus, the effect of IECs on PpyA synthesis cannot be ruled out. Future studies using *aat* deficient *B. breve* MCC1274 or IECs will determine the main producer of PpyA.

While we attempted to mimic the *in vivo* gut environment to provide relevant results, several limitations remain to be addressed. Firstly, our model lacked some important factors present *in vivo*, such as the microbiota and bile acids present in the small intestinal environment. Furthermore, the *in vitro* three-dimensional structure of IECs, such as the villus, differs from that *in vivo*. Therefore, future studies should use a system that best mimic the *in vivo* environment. Additionally, as indicated by RNA-seq analysis, in the co-culture environment, there was an enhancement in the expression of genes involved in amino acid biosynthesis. However, the observed changes in the gene expression were not directly linked with extracellular metabolic shift in this study. Investigating the intracellular metabolites in *B. breve* MCC1274 will provide insights into this enhancement. With this regard, collection of an appropriate bacteria cell numbers is the most challenging issue when using this co-culture system.

In conclusion, we demonstrated that IECs could affect the metabolism of the probiotic strain *B. breve* MCC1274 using a host-microbe co-culture system. Among the various metabolites, the amounts of the immunomodulatory metabolites PLA and ILA increased in the presence of IECs. However, compared with concentrations in monoculture conditions, the concentrations of aromatic amino acids, which are precursor metabolites of ALAs, were not increased in co-culture conditions. Using the metabolomics analysis data, RNA-seq analysis of *B. breve* MCC1274 was conducted. Our findings revealed an enhancement in the expression of some *B. breve* MCC1274 genes in the presence of IECs. This included amino acid synthesis, cofactor synthesis, and transaminase gene expression. Furthermore, hypoxanthine and xanthine derived from IECs was linked with increased ILA production by *B. breve* MCC1274. These results suggest that IECs-derived factors may enhance the synthesis of beneficial metabolites, such as PLA and ILA, by altering the metabolic activity of *B. breve* MCC1274.

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.

Author contributions

AS: conceptualization, investigation, visualization, and writing of the original draft. TN: validation and investigation. SY: supervision. KY: data curation. AG: co-cultivation device development. ToK and TaK: co-cultivation device development and supervision and editing and reviewing manuscript. YY and TH: co-cultivation device development. J-ZX: supervision, editing, and reviewing manuscript. TO: project administration, supervision, editing, and reviewing manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of interest

AS, TN, SY, KY, J-ZX, and TO were employed by Morinaga Milk Industry Co., Ltd. YY and TH were employed by Shimadzu Corp.

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/fmicb.2023.1155438/full#supplementary-material

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Complex probiotics alleviate ampicillin-induced antibiotic-associated diarrhea in mice

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Aim: Antibiotic-associated diarrhea (AAD) is a common side effect during antibiotic treatment, which can cause dysbacteriosis of the gut microbiota. Previous studies have shown beneficial effects in AAD treatment with *Bifidobacterium lactis* XLTG11, *Lactobacillus casei* Zhang, *Lactobacillus plantarum* CCFM8661, and *Lactobacillus rhamnosus* Probio-M9. However, no studies have been conducted on the immunomodulatory effects and protective intestinal barrier function of four complex probiotics. The aim of our study is to investigate the alleviation effects of complex probiotics on ampicillin-induced AAD.

Methods: Thirty-six BALB/c mice were randomly divided into six groups: normal control group (NC), model control group (MC), low-, medium-, and high-dose probiotics groups (LD, MD, and HD), and positive drug (Bifico, 1×10^7 cfu) control group (PDC; Bifico, also known as Bifidobacterium Triple Live Capsule, is composed of *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*). An AAD model was established by intragastric administration of ampicillin, by gavage of different doses of complex probiotics and Bifico. The weight gain, fecal water content, loose stool grade, intestinal permeability, total protein and albumin levels, intestinal barrier, cytokine levels, and gut microbiota were determined.

Results: The results showed that complex probiotics significantly decreased the fecal water content, loose stool grade, intestinal permeability, and ileum tissue damage. Their application increased the weight gain, SIgA, TP, and ALB levels. Additionally, complex probiotics significantly decreased the levels of pro-inflammatory cytokines and increased those of anti-inflammatory cytokines. Meanwhile, the mRNA expression levels of ZO-1, occludin, claudin-1, and MUC2 were significantly upregulated in the probiotic-treated group. Furthermore, the complex probiotics increased the gut microbiota diversity and modulated the changes in the gut microbiota composition caused by ampicillin. At the phylum level, the abundance of *Proteobacteria* in the HD group was lower than that in the MC group, whereas that of *Bacteroidetes* was higher. At the genus level, the abundances of *Klebsiella* and *Parabacteroides* in the HD group were lower, whereas those of *Bacteroides*, *Muribaculaceae*, and *Lactobacillus* were higher than those in the MC group. Moreover, Spearman's correlation analysis also found that several specific gut microbiota were significantly correlated with AAD-related indicators.

Conclusion: We found that complex probiotics improved the diarrhea-related indexes, regulated gut microbiota composition and diversity, increased the expression levels of intestinal protective barrier-related genes, preserved the intestinal barrier function, and relieved inflammation and intestinal injury, thereby effectively improving AAD-associated symptoms.

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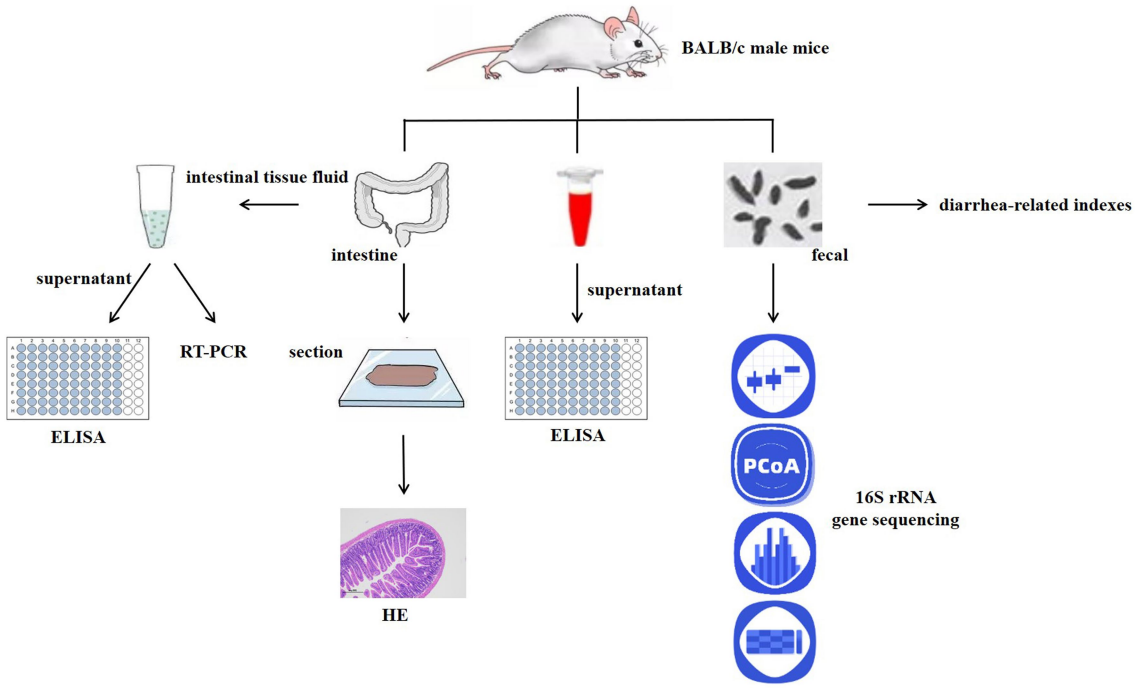

GRAPHICAL ABSTRACT

KEYWORDS

complex probiotics, antibiotic-associated diarrhea, immunomodulatory, 16S rRNA gene sequencing, gut microbiota

1. Introduction

Antibiotics are used to treat illnesses caused by pathogenic infections. Antibiotic-associated diarrhea (AAD) is diarrhea that occurs after the use of antibiotics. It is a common adverse drug reaction (Shao et al., 2020; Yang et al., 2022). Approximately 5–35% of the patients receiving antibiotics have AAD, whose severity ranges from mild to life-threatening (Goodman et al., 2021). The pathogenesis of AAD varies, but the most common mechanism is by damage of the intestinal physiology and microbial community structure caused by antibiotics, resulting in gut dysbiosis (Silverman et al., 2017; Zeng et al., 2019; Mekonnen et al., 2020). Conventional AAD therapy is based on the use of metronidazole and vancomycin and has serious side effects (Silverman et al., 2017). Therefore, it is important to find effective drugs with fewer side effects to alleviate AAD symptoms.

The intestinal tract is the body's digestive system, and the gut microbiota is the largest and most complex microecological system in the human body. It exerts important immune functions and contributes to host defense against pathogens, plays an important role in the maintenance of intestinal homeostasis, and the development and activation of the host immune system (Nishida et al., 2018; Xu et al., 2023). Probiotics are active microorganisms that are beneficial to the host; they colonize the human body and change the composition of the gut microbiota (Azad et al., 2018; Plaza-Diaz et al., 2019). Probiotics can regulate the gut microbiota environment to improve intestinal health, improve the immune system, and play a beneficial role (Stavropoulou and Bezirtzoglou, 2020; Li Y. et al., 2021). The main immune mechanisms of probiotics include enhancing gastrointestinal mucosal barrier function, activating immune cells, modulating the secretion of immunoglobulins, etc. (La Fata et al., 2018). The supplementation with some probiotics can be done by oral intake; they are safe to use and reportedly had a 7% annual market growth rate worldwide (Zawistowska-Rojek and Tyski, 2018; Peng X. et al., 2022). Probiotics have been confirmed to prevent and improve digestive system diseases, such as acute diarrhea, irritable bowel syndrome (IBS), and AAD (Hempel et al., 2012; Li Z. et al., 2021; Skrzydło-Radomańska et al., 2021; Chen Y. et al., 2022). *Bifidobacteria* and *Lactobacilli* are the most important probiotic bacteria of the gut microbiota (Turroni et al., 2018; Fernández-Ciganda et al., 2022). *Bifidobacteria* was found to exert a protective role in intestinal barrier function in NEC mice by inhibiting proinflammatory cytokine secretion and zonulin protein release, and improving intestinal TJ integrity (Ling et al., 2016). *Bifidobacterium pseudocatenulatum* regulates the gut microbiota activities and immune functions, participates in the intestinal protective barrier, and inhibits the TLR4/NF-κB pathway (Chen et al., 2021). Oral *Lactobacillus* administration increased the Paneth cells counts and the intestinal antimicrobial activity in healthy mice (Cazorla et al., 2018).

Previous research has shown that *Bifidobacterium lactis* XLTG11, *Lactobacillus casei* Zhang, *Lactobacillus plantarum* CCFM8661, and *Lactobacillus rhamnosus* Probio-M9 (Yu et al., 2021; Yao G. et al., 2021; Xu et al., 2022; Zhang et al., 2022) promote immunity and the intestinal protective barrier function. Some studies have shown that probiotic combinations exhibit superior functions in protecting the intestines and regulating the immune system compared to single strains (Chapman et al., 2011). However, the enhancement effects and potential mechanism of complex probiotics consisting of *Bifidobacterium lactis* XLTG11, *Lactobacillus casei* Zhang, *Lactobacillus plantarum* CCFM8661, and *Lactobacillus rhamnosus* Probio-M9 have not been reported. Broad-spectrum penicillins, cephalosporins, and clindamycin have been observed to cause a high occurrence rate of diarrhea. Ampicillin is a broad-spectrum penicillin antibiotic with antibacterial activity against both Gram-positive and Gram-negative bacteria, and can be used for the development of a model of dysbacteriosis diarrhea (Zhang et al., 2019; Xu et al., 2022). The aim of our study was to investigate the alleviation effects of complex probiotics on the gut microbiota of an AAD mouse model. This study will provide a reference for the development of complex probiotics preparations.

2. Materials and methods

2.1. Experimental materials

2.1.1. Bacterial strain and culture

Complex probiotics powder (*Bifidobacterium lactis* XLTG11, *Lactobacillus casei* Zhang, *Lactobacillus plantarum* CCFM8661, and *Lactobacillus rhamnosus* Probio-M9) was obtained from Jinhua Yinhe Biological Technology Co., Ltd. (Jinhua, China). The complex probiotics powder was stored in a refrigerator at −80°C before the experiment. Normal saline was added to prepare 2.5×10^6, 5×10^6, and 1×10^7 cfu complex probiotics solutions for immediate use.

2.1.2. Animals

Six-week-old specific pathogen-free (SPF) BALB/c male mice were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, China). The animals were raised in an animal room with a barrier environment, kept in a room under controlled temperature (20–23°C) and humidity (30–60%) under a 12:12-h light–dark cycle, with free access to feed and water. All animal procedures were carried out in compliance with Heilongjiang University of Traditional Chinese Medicine's Regulations on the Administration of Laboratory Animals, and the university's Animal Ethics Committee approved all experiments (ethic approval code: 2021121201).

2.2. Experimental methods

2.2.1. Experimental design

After 1 week of acclimatization, BALB/c mice were randomly divided into six groups ($n=6$), including the normal control group (NC), model control group (MC), positive drug control group (PDC), low-, medium-, and high-dose complex probiotics groups (LD, MD, and HD). The mice in the MC, PDC, LD, MD, and HD groups (except for those in the NC group) received ampicillin by gavage twice daily at a dose of 11.2 g·kg^{-1} each time. The mice in the NC group were given the same volume of normal saline at the same time every day for 3 consecutive days. After modeling, the mice in the PDC group were given Bifico (1×10^7 cfu) by gavage, and those in the LD, MD, and HD groups were given complex probiotics (2.5×10^6, 5×10^6, and 1×10^7 cfu). For 14 days, the mice in the NC and the MC groups received the same amount of normal saline. After the experiment, blood was sampled from the eyes and stored at −80°C. Fresh feces were collected under sterile conditions and stored at −80°C for gut microbiota analysis. Then, ileum tissue was collected for histopathological analysis and colon tissue for real-time quantitative polymerase chain reaction and ELISA assays.

