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Preparation, function and application of postbiotics

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Published in
Frontiers