2.2.2. Weight gain, degree of loose stool, and fecal water content

At 8:00 a.m. on the first day of diarrhea model development, the body weight of the mice in each group was measured and recorded, and then the mice were given ampicillin. At 18:00 every day, the body weight of all mice was also measured and recorded. On the 3rd and 14th day of the experiment, fresh feces of mice in each group were weighed and dried and then weighed again to obtain their dry weight and wet weight, and the degree of loose stool was established using predefined criteria (Table 1).

$$\text{Fecal water content}\,(\%)=\frac{\text{wet fecal weight}-\text{dry fecal weight}}{\text{wet fecal weight}}\times100\%$$

2.2.3. The levels of total protein and albumin in the serum

Further, 1 h after the last administration, blood was sampled from the eyes and let stand for about 1 h, 3,000 r/min^{-1} centrifuged for 10 min, and then separated the serum. The levels of TP and ALB in the serum samples were determined by ELISA kits (Jiangsu Meimian industrial Co., Ltd., Jiangsu, China).

2.2.4. Assays of intestinal permeability in the serum

The levels of LPS and D-LA in the serum samples were determined by ELISA kits (Jiangsu Meimian industrial Co., Ltd., Jiangsu, China).

2.2.5. Histopathological analysis of ileum

Ileum tissue was fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin (Wuhan Junjie Electronics Co., Ltd., Wuhan, China), and cut into 4-μm sections (Leica Instrument Shanghai Ltd., Shanghai, China). These slices were dewaxed and then stained with hematoxylin–eosin (HE; Servicebio Co., Ltd., Wuhan, China), then photographed and observed under a light

TABLE 1 Criteria to determine the degree of loose stool.

Filter paper surface stain diameter/cm	Degree of loose stool
<1	1
1–1.9	2
2–2.9	3
3–3.9	4
4–4.9	5

TABLE 2 Design of RT-PCR primer sequences.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
ZO-1	GCGAACAGAAGGAGCGAGAAGAG	GCTTTGCGGGCTGACTGGAG
Occludin	TGGCTATGGAGGCGGCTATGG	AAGGAAGCGATGAAGCAGAAGGC
Claudin-1	GCTGGGTTTCATCCTGGCTTCTC	CCTGAGCGGTCACGATGTTGTC
MUC2	TGCTGACGAGTGGTTGGTGAATG	TGATGAGGTGGCAGACAGGAGAC
β-actin	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

microscope (Nikon Eclipse Ci-L, 100× magnification; Nikon, Tokyo, Japan).

2.2.6. Real-time quantitative PCR

Real-time quantitative PCR (RT-PCR) was used to determine the relative mRNA levels of tight junction protein genes (ZO-1, claudin-1, and occludin) and MUC2. Appropriate samples of colon tissue were taken and homogenized. Using the TaKaRa Kit, total RNA was extracted from the colon tissue. Then, mRNA was subjected to reverse transcription into cDNA with PrimeScriptTmRT reagent kit with gDNA Eraser manufactured (TaKaRa). The TB Green® Premix Ex Taq™II kit (TaKaRa) was used to prepare the reaction solution and amplification was performed using a real-time fluorescent quantitative PCR system (QuantStudio 3). The sequences of the primers are presented in Table 1. The following reaction conditions were applied: pre-denaturation 95°C (30 s), PCR 95°C (5 s), 60°C (34 s), repeated for 40 cycles to determine the Ct value of specific gene and the Ct value of β-actin. The expression of genes was examined using the $2^{-\Delta\Delta Ct}$ method (Table 2).

2.2.7. ELISA measurement of inflammatory cytokines of colon

Colon tissues (100 mg) were homogenized in 900 μL of ice-cold PBS using a homogenizer, and the supernatant was then transferred into sterile tubes after centrifugation at 10,000 rpm for 10 min at 4°C. The levels of SIgA, IL-6, IL-1β, and TNF-α in the colon tissue were determined with ELISA kits (Jiangsu Meimian industrial Co., Ltd., Jiangsu, China).

2.2.8. 16S rRNA gene sequencing

The 16S amplicon sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). Next, we utilized the MagPure Soil DNA LQ Kit (Shanghai Magen Biotechnology Co., Ltd., Shanghai, China) to extract genomic DNA from the fecal samples of each group ($n = 6$). The purity and quantity of DNA were confirmed using agarose gel and NanoDrop. Afterward, the bacterial 16S rRNA gene V3-V4 region was amplified using the method/manual of the manufacturer by polymerase chain reaction (PCR) with the forward primer 343F (5′- TACGGRAGGCAGCAG −3′) and the reverse primer 798R (5′- AGGGTATCTAATCCT-3′). Amplicon quality was visualized using gel electrophoresis, purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN), and amplified for another round of PCR. The final amplicon was measured using the dsDNA HS Assay Kit for Qubit after being once more purified with AMPure XP beads (Yeasen Biotechnology Co., Ltd., Shanghai, China). Sequencing was performed on the Illumina Miseq platform (Illumina Inc., San Diego, CA, United States).

2.2.9. Statistical analysis

The data of the present study were statistically analyzed using SPSS 26.0 software (IBM, Armonk, NY, United States) and are expressed as mean ± SD. One-way ANOVA was employed for comparing differences between groups, followed by Student–Newman–Keuls (S-N-K) test. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Effects of complex probiotics on general mice effects in AAD model mice

3.1.1. Effects of complex probiotics on weight gain, fecal water content, and loose stool grade

Significant differences in the weight gain, fecal water content, and loose stool grade were observed between the NC group and the MC group ($p < 0.001$). Compared with the MC group, the fecal water content of mice in the PDC, LD, MD, and HD groups decreased in a dose-dependent manner by 25.40% ($p < 0.05$), 18.15% ($p > 0.05$), 27.16% ($p < 0.05$), 37.52% ($p < 0.01$), respectively; the loose stool grade decreased by 31.49% ($p < 0.001$), 21.55% ($p < 0.001$), 27.62% ($p < 0.001$), and 33.70% ($p < 0.001$), respectively. Compared with the MC group, the weight gain of the PDC, MD, and HD groups increased by 69.46% ($p < 0.05$), 62.83% ($p > 0.05$), and 125.67% ($p < 0.001$). The weight gain in the LD group was by 46.61% lower ($p < 0.05$) than that in the PDC group. The results showed that the high-dose of the complex probiotics not only significantly improved the diarrhea symptoms of the AAD model mice, but also promoted their growth. The therapeutic effect of Bifico was better than that of the low-dose complex probiotic (Figures 1A–C).

3.1.2. Effects of complex probiotics on intestinal permeability

The contents of LPS and D-LA in the MC group were higher than those in the NC group by 24.87 and 18.59% ($p < 0.01$ or $p < 0.001$), respectively, indicating that ampicillin changed the intestinal permeability of the mice. The content of LPS in the PDC, MD, and HD groups was lower than that in the MC group by 19.15% ($p < 0.01$), 15.46% ($p < 0.05$), and 16.88% ($p < 0.05$), correspondingly; the contents of D-LA in these three groups were lower by 12.14% ($p < 0.01$), 5.99% ($p > 0.05$), and 11.44% ($p < 0.01$), respectively, than that in the MC group. Compared with the PDC group, the content of D-LA in the LD group increased by 12.26% ($p < 0.01$). These findings revealed that the high-dose of complex probiotic had a good effect on reducing intestinal barrier

permeability. The therapeutic effect of the low dose of the complex probiotic was weaker than that of Bifico (Figures 1D,E).

3.2. Effects of complex probiotics on the index of intestinal immune barrier in AAD model mice

3.2.1. Effects of complex probiotics on serum total protein and albumin

Compared with the NC group, the levels of TP and ALB in the serum in the MC group were reduced by 25.09% ($p < 0.01$) and 16.45% ($p < 0.05$). Compared with the MC group, the level of TP in the PDC, MD, and HD groups increased by 28.12% ($p < 0.05$), 36.40% ($p < 0.01$), 55.06% ($p < 0.001$), and the level of ALB increased by 19.32% ($p < 0.05$), 35.23% ($p < 0.001$), and 37.28% ($p < 0.001$). The levels of TP in the HD group were higher than those in the PDC group by 21.02% ($p < 0.05$), indicating that the high-dose complex probiotic have a better therapeutic effect than Bifico (Figures 2A,B).

3.2.2. Effects of complex probiotics on inflammatory cytokines in the colon tissue

We found that the levels of IL-6, IL-1β, and TNF-α in the colon tissue in the MC group were higher than those in the NC group by 37.66% ($p < 0.001$), 27.95% ($p < 0.01$), 23.89% ($p < 0.01$), respectively, whereas the levels of IL-10 and SIgA were lower by 15.01% ($p < 0.001$) and 20.58% ($p < 0.001$), correspondingly. These results indicated that ampicillin triggered inflammation by increasing the secretion of pro-inflammatory factors and decreasing the secretion of anti-inflammatory factors. Compared with the MC group, the levels of SIgA in the PDC, LD, MD, and HD groups increased by 12.33% ($p < 0.05$), 10.66% ($p < 0.05$), 13.67% ($p < 0.05$), and 20.15% ($p < 0.001$), respectively. The levels of IL-6 decreased by 16.54% ($p < 0.05$), 10.31% ($p > 0.05$), 14.97% ($p < 0.05$), and 18.59% ($p < 0.01$), correspondingly. The levels of IL-1β decreased by 21.42% ($p < 0.01$), 10.28% ($p > 0.05$), 13.81% ($p < 0.05$), and 19.34% ($p < 0.01$), respectively, whereas the levels of IL-10 increased by 14.39% ($p < 0.01$), 4.19% ($p > 0.05$), 10.90% ($p < 0.05$), and 16.86% ($p < 0.01$), correspondingly. These results suggested that the complex probiotics decreased the levels of IL-6, IL-1β, and increased the levels of IL-10 in the colon tissue in a dose-dependent manner. Compared with the MC group, the levels of TNF-α in the HD group decreased by 14.29% ($p < 0.05$). However, there was no significant difference in the levels of TNF-α between the MD group and the MC group ($p > 0.05$). Compared with the PDC group, the levels of TNF-α in the LD group increased by 14.80% ($p < 0.05$), the levels of IL-10 decreased by 8.92% ($p < 0.05$). These results showed that the high-dose complex probiotics regulated the level of inflammatory factors and promoted the immune function of the AAD model mice. The therapeutic effect of the low-dose complex probiotic was weaker than that of Bifico (Figures 2C–G).

3.3. Effects of complex probiotics on the intestinal in AAD model mice

3.3.1. Effects of complex probiotics on ileum histopathological analysis

The photomicrographs of (HE)-stained intestinal sections are displayed in Figure 3A. In the NC group, the small intestinal mucosa structure was intact, and the villi were arranged closely and neatly. In the MC group, the small intestinal mucosa was damaged severely, and the villi were arranged sparsely. The probiotic administration

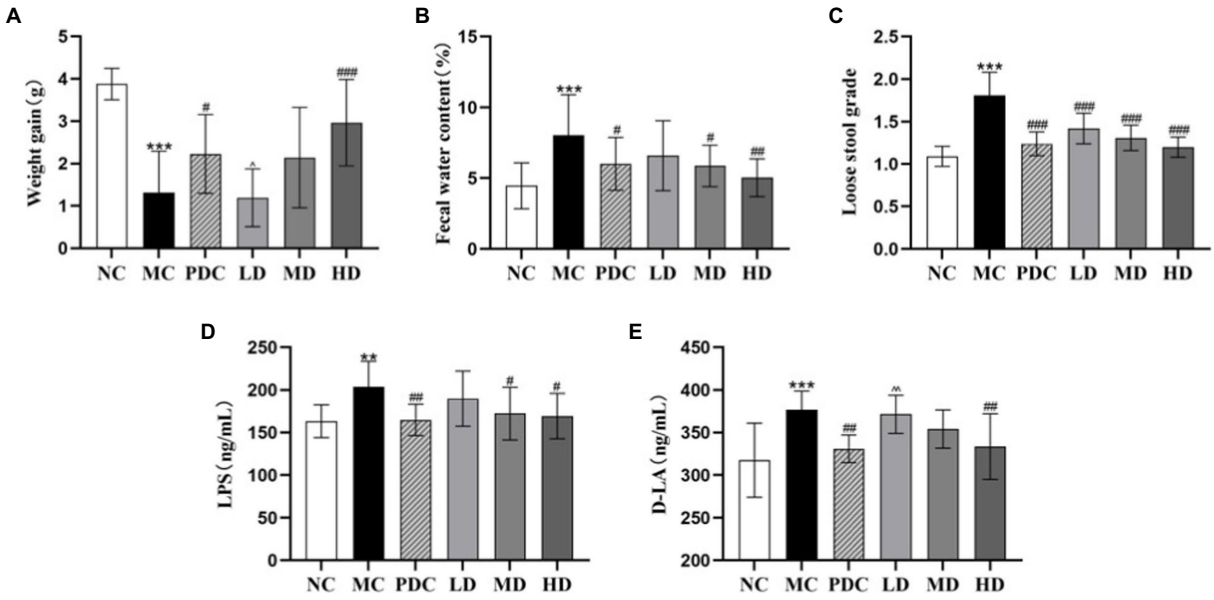

FIGURE 1
Effects of complex probiotics on general mice effects in AAD model mice (n=6). **(A)** Weight gain; **(B)** Fecal water content; **(C)** Loose stool grade; **(D)** LPS; **(E)** D-LA. NC, normal control group; MC, model control group; PDC, Positive drug control group; LD, low-dose complex probiotics group; MD, medium-dose complex probiotics group; HD, high-dose complex probiotics group. All data are expressed as mean±SD. Compared with the NC group **p<0.01, ***p<0.001; Compared with the MC group $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001; Compared with the PDC group $^{\wedge}p$<0.05, $^{\wedge\wedge}p$<0.01.

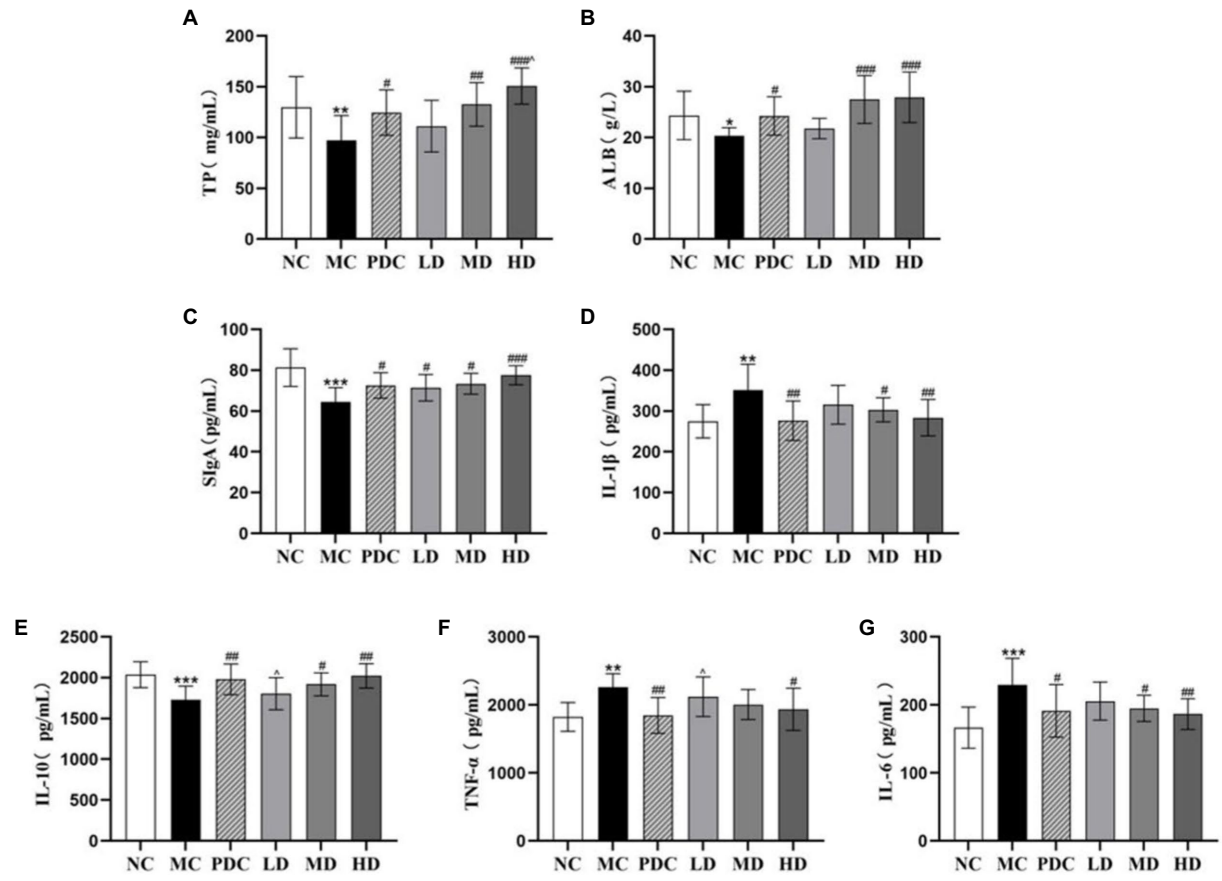

FIGURE 2
Effects of complex probiotics on the index of intestinal immune barrier in AAD model mice (n=6). **(A)** TP; **(B)** ALB; **(C)** SIgA; **(D)** IL-1β; **(E)** IL-10; **(F)** TNF-α; **(G)** IL-6. NC, normal control group; MC, model control group; PDC, Positive drug control group; LD, low-dose complex probiotics group; MD, medium-dose complex probiotics group; HD, high-dose complex probiotics group. All data are expressed as mean±SD. Compared with the NC group *p<0.05, **p<0.01, ***p<0.001; Compared with the MC group $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001; Compared with the PDC group $^{\wedge}p$<0.05.

attenuated the damage degree of the small intestinal mucosa in different degrees in a dose-dependent manner. These findings showed that ampicillin can damage the intestinal structural integrity, whereas the complex probiotic effectively prevented and ameliorated the intestinal damage in a dose-dependent fashion.

3.3.2. Effects of complex probiotics on ZO-1, occludin, claudin-1, and MUC2 mRNA expression in the colon tissue

The mRNA expression levels of ZO-1, occludin, claudin-1, and MUC2 in the MC group were lower than those in the NC group by 60.74% ($p < 0.001$), 51.20% ($p < 0.001$), 51.83% ($p < 0.001$), and 61.24% ($p < 0.001$), respectively, indicating that the AAD model was successfully established. Compared with the MC group, the mRNA expression levels of ZO-1 in the PDC, LD, MD, and HD groups increased by 72.17% ($p < 0.01$), 44.26% ($p > 0.05$), 64.58% ($p < 0.01$), and 119.76% ($p < 0.001$), the mRNA expression levels of occludin increased by 86.40% ($p < 0.001$), 42.83% ($p < 0.05$), 55.74% ($p < 0.01$), and 98.03% ($p < 0.001$), the mRNA expression levels of claudin-1 increased by 86.55% ($p < 0.001$), 33.02% ($p < 0.05$), 36.27% ($p < 0.05$), and 82.05% ($p < 0.001$), and the mRNA expression levels of MUC2 in the PDC and HD groups increased by 47.44% ($p < 0.01$) and 81.03% ($p < 0.001$). However, there was no significant difference in the mRNA expression levels of MUC2 between the MD group and the MC group. Compared with the PDC group, the mRNA expression levels of ZO-1 in the HD group were increased by 27.24% ($p < 0.05$), the mRNA expression levels of occludin and MUC2 in the LD group were reduced by 23.37% ($p < 0.05$) and 35.35% ($p < 0.01$). The mRNA expression levels of MUC2 in the HD group were 22.78% higher ($p < 0.05$), whereas the mRNA expression levels of claudin-1 in the LD and MD groups were lower by 28.69% ($p < 0.001$) and 26.95% ($p < 0.001$), respectively. The aforementioned results indicated that the high dose of the complex probiotics effectively alleviated the damage of the intestinal barrier functional protein in the AAD model mice (Figures 3B–E).

3.4. Effects of complex probiotics on the diversity and structure of gut microbiota in AAD model mice

3.4.1. Alpha diversity analysis

The species richness and evenness of distribution within a sample were assessed by alpha-diversity analysis. The Chao1, Shannon, and Simpson indices were employed for the evaluations in this experiment. The Chao1, Shannon, and Simpson indices in the MC group were significantly lower than those in the NC group ($p < 0.01$). Notably, the

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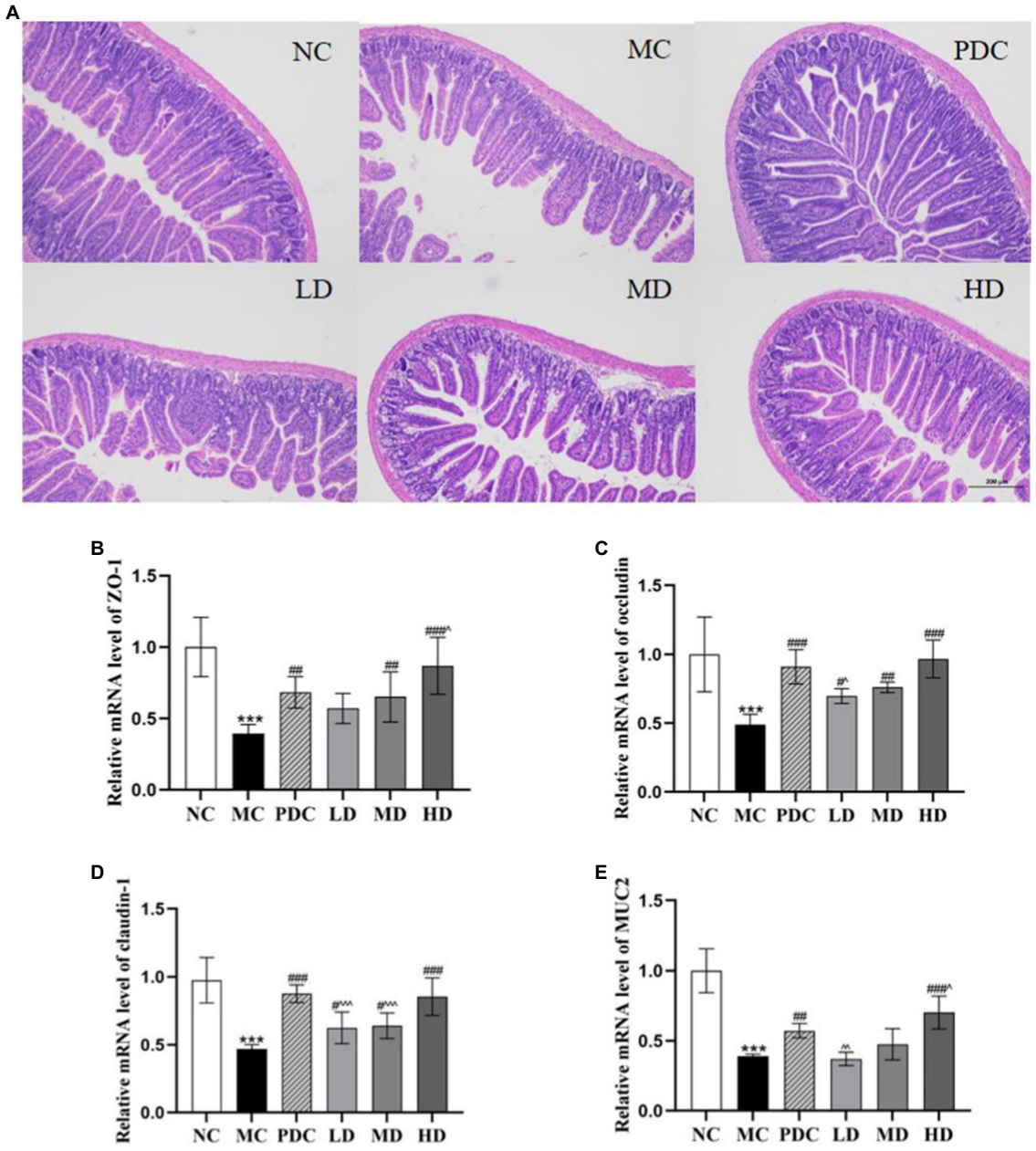

FIGURE 3
Effects of complex probiotics on the intestinal in AAD model mice (n=6). **(A)** HE staining of jejunum section (×100), **(B)** ZO-1, **(C)** occludin, **(D)** claudin-1, **(E)** MUC2. NC, normal control group; MC, model control group; PDC, Positive drug control group; LD, low-dose complex probiotics group; MD, medium-dose complex probiotics group; HD, high-dose complex probiotics group. All data are expressed as mean±SD. Compared with the NC group ***p<0.001; Compared with the MC group $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001; Compared with the PDC group $^{\wedge}p$<0.05, $^{\wedge\wedge}p$<0.01, $^{\wedge\wedge\wedge}p$<0.001.

administration of the high-dose complex probiotics significantly increased the values of the Chao1, Shannon, and Simpson indices ($p<0.05$ or $p<0.01$). The combined use of the complex probiotics effectively promoted the diversity and uniformity of the gut microbiota (Figures 4A–C).

3.4.2. Beta diversity analysis

Principal coordinates analysis was applied to assess community similarity and difference (PCoA). The results showed that the gut microbiota of the MC and NC groups were significantly different, indicating that the AAD model was successfully developed. Significant differences were found between the microflora structure of the MC group and the complex probiotics groups (Figure 4D).

3.4.3. Effects of complex probiotics on the gut microbiota composition in the AAD model mice

At the phylum level, the composition of the intestinal microorganisms in each group at the phylum level included *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Epsilonbacteraeota*, and *Actinobacteriota*. Of them, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the dominant flora representatives in the

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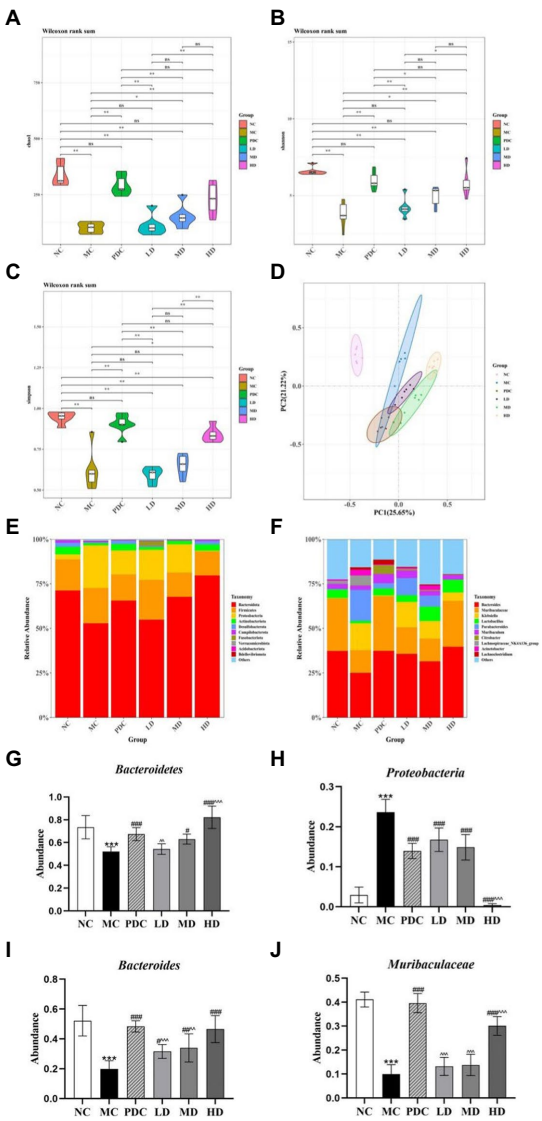

FIGURE 4
Effects of complex probiotics on the diversity and structure of gut microbiota in AAD model mice (n=6). **(A)** Chao1; **(B)** Shannon; **(C)** Simpson; **(D)** Principle coordinate analysis (PCoA); **(E)** Gut microbiota composition at the phylum level; **(F)** Gut microbiota composition at the genus level; **(G)** The abundance of *Bacteroidetes*; **(H)** The abundance of *Proteobacteria*; **(I)** The abundance of *Bacteroides*; **(J)** The abundance of *Muribaculaceae*. NC, normal control group; MC, model control group; PDC, Positive drug control group; LD, low-dose complex probiotics group; MD, medium-dose complex probiotics group; HD, high-dose complex probiotics group. All data are expressed as mean±SD. Compared with the NC group ***p<0.001; Compared with the MC group $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001; Compared with the PDC group $^{\wedge\wedge}p$<0.01, $^{\wedge\wedge\wedge}p$<0.001.

intestinal tract. The relative abundance of *Bacteroidetes* in the MC group ($p < 0.001$) was lower, whereas the relative abundance of *Proteobacteria* was higher ($p < 0.001$). The obtained results indicated that ampicillin transformed the composition of the gut microbiota in the various groups, leading to the overgrowth of *Proteobacteria* but restricted growth of *Bacteroidetes*, which was generally considered to be a feature of dysbiosis. Compared with the MC group, the relative abundance of *Bacteroidetes* in the MD and HD groups increased by 20.93% ($p < 0.05$), 57.76% ($p < 0.001$), and the relative abundance of *Proteobacteria* decreased by 37.06% ($p < 0.001$) and 98.25% ($p < 0.001$). Compared with the PDC group, the relative abundance of *Bacteroidetes* in the LD group decreased by 19.46% ($p < 0.01$), the relative abundance of *Bacteroidetes* in the HD group increased by 21.96% ($p < 0.001$), and the relative abundance of *Proteobacteria* in the HD group decreased by 97.03% ($p < 0.001$). Therefore, the complex probiotics were found to significantly alter the relative abundance of various phyla after antibiotic administration (Figures 4E,G,H).

At the genus level, the composition of the intestinal microorganisms in each group at the genus level included *Bacteroides*, *Muribaculaceae*, *Klebsiella*, *Lactobacillus*, and *Parabacteroides*. Compared with the NC group, the relative abundance of *Bacteroides* and *Muribaculaceae* in the MC group decreased by 62.06% ($p < 0.001$) and 75.78% ($p < 0.001$). The relative abundance of *Klebsiella* and *Parabacteroides* in the MC group were increased. Compared with the MC group, the abundance of *Klebsiella* and *Parabacteroides* in the HD group were reduced, the relative abundance of *Bacteroides* in the MD and HD groups increased by 71.51% ($p < 0.01$), 135.12% ($p < 0.001$), and the relative abundance of *Muribaculaceae* increased by 38.69% ($p > 0.05$), 202.28% ($p < 0.001$). Compared with the PDC group, the relative abundance of *Bacteroides* in the LD and MD groups decreased by 34.77% ($p < 0.001$) and 29.83% ($p < 0.01$), the relative abundance of *Muribaculaceae* in the LD, MD, and HD groups decreased by 66.72% ($p < 0.001$), 65.11% ($p < 0.001$), and 23.97% ($p < 0.001$). The complex probiotics supplementation after antibiotic administration significantly altered the relative intestinal microbial abundance at the genus level (Figures 4F,I,J).

3.5. Correlation analysis between diarrhea-related indexes, cytokine levels, intestinal protective barrier-related genes expression, and dominant gut microbiota

To understand the involved and interacted roles of the intestinal microbiota in probiotics-mediated benefits, the correlation analysis between diarrhea-related indexes, cytokine levels, intestinal protective barrier-related genes expression, and dominant gut microbiota at the genus levels was analyzed. The relative abundance of *Bacteroides* and *Muribaculaceae* were significantly positively correlated with weight gain, TP, ALB, tight junction proteins (ZO-1, occludin, and claudin-1), MUC2, SIgA, and IL-10, while was significantly negatively correlated with fecal water content, loose stool grade, LPS, D-LA, and pro-inflammation cytokines (IL-1β, TNF-α, and IL-6). *Klebsiella* and *Parabacteroides* were positively associated with fecal water content, loose stool grade, LPS, D-LA, and pro-inflammation cytokines (IL-1β, TNF-α, and IL-6), while were negatively associated with weight gain, TP, ALB, tight junction proteins (ZO-1, occludin, and claudin-1), MUC2, SIgA, and IL-10. However, the *Lactobacillus* was positively associated with weight gain, TP, ALB, tight junction proteins (ZO-1, occludin, and claudin-1), MUC2, SIgA, and IL-10, but was significantly negatively correlated with fecal water content, LPS,

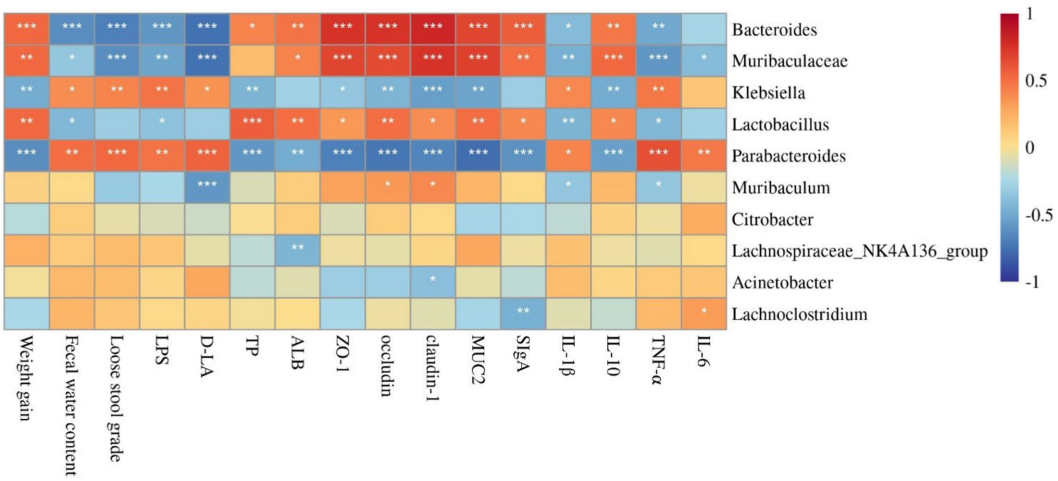

FIGURE 5
Correlation analysis between diarrhea-related indexes, cytokine levels, intestinal protective barrier-related genes expression, and dominant gut microbiota by Spearman. *, **, and *** indicate the associations significant ($p<0.05$, $p<0.01$, and $p<0.001$, respectively).

IL-1β, and TNF-α. The relative abundance of *Muribaculum* was positively associated with occludin and claudin-1, while was significantly negatively correlated with D-LA, IL-1β, and TNF-α. The relative abundance of *Lachnospiraceae NK4A136 group* was negatively associated with ALB. The relative abundance of *Acinetobacter* was significantly negatively correlated with claudin-1. The relative abundance of *Lachnoclostridium* was positively correlated with IL-6, but was negatively correlated with SIgA (Figure 5).

4. Discussion

Antibiotic use over an extended period alters the balance of the intestinal microbiota, causes an imbalance in the gut microbiota, and can result in AAD (Overeem et al., 2008; Cui et al., 2020). Probiotics, on the other hand, regulate the gut microbiota, increase the integrity of the intestinal barrier, and enhance immunity (Wang X. et al., 2021). Therefore, finding complex probiotics that can better regulate gut microbiota is the first task of anti-AAD researchers.

Different types of probiotics exist, but the most common of them include Lactobacilli and Bifidobacteria, which are widely used in many functional foods and dietary supplements (Turroni et al., 2011; Xue et al., 2017). Studies have demonstrated that *Bifidobacterium lactis* XLTG11 can regulate the secretion of inflammatory cytokines and gut microbiota activities. Additionally, it produces substantial amounts of acetic, propionic, and butyric acid, which lower the intestinal pH, inhibit the proliferation of harmful bacteria, and strengthen gut protection, thereby improving AAD (Wang N. et al., 2021; Xu et al., 2022; Ma W. et al., 2023; Ma Y. et al., 2023). Additionally, *Lactobacillus casei* Zhang was established to regulate tight-junction proteins and inflammatory factor expression, alleviating gut microbiota disorders (Wang et al., 2019; Li X. et al., 2021). In addition, it also enhanced immunity by increasing the production of IgA and IFN-γ (Wang et al., 2013), and induced gut mucosal responses by promoting the production of SIgA (Ya et al., 2008). Studies have shown that *Lactobacillus plantarum* CCFM8661 can regulate the diversity and composition of the gut microbiota, alleviate the pathological damage to the colon and ileum, increase jejunum villus height-crypt depth ratio (Tian et al., 2012; Wang et al., 2019; Yu et al., 2021). *Lacticaseibacillus rhamnosus* Probio-M9 is a novel probiotic strain isolated from human breast milk of healthy women (Zhang et al., 2022). Oral Probio-M9 administration regulated the stability of the gut microbiota (Zheng et al., 2021). Probio-M9 has demonstrated excellent tolerance to gastrointestinal digestive fluids. Its survival rate after being subjected to artificial gastric fluid for 3 h is 83.72%, and after being subjected to artificial intestinal fluid for 11 h, it is 78.33%. Even when exposed to a pH of 2.5, Probio-M9 can pass through the stomach and reach the intestines to exert its beneficial effects (Liu et al., 2020). Furthermore, Probio-M9 can improve the intestinal environment by increasing the diversity of gut microbiota and regulating metabolic pathways (Xu et al., 2021). Furthermore, research has demonstrated that the symbiotic relationship between Lactobacillus and Bifidobacterium can synergistically enhance the production of metabolites, bolster their antibacterial capabilities, suppress the growth of pathogenic microorganisms, reinforce the integrity of the mucosal barrier, and promote the upregulation of immune responses (Chang et al., 2017). These studies showed that single species can regulate immunity and gut through different mechanisms, and we speculated that the combined use of multiple strains can enhance the immunomodulatory effects and protect the intestinal barrier function. In this experiment, *Bifidobacterium lactis* XLTG11, *Lactobacillus casei* Zhang, *Lactobacillus plantarum* CCFM8661, and *Lactobacillus rhamnosus* Probio-M9 were mixed at a ratio of 1:1:1:1 to prepare the complex probiotics utilized to study their alleviative effect on ampicillin-induced AAD.

Commonly used indicators to assess the severity of diarrhea include body weight gain, fecal water content, and fecal consistency

(Hu et al., 2020; Ma Y. et al., 2023). *Bifidobacterium animalis* subsp. *lactis* XLTG11 not only significantly improved the diarrhea symptoms of AAD model mice, but also promoted the growth of mice (Ma Y. et al., 2023), which was consistent with our results. LPS and D-LA are important indicators to detect the degree of intestinal damage and permeability (Li et al., 2020; Ma Y. et al., 2023). Our results showed that the high-dose of the complex probiotics reduced the contents of LPS and D-LA to decrease intestinal permeability, which was similar to the results of Xu et al. (2022). TP can be divided into albumin and globulin. Serum protein can maintain the normal colloid osmotic pressure and PH of the blood, transport various metabolites, and regulate immune function. The results we obtained showed that the complex probiotics increased the levels of TP and ALB in the serum. These findings are similar to those of Jiang et al., who found that *Smilax glabra* extract elevated the levels of TP and ALB in antibiotic-associated diarrhea mice, and repaired the damage of the intestinal immune barrier (Jiang et al., 2022).

A primary function of the intestinal epithelium is to form a biological barrier that prevents the invasion of pathogenic antigens and maintains intestinal homeostasis (Feng et al., 2019; Jiang et al., 2021). Tight junctions are the main connection type between intestinal epithelial cells (Peterson and Artis, 2014). ZO-1, occludin, and claudin-1 are the major intestinal barrier proteins. MUC2 participates in the synthesis and secretion of goblet cells, which affect the permeability and integrity of the intestinal mucosa (Zhang et al., 2018; Zhuang et al., 2019). Lack of tight junction protein and MUC2 can induce intestinal epithelial dysfunction, increase intestinal barrier permeability, and increase the entry of harmful bacteria, which can lead to inflammation (Furuse et al., 1993; Van der Sluis et al., 2006; Al-Sadi et al., 2011; Pope et al., 2014; Kuo et al., 2021; Yao D. et al., 2021). Our results showed that the expression levels of ZO-1, claudin-1, occludin, and MUC2 in the colon of the MC group were decreased, indicating that ampicillin caused impairment of intestinal barrier function. However, the supplementation with complex probiotics improved intestinal epithelial permeability. In addition, studies have shown that *Lactobacillus plantarum* can increase the expression levels of ZO-1, occludin, and claudin-1, strengthen epithelial defense functions, and reduce intestinal mucosal permeability (Wang et al., 2018), *Bacteroides fragilis* can increase the expression levels of MUC2 and maintain the integrity of the intestinal barrier, alleviating symptoms of AAD in mice (Zhang et al., 2018), which are consistent with our results.

Antibiotic-associated diarrhea occurrence is accompanied by systemic inflammation (Ling et al., 2015). IL-6 can induce the differentiation of B cells. IL-6 promotes host defense through the stimulation of acute phase responses, immune reactions, and hematopoiesis (Popko et al., 2010; Tanaka et al., 2014). IL-1β is a major pro-inflammatory cytokine, it will aggravate the damage during chronic disease and acute tissue damage (Lopez-Castejon and Brough, 2011). TNF-α is an inflammatory cytokine, which can cause inflammation or cell apoptosis. TNF-α induces the release of a large number of cytokines, including IL-6, IL-8, and IL-1β by stimulating macrophages (Idriss and Naismith, 2000; Ma et al., 2016). IL-10 is a cytokine with a significant anti-inflammatory effect. It inhibits the release of pro-inflammatory factors, and plays an essential role in maintaining gastrointestinal homeostasis (Iyer and Cheng, 2012; Li et al., 2014). The results of this experiment showed that complex probiotics significantly reduced the levels of IL-6, IL-1β, and TNF-α while increasing the level of IL-10 in the colon. Therefore, complex probiotics have a good anti-inflammatory effect in AAD. Studies have shown that echinacin attenuated LPS-induced secretion and mRNA expression of TNF-α and IL-6, enhanced the secretion and mRNA expression of IL-10, and weakened the inflammatory response, which is consistent with our previous results (Li et al., 2018). SIgA is the main component of the mucosal defense system of the body and plays a decisive role in the first line of defense against diseases (Kumar et al., 2020; Chen S. et al., 2022). Our present results showed that complex probiotics increased the SIgA level in the colon tissue, which was similar to the results of Peng C. et al. (2022).

In this study, we further analyzed the diversity and richness of the intestinal microorganisms at the phylum and genus levels. At the phylum level, *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* were the dominant phyla (Shin et al., 2015; Guan et al., 2018). *Bacteroides* and *Firmicutes* were also established to be abundant in the intestinal tract of healthy people (Schultz et al., 2017). An increase in the relative abundance of *Proteobacteria* led to a decrease in short-chain fatty acid (SCFA) production (Meng et al., 2020), which indicates a risk of infection and metabolic disorders (Shao et al., 2020). Our results showed that ampicillin administration transformed the composition of the gut microbiota in the examined groups, leading to *Proteobacteria* overgrowth and restricted growth of *Bacteroidetes*. Notably, the complex probiotic treatment reversed these changes, which was similar to the results obtained by Wang N. et al. (2021). At the genus level, *Bacteroides* is a Gram-negative, obligate anaerobic bacteria that controls lymphocyte and cytokine expression and suppresses inflammation (Tan et al., 2019). *Muribaculateae* can reduce cholesterol content and prevent excessive cholesterol levels by regulating intestinal microbiota and its metabolites (Li et al., 2022). *Klebsiella* is the pathogen of antibiotic-associated hemorrhagic colitis (Högenauer et al., 2006), it was positively correlated with inflammatory factors such as TNF-α and IL-6 (Liang et al., 2022). Lactic acid and other metabolites produced by the *Lactobacillus* living in the intestine can kill pathogenic microorganisms. In addition, such microbial metabolites also regulate gut microbiota diversity, enhance immunity, improve intestinal function, and maintain normal intestinal homeostasis (Zafar and Saier, 2020). The results of this experiment showed that the treatment with complex probiotics regulated the abundance of *Bacteroides*, *Muribaculateae*, *Klebsiella*, *Lactobacillus*, and *Parabolides*, which was similar to earlier results (Xu et al., 2022). All of the aforementioned experimental findings imply that complex probiotics may exert therapeutic effects in patients with diarrhea and gut microbiota imbalance caused by antibiotics.

This study used mice as the animal model for diarrhea because mice share a high genetic similarity with humans; mice have pure strains, which can reduce interference from different strains; mice are small and easy to breed indoors on a large scale. However, there are significant differences between mice and humans in anatomy and physiology. Moreover, the mice model cannot reflect how human lifestyle, diet, age, and other factors affect the gut microbiota and immune system. These limitations may reduce the generalizability and validity of the experimental results, making it hard to apply the research on complex probiotics for treating AAD to clinical practice.

Therefore, future research should consider using animal models closer to humans or testing the findings of this study in clinical trials.

5. Conclusion

In summary, complex probiotics composed of *Bifidobacterium lactis* XLTG11, *Lactobacillus casei* Zhang, *Lactobacillus plantarum* CCFM8661, and *Lactobacillus rhamnosus* Probio-M9 could alleviate ampicillin-induced AAD in mice including protecting intestinal barrier function, regulating the secretion of proinflammatory factors, the gut microbiota diversity and composition. Our research will provide further evidence and theoretical support for the treatment of antibiotic-associated diarrhea with complex probiotics. In the future, it will be feasible to study the differential therapeutic efficacy among various combinations of microbial strains and explore which strains or combinations are more effective in treating AAD, thereby providing more robust evidence for their clinical application.

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 all animal procedures were carried out by Heilongjiang University of Traditional Chinese Medicine's Regulations on the Administration of Laboratory Animals, and the University's Animal Ethics Committee approved all experiments (Ethic approval code: 2021121201).

Author contributions

WL and SZ participated in the analysis of experimental data, analyzed and discussed the results, drew graphs, and wrote the paper. YW, HB, and SY conducted experiments, analyzed and discussed the results, and helped to revise the manuscript. LH and WM were mainly responsible for conceiving and designing the study, directing and supervising the experiment, and helping to revise 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/fmicb.2023.1156058/full#supplementary-material

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Lactobacillus paracasei ATG-E1 improves particulate matter 10 plus diesel exhaust particles (PM_{10}D)-induced airway inflammation by regulating immune responses

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Particulate matter (PM) exposure can adversely affect respiratory function. Probiotics can alleviate the inflammatory responses in respiratory diseases. We examined the protective effects of *Lactobacillus paracasei* ATG-E1 isolated from the feces of a newborn baby against airway inflammation in a PM_{10} plus diesel exhaust particle (DEP) (PM_{10}D)-induced airway inflammation model. BALB/c mice were exposed to PM_{10}D by intranasal injection three times at 3-day intervals for 12 days, and *L. paracasei* ATG-E1 was administered orally for 12 days. Analysis of immune cell population and expression of various inflammatory mediators and gut barrier-related genes were determined in bronchoalveolar lavage fluid (BALF), lung, peyer's patch, and small intestine. A histological analysis of the lungs was performed. In addition, the *in vitro* safety and their safety in genomic analyses were examined. *L. paracasei* ATG-E1 was found to be safe *in vitro* and by genomic analysis. *L. paracasei* ATG-E1 suppressed neutrophil infiltration and the number of $CD4^+$, $CD4^+CD69^+$, $CD62L^-CD44^{+high}$, $CD21/35^+B220^+$, and $Gr\text{-}1^+CD11b^+$ cells, as well as the expression of inflammatory mediators, including chemokine (C-X-C motif) ligand (CXCL)-1, macrophage inflammatory protein (MIP)-2, interleukin (IL)-17a, tumor necrosis factor (TNF)-α, and IL-6 in BALF and lungs in PM_{10}D-induced airway inflammation. It protected against histopathological damage in the lungs of mice with PM_{10}D-induced airway inflammation. *L. paracasei* ATG-E1 concomitantly increased the expression levels of the gut barrier function-related genes occludin, claudin-1, and IL-10 in the small intestine, with an increased number of $CD4^+$ and $CD4^+CD25^+$ immune cells in the peyer's patch. *L. paracasei* ATG-E1 suppressed immune activation and airway inflammatory responses in the airways and lungs by restoring the lung damage by PM_{10}D. It also regulated intestinal immunity and ameliorated the gut barrier function in

the ileum. These results indicate the potential of *L. paracasei* ATG-E1 as an protective and therapeutic agent against airway inflammation and respiratory diseases.

KEYWORDS

probiotics, *Lactobacillus paracasei*, particulate matter, diesel exhaust particles, airway inflammation

Introduction

With increasing industrialization and urbanization, air pollution is steadily increasing and has become a great concern because it induces various adverse health effects and rises the risks of mortality and morbidity through damage to the lungs and respiratory system (Brauer et al., 2012; Kim et al., 2013). Particulate matter (PM) is one of air pollutant that health threats (Gehring et al., 2010; Nkhama et al., 2017). PM consists of a heterogeneous mixture of solid and liquid granular substances in the atmosphere (Kim et al., 2015), and its chemical composition varies depending on its source, including metals, ions, nitrates, sulfates, elemental and organic carbon, and polycyclic aromatic hydrocarbons (van der Valk et al., 2015; Winterbottom et al., 2018; Riediker et al., 2019). According to the diameter, PM is divided into coarse particles (diameter $\leq$ 10 μm, PM_{10}), fine particles (diameter $\leq$ 2.5 μm, $PM_{2.5}$), and ultrafine particles (diameter $\leq$ 0.1 μm, $PM_{0.1}$) (Gehring et al., 2010), and the size of PM may differently affect the respiratory system (Kim et al., 2015; Riediker et al., 2019). When PM is inhaled, the body eliminates it through processes such as sneezing and coughing (Cadelis et al., 2014). PM less than 10 μm in diameter is deposited on epithelial cells and alveolar macrophages in the lung, which causes an inflammatory response and injury in the airway and lung through stimulation of oxidative stress and the immune system. This leads to cell death, bronchial fibrosis, and a decline in respiratory functions, but it has also been reported to make worsen the asthma and chronic obstructive pulmonary disease (COPD) (Leikauf et al., 2020). Therefore, despite the interest in PM and related research is growing, there is a need for novel agents to prevent and treat PM-induced inflammation and respiratory damage.

A probiotic is defined as "a live microorganism that when administrated in adequate amounts confers a health benefit on the host" (FAO/WHO, 2002). Probiotics were originally known to regulate the intestinal microbial balance, and they have been shown to improve the inflammatory response in hosts due to their immunomodulatory effects (Forsythe, 2011, 2014). *Lactobacillus rhamnosus* GG, *Lactobacillus gasseri, Lactobacillus reuteri, and Bifidobacterium lactis Bb12* have been shown to decrease airway inflammatory responses by regulating immune properties in lung diseases, such as asthma, allergic rhinitis, and COPD (Feleszko et al., 2007; Forsythe et al., 2007; Carvalho et al., 2020). Furthermore, it has recently been reported that *Bifidobacterium lactis* can inhibit PM-induced lung inflammation (Wang et al., 2017). Therefore, these studies suggest that probiotics can alleviate respiratory diseases by modulating immune functions. However, few studies to date have examined the effects of probiotic strains in pathological conditions caused by air pollution, especially PM and DEP, for which there have been few studies so far. In the present study, we investigated whether *L. paracasei* ATG-E1 has beneficial effects on respiratory function through regulation of the immune and inflammatory responses in the airway and lung, and we also examined the probiotics properties including their safety and functionalities in genomic analyses as potential probiotics for improving respiratory health.

Materials and methods

Isolation and identification of *L. paracasei* ATG-E1

Lactobacillus paracasei ATG-E1 was isolated from an anonymous donor in Daejeon, Republic of Korea, who kindly provided fecal samples from a newborn baby (January 2016). In brief, fecal samples were blended with NaCl (0.85% w/v) in a sterile bag and then serially diluted with NaCl (0.85%). The serially diluted fecal samples were plated on De Man Rogosa Sharpe broth (MRS, Difco Laboratories Inc., Franklin Lakes, NJ, USA) agar plates and then incubated at 37°C for 24—48 h. Colonies grown on MRS agar plates were selected for isolation and purification based on colony morphology by microscopy and catalase activity. A catalase-negative strain was identified by 16S rRNA sequencing and analyzed using the BLAST database with a sequence-matching program and was called *L. paracasei* ATG-E1. Isolated *L. paracasei* ATG-E1 was deposited in the Korean Collection for Type Cultures (KCTC 14245BP).

Probiotic properties of *L. paracasei* ATG-E1

Carbohydrate fermentation patterns

The fermentation patterns of *L. paracasei* ATG-E1 were examined using an API® 50 CHL test (bioMérieux, Inc., Marcy l'Etoile, France), according to the manufacturer's protocols. Briefly, cultures of *L. paracasei* ATG-E1 were suspended in API® 50 CHL medium, and then applied to cupules on an API® 50CH test strip. The patterns of fermentation were observed for up 72 h at 37°C.

Acid and bile tolerance

An aliquot of *L. paracasei* ATG-E1 culture was inoculated into MRS broth adjusted to pH 3.0 and incubated at 37°C for 1 h. For the

bile tolerance test, *L. paracasei* ATG-E1 culture was inoculated into MRS broth containing 0.3% (w/v) oxgall (Sigma-Aldrich, St. Louis, MO, USA) that was incubated at 37°C for a further 3 h. The number of viable bacterial cells was determined by MRS agar plating after 0, 1, and 3 h and expressed as log CFU/mL.

Bile salt hydrolase (BSH) activity

L. paracasei ATG-E1 was spotted onto MRS agar plates containing the 0.5% (w/v) sodium salt taurodeoxycholic acid (TDCA, Sigma-Aldrich, St. Louis, MO, USA) and incubated under anaerobic conditions at 37°C for 48 h. The presence of halos around opaque white colonies indicated BSH activity. An MRS agar plate without *L. paracasei* ATG-E1 was used as a negative control.

Safety test assessment of *L. paracasei* ATG-E1

Antibiotic resistance testing

A culture of *L. paracasei* ATG-E1 was diluted in 0.9% saline to an OD_{600} of 0.8. The bacterial suspensions were spread onto counting agar plates (PCA; Difco Laboratories Inc., Franklin Lakes, NJ, USA), and E-test strips (BioMérieux, Inc., Marcy-l'Étoile, France) were placed on the agar plates. The minimal inhibitory concentration (MIC) values were compared with the breakpoint values, as suggested by the EFSA and Panel on Additives and Products or Substances used in Animal Feed [FEEDAP], 2012. The antibiotics erythromycin, gentamycin, ampicillin, tetracycline, chloramphenicol, streptomycin, ciprofloxacin, and penicillin G were used in this experiment.

Hemolysis

L. paracasei ATG-E1 was streaked on a blood agar plate containing 5% sheep blood (Asanpharm Co., Ltd., Seoul, Republic of Korea) and incubated at 37°C for 24–48 h. The ability to produce hemolysins was measured by the formation of any clear (β-hemolysis) or greenish (α-hemolysis) hemolytic zones and no such zone (γ-hemolysis) around the *Lactobacillus* colonies. Biogenic amine formation *L. paracasei* ATG-E1 was cultured in MRS broth containing the precursor amino acids tyrosine, histidine, ornithine, and lysine (Sigma-Aldrich, St. Louis, MO, USA) to identify the production of the biogenic amines such as tyramine, histamine, putrescine, and cadaverine. Production of biogenic amines was determined by the color change of the pH indicator bromocresol purple. MRS broth was used as a negative control.

Whole-genome analysis of *L. paracasei* ATG-E1

A single colony of *L. paracasei* ATG-E1 was transferred into MRS broth in a shaking incubator at 37°C for 6 h. Genomic DNA was extracted from the culture broths after 6 h using a QIAmp DNA mini Kit (QIAGEN, Stanford, CA, USA) and the concentration was determined using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and then the DNA quality and integrity were checked using a LabChip® GX Touch™ nucleic acid analyzer (PerkinElmer, Waltham, MA, USA). Whole genomes were sequenced on a MinION platform (Oxford Nanopore Technologies, Oxford, UK) with an R9.4.1-flow cell.

Genome assembly, gene prediction, and safety assessment

Sequence reads (fastq files) were base-called using Guppy v3.4.5[1] with the high-accuracy base-calling algorithm. Fastq files were assembled with Flye v. 2.9 (Kolmogorov et al., 2019) using the default settings. Complete genomes were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (Tatusova et al., 2016) and Average Nucleotide Identity (ANI) values were obtained using the ANI Calculator tool on EzBiocloud[2] (Yoon et al., 2017). PathogenFinder was used to determine pathogenic potential (Cosentino et al., 2013) and ResFinder was used to determine antimicrobial susceptibility (Bortolaia et al., 2020).

Animal experiments

Sample preparation

L. paracasei ATG-E1 was incubated in MRS broth at 37°C for 16 h, and cells were then obtained by centrifugation (3,000 × g, 10 min, 4°C) and washed three times with phosphate-buffered saline (PBS; pH 7.4). The cell pellets were resuspended in a cryoprotectant solution and lyophilized using an FD8508 freeze-dryer (ilShinBioBase, Seoul, Republic of Korea). The freeze-dried *L. paracasei* ATG-E1 powder was resuspended in saline and prepared daily for the animal experiments.

Animals and experimental procedures

BALB/c mice (male, 6–8 weeks old) were obtained from Orient Bio Co., Ltd. (Gyeonggi-do, Republic of Korea). The mice were housed in animal facility with environmental conditions (temperature of 22 ± 2°C, a humidity of 60 ± 10%, and a 12-h light/dark cycle), with free access to food and water. This experimental protocol was approved by the Committee for Animal Welfare at Daejeon University (DJUARB2019-021) and was performed in accordance with the committee guidelines. The mice received intranasal administration of a mixture of PM_{10} (ERM CZ-120, Sigma-Aldrich, USA) and diesel exhaust particles (DEP, SRM 2975, Sigma-Aldrich, St. Louis, MO, USA) on days 4, 7, and 10 and were orally treated with *L. paracasei* ATG-E1 every other day for 12 days. PM_{10} (3 mg/mL) and DEP (0.6 mg/mL) were dissolved in 1% aluminum hydroxide gel adjuvant. The mice were divided into following groups (n = 8 per group): (1) 1% aluminum hydroxide gel adjuvant-treated mice (NC), (2) PM_{10} + DEP-sensitized mice (PM_{10}D-CTL), (3) 4×10^{10} CFU of ATG-E1-treated PM_{10} + DEP-sensitized mice (PM_{10}D-ATG-E1), and (4) 3 mg/kg dexamethasone-treated PM_{10} + DEP-sensitized mice (PM_{10}D-Dexa, positive control). All mice were sacrificed and blood, bronchoalveolar lavage fluid (BALF), and lung and intestinal tissues were harvested on day 12.

1 https://community.nanoporetech.com/downloads
2 http://www.ezbiocloud.net/

BALF collection and cytological analysis

Bronchoalveolar lavage fluid was harvested by cannulation into the trachea and airways and centrifuged (400 × *g*, 5 min, 4°C). The collected BALF cells were washed and suspended in PBS and then used by fluorescence-activated cell sorting (FACS). For cytological analysis, cells from the BALF were collected on glass slides by centrifugation (400 × *g*, 4 min), fixed, stained with Diff-Quick® Stain (Baxter Healthcare Corp., Miami, FL, USA), and the number of neutrophils was counted.

Digestion of pulmonary tissue and cell preparations

Lung tissues were minced and incubated in PBS containing collagenase IV (1 mg/mL) and dispase (2 mg/mL) at 37°C on a shaker. After incubation, the dissolved lung tissues were filtered through a cell strainer and centrifuged (2,000 × *g*, 20 min). The total cell numbers were measured with a hemocytometer.

Flow-cytometric analysis

Cells from the BALF and lung tissue were incubated with monoclonal antibodies against CD3, CD4, CD8, CD69, CD62L, CD44, CD25, granulocytic marker Gr-1, and CD11b, CD11c, and B220, washed with FACS buffer, fixed with 0.5% paraformaldehyde, washed again with FACS buffer, and analyzed on a FACSCalibur™ with the CellQuest™ software (BD Biosciences, San Diego, CA, USA).

Analysis of inflammatory mediators in BALF

The levels of CXCL-1, MIP-2, IL-17a, and TNF-α in BALF were measured using ELISA kits, according to the manufacturer's instructions (R&D Systems, St. Louis, MO, USA).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA from the lung and small intestine was extracted using TRIzol® reagent (Thermo Fisher Scientific), and the total RNA (1 μg) was reverse-transcribed into cDNA using a reverse transcription system (AccuPower RT PreMix, Bioneer, Daejeon, Republic of Korea), according to the manufacturer's instructions. qRT-PCR was carried out using SYBR™ Green PCR Master Mix on the Applied Biosystems 7,500 Real-Time PCR system, follow a manufacturer's instructions. The primer sequences are presented in **Supplementary Table 1**. All the samples were normalized for the corresponding expression of GAPDH, and the expression levels of the gene of interest in treated groups relative to its expression level of the PM_CTL group.

Histopathological analysis of lung tissues

Lung tissues were fixed with 10% neutral-buffered formalin, embedded in paraffin, and sliced into 5 μm sections. The tissue sections were stained with hematoxylin–eosin (H&E), Masson's trichrome (M-T), and periodic acid-Schiff (PAS). Tissue damage were quantified in a blinded manner using a 5-point (0—4) grading system, as previously described (Ozaki et al., 1996).

Statistical analysis

The data are presented as means ± the standard error of the mean (SEM) and were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test using Prism 8.0 software (GraphPad Inc., San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Results

Whole-genome sequence assembly and annotation of *L. paracasei* ATG-E1

The genome of *L. paracasei* ATG-E1 was sequenced using the MinION platform with 320× coverage. The raw sequencing data of ATG-E1 was assembled, results shows a circular chromosome with two plasmids (a total genome size of 3,221,112 bp, **Figure 1**). The genome was annotated using NCBI PGAAP and uploaded in GenBank under accession number GCA_026013725.1. A total of 3,159 open reading frames (ORFs) were predicted from the genome of strain ATG-E1; 3,081 were protein-coding genes, and 78 were RNA genes (**Table 1**). Based on ANI analysis, ATG-E1 exhibited 98.37% sequence identity with the type strain *Lactobacillus paracasei* ATCC 25302.

Characterization of *L. paracasei* ATG-E1

The fermentation patterns of carbohydrate sources by *L. paracasei* ATG-E1 are presented in **Supplementary Table 2**. Based on the pattern identification through the APIWEB database of bioMérieux, the identities (%) of *L. paracasei* ATG-E1 were 99.5% in the *L. paracasei* control group. The gastrointestinal tract is considered to be the main location that affects the viability of probiotics. The survival rate of *L. paracasei* ATG-E1 was 128.13% after 1 h incubation in the environment with a pH = 3.0. As for the simulated 0.3% oxgall solution, the survival rate of *L. paracasei* ATG-E1 was 51.48% (**Supplementary Table 3**). When *L. paracasei* ATG-E1 was cultured on MRS agar plates supplemented with TDCA, no visible halo surrounding colonies or white precipitates with colonies were observed, indicating that *L. paracasei* ATG-E1 did not have BSH activity (**Supplementary Figure 1**).

Safety of *L. paracasei* ATG-E1 confirmed through *in vitro* and whole genome sequence analysis

As shown in **Table 2**, *L. paracasei* ATG-E1 was sensitive to ampicillin, gentamicin, vancomycin, kanamycin, erythromycin, streptomycin, tetracycline, clindamycin, and chloramphenicol compared to the MIC suggested by the EFSA and Panel on Additives and Products or Substances used in Animal Feed [FEEDAP] (2012). When *L. paracasei* ATG-E1 was grown on sheep blood agar, there was no colorless zone around the colonies, which is considered to indicate γ-hemolytic activity (**Table 2**), *L. paracasei* ATG-E1 did not produce any biogenic amines, cadaverine, histamine, putrescine, or tyramine from lysine, histidine, ornithine, or tyrosine (**Table 2** and **Supplementary Figure 2**). In further safety tests, the *L. paracasei* ATG-E1 strain was predicted to be a non-human pathogen with PathogenFinder1.1 and did not have

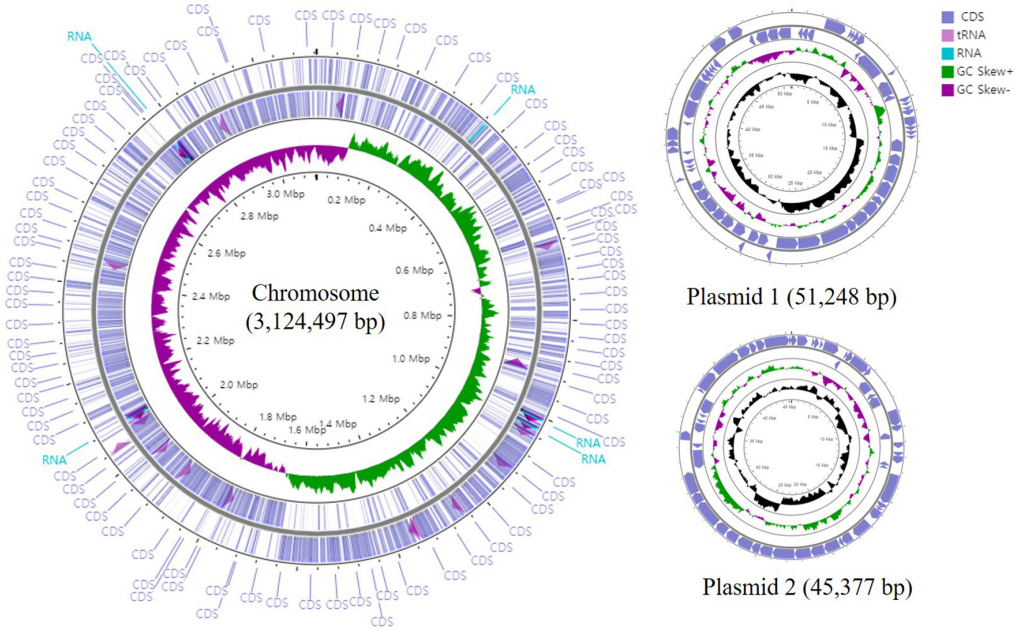

FIGURE 1
Circular genome map of *L. paracasei* ATG-E1.

antibiotic resistance genes with ResFinder 4.1 (**Supplementary Datasheet 1, 2**).

Effects of *L. paracasei* ATG-E1 on the number of total cells and neutrophils in BALF and lung tissue

The total number of cells in the BALF and lungs was higher in the CTL group than in the NC group, but it did not differ in the lungs and BALF after *L. paracasei* ATG-E1 and dexamethasone treatment (**Figures 2A, B**). The number of neutrophils in BALF in the CTL group was increased than that in the NC group, but the *L. paracasei* ATG-E1- and dexamethasone-treated groups had significantly lower neutrophil infiltration than the CTL group (**Figures 2C, D**).

TABLE 1 General features of *L. paracasei* ATG-E1.

Attribute	Value of strain ATG-E1
Total genome size (bp)	32,21,122
Chromosome size	31,24,497
Plasmid number	2
DNA G+C (%)	46
Total genes	3,159
Protein coding genes	2,910
rRNA genes	5,5,5 (5S, 16S, 23S)
tRNA genes	60
ncRNA genes	3
Pseudo genes	171
GenBank accession	GCA_02613725.1

Effects of *L. paracasei* ATG-E1 on lung tissue damage

As shown in **Figure 3A**, various immune cells, including neutrophils and macrophages, infiltrated into the airway, and destruction of alveolar cells, collagen deposition, and increase of goblet cells were observed in the CTL group compared with the NC group. *L. paracasei* ATG-E1 and dexamethasone treatment recovered these histological scores compared with the CTL group (**Figure 3B**).

Effects of *L. paracasei* ATG-E1 on the number of immune cells in BALF and lung tissues

The absolute numbers of lymphocytes, neutrophils, neutrophils to eosinophils ratio, $CD4^+$, $CD8^+$, $CD4^+CD69^+$, $CD62L^-CD44^{+high}$, and $Gr\text{-}1^+CD11b^+$ cells were increased in the BALF of the CTL group compared with the NC group (**Table 3** and **Supplementary Figures 3-5**). *L. paracasei* ATG-E1 treatment decreased the absolute number of neutrophils and $CD4^+$, $CD4^+CD69^+$, and $CD62L^-CD44^{+high}$ in BALF compared with the CTL group, but there was no change in the absolute number of lymphocytes, neutrophils to eosinophils ratio, $CD8^+$, and $Gr\text{-}1^+CD11b^+$ cells. The absolute numbers of eosinophils did not differ among treated groups. Dexamethasone treatment did not affect the number of immune cells. In the

TABLE 2 *In vitro* safety test of *L. paracasei* ATG-E1.

		MIC (μg/ml)									Biogenic amines			
	Hemolysis	AMP	VAN	GEN	KAN	STR	ERY	TET	CLI	CHL	Tyramine	Histamine	Putrescine	Cadaverine
Lactobacillus paracasei ATG-E1	γ	2	N.R	24	48	16	0.125	0.75	0.19	4	–	–	–	–
EFSA and Panel on Additives and Products or Substances used in Animal Feed [FEEDAP], 2012		4	N.R	32	64	64	1	1	4	4				

AMP, ampicillin; VAN, vancomycin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; TET, tetracycline; CLI, clindamycin; CHL, chloramphenicol; N.R, not required.

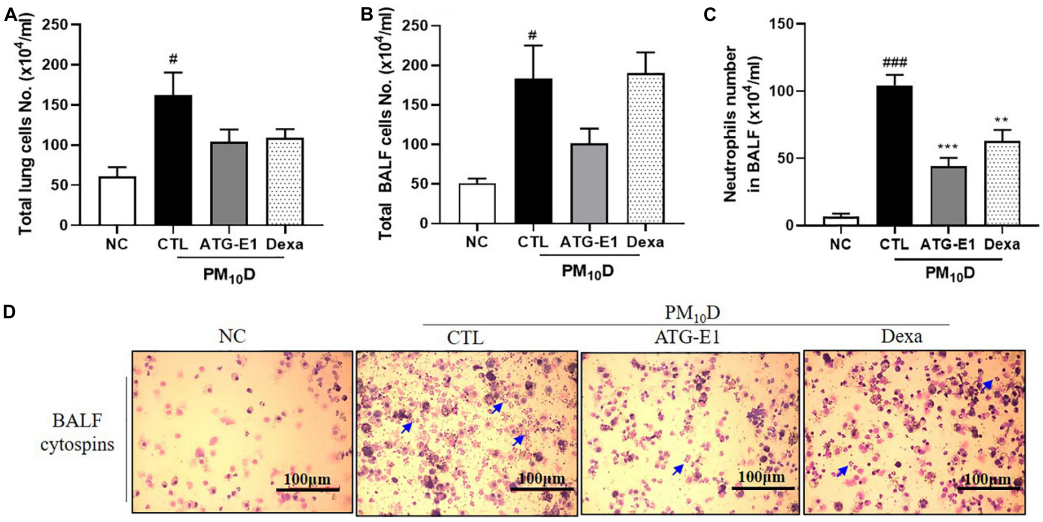

FIGURE 2
Effects of *L. paracasei* ATG-E1 on cell numbers in a PM_{10}D-induced airway inflammation animal. Total number of **(A)** lung cells, **(B)** BALF cells, **(C)** neutrophil number in BALF cytospins, and **(D)** photomicrograph of BALF cytospins. The data are presented as means ± SEM (n = 8). Blue arrow represents the neutrophils. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.005$ vs. NC; $^{**}p < 0.01$ and $^{***}p < 0.005$ vs. CTL. NC: normal mice; CTL: PM_{10}D-sensitized control mice; Dexa: 3 mg/kg dexamethasone-treated PM_{10}D-sensitized mice; ATG-E1: 4×10^9 CFU/day of *L. paracasei* ATG-E1-treated PM_{10}D-sensitized mice.

lungs, the absolute numbers of neutrophils, eosinophils, $CD4^+$, $CD8^+$, $CD4^+CD69^+$, $CD62L^-CD44^{+high}$, and $Gr\text{-}1^+CD11b^+$ cells were higher in the CTL group than in the NC group, whereas the absolute numbers of these cells, including neutrophils, $CD4^+$, $CD4^+CD69^+$, $CD62L^-CD44^{+high}$, $CD21/35^+B220^+$, and $Gr\text{-}1^+CD11b^+$ were decreased by *L. paracasei* ATG-E1 and dexamethasone treatment (Table 3). These findings indicate that *L. paracasei* ATG-E1 effectively inhibited the hyperactivation of the immune system caused by PM_{10}D.

Effects of *L. paracasei* ATG-E1 on inflammatory mediators in BALF and lung

CXCL-1, MIP-2, IL-17a, and TNF-α levels in BALF of the CTL group were increased than those of the NC group, but *L. paracasei* ATG-E1 and dexamethasone treatment significantly suppressed the levels of these cytokines and chemokines compared with the CTL group (Figures 4A–D). In the lungs, CXCL-1 and MIP-2 mRNA expression levels showed a reduction tendency, although there were no significant differences (Figures 4E, F). IL-6 and TNF-α mRNA expression levels were higher in the CTL group than in the NC group, but these gene expression levels were lowered by administration of *L. paracasei* ATG-E1 and dexamethasone (Figures 4G, H). These results demonstrate that *L. paracasei* ATG-E1 may downregulate cytokine expression during PM_{10}D-induced airway inflammation.

Effects of *L. paracasei* ATG-E1 on intestinal immunity

In peyer's patch, the absolute numbers of $CD4^+$, $CD4^+CD25^+$, and $B220^+CD69^+$ cells were decreased in the CTL group compared with the NC group. However, the absolute number of $CD4^+$ and $CD4^+CD25^+$ cells increased, and the absolute number of $B220^+CD69^+$ cells increased in the *L. paracasei* ATG-E1-treated

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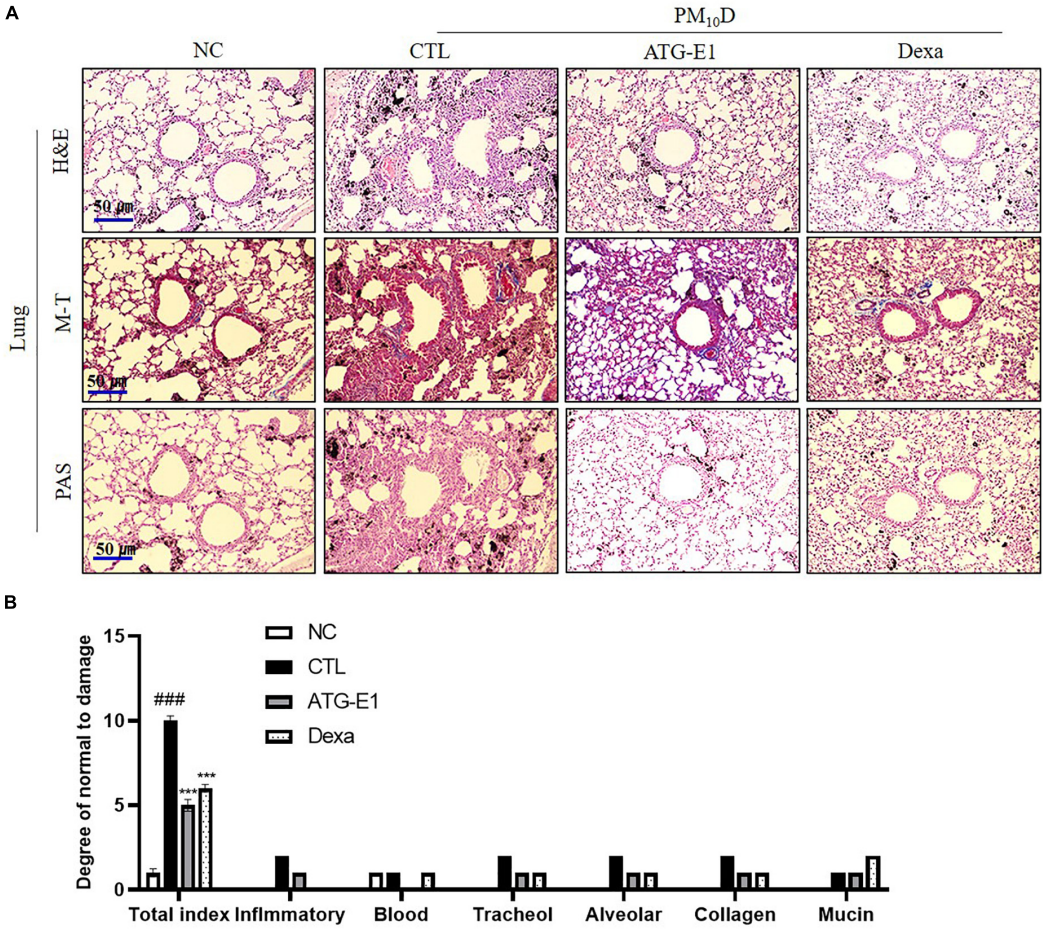

FIGURE 3
Effects of *L. paracasei* ATG-E1 on histological analysis in a PM_{10}D-induced airway inflammation animal. **(A)** H&E, MT, and PAS staining of lung tissues and **(B)** quantitative analyses of the degree of lung tissue damage. The data are presented as means ± SEM (n = 8). $^{###}p < 0.001$ vs. NC; $^{***}p < 0.005$ vs. CTL. NC: normal mice; CTL: PM_{10}D-sensitized control mice; Dexa: 3 mg/kg dexamethasone-treated PM_{10}D-sensitized mice; ATG-E1: 4×10^9 CFU/day of *L. paracasei* ATG-E1-treated PM_{10}D-sensitized mice.

group (Figures 5A–C). In the case of $CD11c^+CD69^+$, the absolute cell number showed an increasing tendency compared with that in the CTL group after administration of ATG-E1 (Figure 5D). Dexamethasone treatment resulted in a significant increase in the number of $CD4^+$ and $B220^+CD69^+$ cells. Next, we investigated the mRNA expression of inflammatory cytokines in the small intestine. TNF-α mRNA expression levels were upregulated in the CTL group than in the NC group, while were downregulated in the *L. paracasei* ATG-E1- and dexamethasone-treated groups (Figure 5E). IL-10 mRNA expression levels did not differ among the NC-, CTL-, and dexamethasone-treated groups, but their expression levels were increased by *L. paracasei* ATG-E1 (Figure 5F).

Effects of *L. paracasei* ATG-E1 on gut barrier function

Claudin-1 mRNA expression levels were downregulated in the ileum of the CTL group compared to the NC group, while they were upregulated by *L. paracasei* ATG-E1 compared to the CTL group (Figure 6A). Occludin mRNA expression levels were also increased by *L. paracasei* ATG-E1, although there was no difference between the NC-, CTL-, and dexamethasone-treated groups (Figure 6B). ZO-1 mRNA expression levels in the *L. paracasei* ATG-E1 group showed a tendency to increase compared with the CTL group, but did not significant (Figure 6C).

Discussion

The application of new probiotic strains as functional health foods and therapies requires careful consideration with regard to safety, although probiotics, especially *Lactobacillus*, are generally considered to be safe. In the present study, we performed genetic and phenotypic analyses of *L. paracasei* ATG-E1 isolated from feces of a newborn baby and *in vitro* safety analysis based on international guidelines for the evaluation of probiotics recommended by the FAO/WHO (2002). *L. paracasei* ATG-E1 did not exhibit antibiotic resistance, toxin production, or hemolytic activity. In addition, there were no antibiotic resistance- or pathogen-related genes in the genome sequence of *L. paracasei* ATG-E1. These results demonstrate that this strain is safe for use in the development of probiotics.

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TABLE 3 The effects of *L. paracasei* ATG-E1 on airway immune cell number in PM_{10}D-induced airway inflammation mouse.

Cell phenotype		PM_{10}D-induced airway inflammation model (Absolute no.)			
		NC	CTL	ATG-E1	Dexa_3 mg/kg
Lymphocytes ($\times 10^7$ cells)	BALF	0.08 ± 0.02	$0.82 \pm 0.05^{###}$	0.87 ± 0.25	0.96 ± 0.53
Neutrophils ($\times 10^7$ cells)		3.59 ± 0.73	$15.09 \pm 3.49^{##}$	$7.58 \pm 1.40^{*}$	15.01 ± 2.14
Eosinophils ($\times 10^7$ cells)		1.18 ± 0.14	1.82 ± 0.49	1.25 ± 1.15	2.29 ± 0.28
Neutrophils: eosinophils ratio		3.22 ± 0.85	$8.52 \pm 0.57^{#}$	5.97 ± 0.61	6.82 ± 1.50
$CD4^+$ ($\times 10^7$ cells)		0.07 ± 0.03	$5.26 \pm 1.14^{###}$	$2.18 \pm 0.48^{**}$	3.43 ± 0.58
$CD8^+$ ($\times 10^7$ cells)		0.14 ± 0.08	$3.72 \pm 0.52^{###}$	2.54 ± 0.52	2.80 ± 0.71
$CD4^+CD69^+$($\times 10^7$ cells)		0.08 ± 0.04	$1.73 \pm 0.52^{##}$	$0.61 \pm 0.10^{*}$	1.40 ± 0.19
$CD62L^-CD44^{+high}$ ($\times 10^7$ cells)		0.99 ± 0.21	$6.40 \pm 1.52^{##}$	$2.54 \pm 0.41^{*}$	5.95 ± 0.85
$Gr\text{-}1^+CD11b^+$ ($\times 10^7$ cells)		0.34 ± 0.06	$1.62 \pm 0.42^{##}$	0.79 ± 0.14	1.38 ± 0.12
Lymphocytes ($\times 10^7$ cells)	Lung	2.60 ± 0.64	3.17 ± 0.51	4.39 ± 0.83	9.34 ± 6.56
Neutrophils ($\times 10^7$ cells)		3.03 ± 0.48	$12.04 \pm 2.26^{##}$	$5.06 \pm 0.67^{*}$	$3.41 \pm 0.83^{**}$
Eosinophils ($\times 10^7$ cells)		1.56 ± 0.20	$6.87 \pm 0.97^{###}$	4.28 ± 1.02	3.41 ± 1.06
$CD4^+$ ($\times 10^7$ cells)		1.97 ± 0.35	$6.13 \pm 1.08^{##}$	$3.39 \pm 0.62^{*}$	$3.32 \pm 0.42^{*}$
$CD8^+$ ($\times 10^7$ cells)		1.11 ± 0.23	$3.46 \pm 0.78^{##}$	1.84 ± 0.28	1.92 ± 0.18
$CD4^+CD69^+$($\times 10^7$ cells)		0.20 ± 0.08	$3.01 \pm 1.08^{#}$	$0.46 \pm 0.11^{*}$	$0.29 \pm 0.07^{*}$
$CD62L^-CD44^{+high}$ ($\times 10^7$ cells)		0.35 ± 0.05	$2.53 \pm 0.50^{##}$	$0.87 \pm 0.06^{*}$	1.27 ± 0.15
$CD21/35^+B220^+$($\times 10^7$ cells)		0.27 ± 0.09	$3.40 \pm 0.72^{###}$	$1.81 \pm 0.35^{*}$	$1.35 \pm 0.32^{**}$
$Gr\text{-}1^+CD11b^+$ ($\times 10^7$ cells)		0.17 ± 0.05	$1.07 \pm 0.09^{###}$	$0.38 \pm 0.06^{***}$	$0.60 \pm 0.08^{***}$

The data are presented as means ± SEM ($n = 8$). $^{#}p < 0.05$, $^{##}p < 0.01$ and $^{###}p < 0.005$ vs. NC; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$ vs. CTL. NC: BALB/c normal mice; CTL: PM_{10}D-sensitized control mice; Dexa: 3 mg/kg dexamethasone-treated PM_{10}D-sensitized mice; ATG-E1: 4×10^9 CFU/day of *L. paracasei* ATG-E1-treated PM_{10}D-sensitized mice.

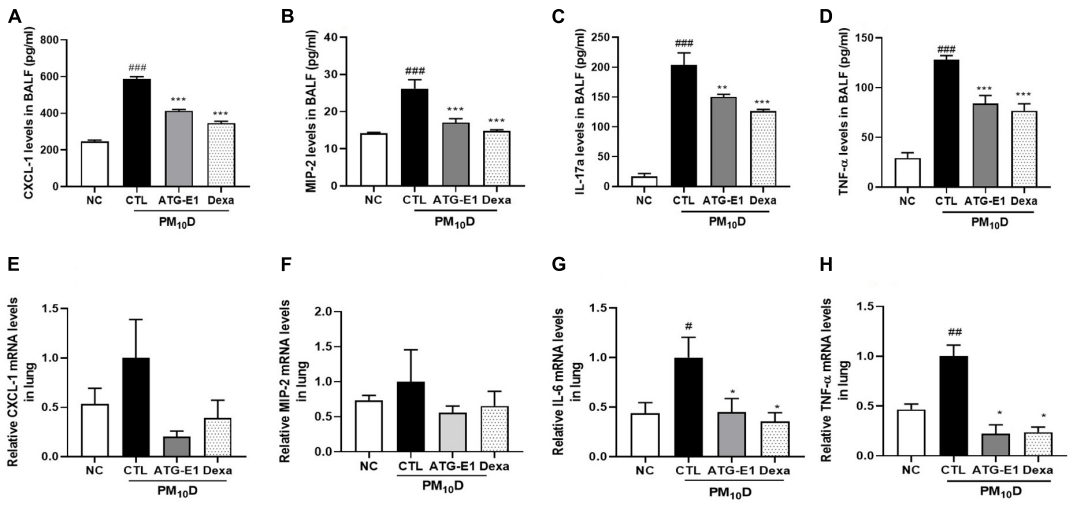

FIGURE 4
Effects of *L. paracasei* ATG-E1 on proinflammatory mediators in BALF and lung of a PM_{10}D-induced airway inflammation animal. **(A)** CXCL-1, **(B)** MIP-2, **(C)** IL-17a, and **(D)** TNF-α levels in the BALF. **(E)** CXCL-1, **(F)** MIP-2, **(G)** IL-6, and **(H)** TNF-α gene expression levels in the lung. The data are presented as means ± SEM ($n = 8$). $^{#}p < 0.05$, $^{##}p < 0.01$, and $^{###}p < 0.005$ vs. NC; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$ vs. CTL. NC: normal mice; CTL: PM_{10}D-sensitized control mice; Dexa: 3 mg/kg dexamethasone-treated PM_{10}D-sensitized mice; ATG-E1: 4×10^9 CFU/day of *L. paracasei* ATG-E1-treated PM_{10}D-sensitized mice.

Exposure to PM and DEP can interact with the immune system and enhance inflammatory responses in the airways and lungs. They cause infiltration of neutrophils and macrophages into the airways and lungs, with increased proinflammatory cytokine levels, in addition to inducing an antigen-presenting cell-mediated inflammatory response and imbalance of T-helper 1 cells (Losacco and Perillo, 2018; Tiotiu et al., 2020). Neutrophils are considered a hallmark of pulmonary inflammation caused by PM since they play an important role in the PM-mediated inflammatory cells infiltration to the airway (Becker et al., 2005; Sarir et al., 2008; Møller et al., 2014). $CD4^+CD69^+$ cells are activated by T cells and suppress the production of proinflammatory cytokines

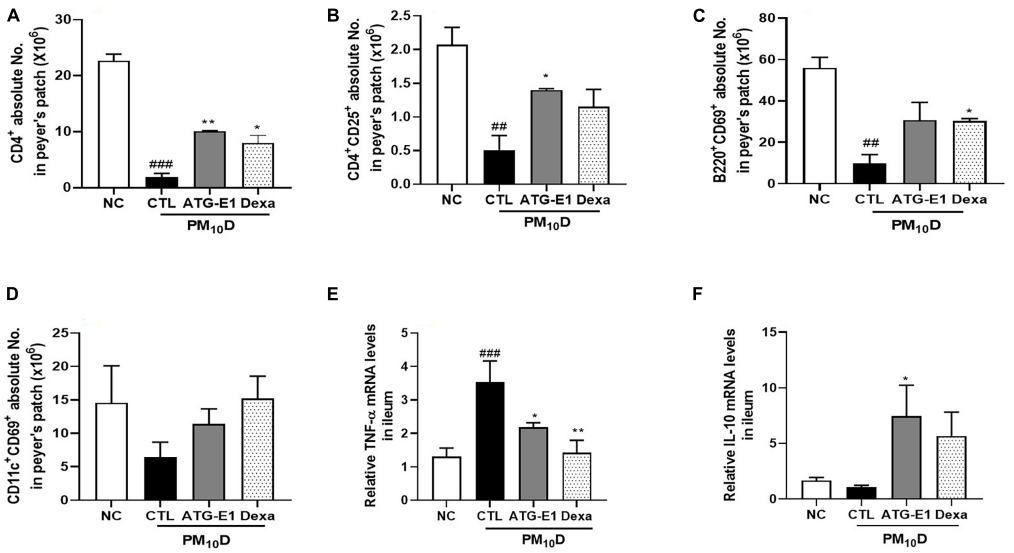

FIGURE 5
Effects of *L. paracasei* ATG-E1 on immune cell numbers and cytokine expression levels in the peyer's patch and the ileum of a PM_{10}D-induced airway inflammation animal. **(A)** $CD4^+$, **(B)** $CD4^+CD69^+$, **(C)** $B220^+CD69^+$, and **(D)** $CD11c^+CD69^+$ absolute cell numbers in peyer's patch. **(E)** TNF-α, and **(F)** IL-10 mRNA expression levels in the ileum. The data are presented as means ± SEM (n = 8). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.05$ vs. NC; $^*p < 0.05$ and $^{**}p < 0.01$ vs. CTL. NC: normal mice; CTL: PM_{10}D-sensitized control mice; Dexa: 3 mg/kg dexamethasone-treated PM_{10}D-sensitized mice; ATG-E1: 4×10^9 CFU/day of *L. paracasei* ATG-E1-treated PM_{10}D-sensitized mice.

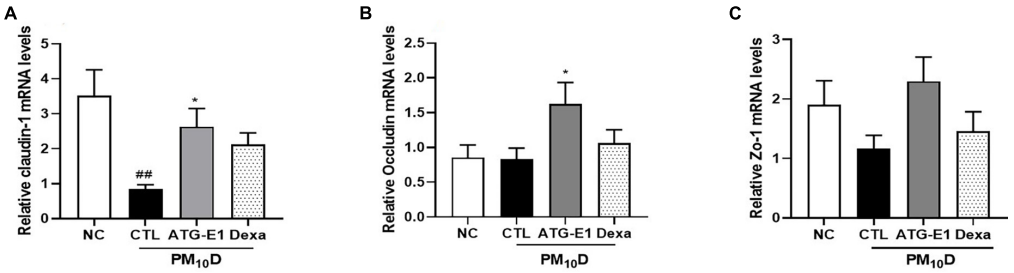

FIGURE 6
Effects of *L. paracasei* ATG-E1 on the expression of tight junction-related genes in the ileum of a PM_{10}D-induced airway inflammation animal. **(A)** Occludin, **(B)** claudin-1, and **(C)** ZO-1 mRNA expression levels in the ileum. The data are presented as means ± SEM (n = 8). $^{\#\#}p < 0.01$ vs. NC; $^*p < 0.05$ vs. CTL. NC: normal mice; CTL: PM_{10}D-sensitized control mice; Dexa: 3 mg/kg dexamethasone-treated PM_{10}D-sensitized mice; ATG-E1: 4×10^9 CFU/day of *L. paracasei* ATG-E1-treated PM_{10}D-sensitized mice.

(Ishikawa et al., 1998). $CD62L^-CD44^{+high}$ markers are increased on naive T cells after activation and have been required for activated T cell extravasation into inflammation site. Additionally, PM has been known to increase the number of $CD62L^-CD44^{+high}$ cells (DeGrendele et al., 1997; Deiuliis et al., 2012). $CD21/35^+B220^+$ are markers of mature B cells and are involved in T cell-dependent responses (Haas et al., 2002). $Gr\text{-}1^+CD11b^+$ markers on the surface of granulocytes are involved in inflammation and cytotoxic T-cell inhibition (Lee et al., 2010). Among chemokines and cytokines, CXCL1, and MIP2 recruit neutrophils and lymphocytes and mediate the neutrophilic inflammatory response in lungs, subsequently promoting the expression of other chemokines (Tsujimoto et al., 2002; Sawant et al., 2015). TNF-α is a pleiotropic cytokine associated with inflammation and immunomodulation and involved in the pathogenesis of lung inflammation (Matera et al., 2010). In the present study, we found that *L. paracasei* ATG-E1 reduced the number of neutrophils and various immune cells, including $CD4^+$, $CD4^+CD69^+$, and $CD62L^-CD44^{+high}$ cells, with a tendency of reduced $Gr\text{-}1^+CD11b^+$ cells in BALF, but also a decreased number of $CD21/35B220^+$ and $Gr\text{-}1^+CD11b^+$ immune cells in the lung. In addition, *L. paracasei* ATG-E1 suppressed the expression of various proinflammatory cytokines, such as CXCL-1, MIP2, IL-17A, and TNF-α, in the BALF and lungs. These results indicated that *L. paracasei* ATG-E1 effectively inhibited PM_{10}D-induced immune activation, consequently suppressing inflammation.

Particulate matter and DEP can result in histopathological changes accompanied by an increase in inflammatory cell infiltration, oxidative products, various cytokines and chemokines, mucus production, and epithelial thickness (Curtis et al., 1991). We observed that airway inflammation and airway wall thickening in the lung were reduced by *L. paracasei* ATG-E1 treatment. These results indicate that *L. paracasei* ATG-E1 protects the lung against PM_{10}D-induced histopathological changes.

Although the lungs are the primary organ exposed to PM and DEP, several studies have reported that exposure to PM is also linked to adverse health effects in the gastrointestinal tract (Kaplan et al., 2010). A part of their exposure can be deposited in the gastrointestinal tract directly or indirectly through swallowing of the PM and DEP deposited in the upper airway (Mutlu et al., 2011). PM and DEP may perturb barrier integrity by affecting the expression of tight junctions and induce inflammation in the gut (Mutlu et al., 2011; Wang et al., 2012). In addition, it has been reported that oral probiotic administration affects immune response in intestine by inducing Treg cells such as $CD4^+CD25^+Foxp3^+$ cells and immunosuppressive cytokines including IL-10 and transforming growth factor-β (Segawa et al., 2008; Lim et al., 2017; Song et al., 2019), Although the exact mechanisms of immunomodulation exerted by probiotics are not fully understood yet, they also improve gut barrier function. *L. paracasei* ATG-E1 increased the number of $CD4^+CD25^+$ cells and IL-10 expression, while decreasing TNF-α expression in the peyer's patch and ileum. These results demonstrate that *L. paracasei* ATG-E1 may have beneficial effects on intestinal immunity by inducing a Treg response; however, further studies are needed in this regard. In addition, we observed that *L. paracasei* ATG-E1 increased the expression of tight junction genes claudin-1 and occludin-1 in the ileum, suggesting that *L. paracasei* ATG-E1 may improve barrier integrity impaired by $PM_{10}D$. These results indicate that *L. paracasei* ATG-E1 has beneficial effects on immunity and barrier function in the intestine during $PM_{10}D$-induced airway inflammation.

In conclusion, *L. paracasei* ATG-E1 was safe and effectively ameliorated lung tissue damage and inhibited airway inflammation by regulation of the immune system. It also regulated intestinal immunity, with improved gut barrier function in $PM_{10}D$-induced airway inflammation. Therefore, the findings of the present study suggest that *L. paracasei* ATG-E1 exerts preventive and protective effects against airway inflammation and respiratory diseases.

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.

Ethics statement

This animal study was reviewed and approved by the Committee for Animal Welfare at Daejeon University.

Author contributions

Y-SL designed and performed the experiments, analyzed the data, and wrote and supervised the drafting of the manuscript. S-HKo and G-SP performed the whole-genome analysis, genome assembly, gene prediction, and safety assessment and wrote the manuscript. W-KY, H-JS, S-HKi, and NJ performed the experiments and analyzed the data. JK supervised the drafting of the manuscript. All authors contributed to the manuscript and approved the final manuscript.

Conflict of interest

Y-SL, G-SP, S-HKo, NJ, and JK were employed by AtoGen 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/fmicb.2023.1145546/full#supplementary-material

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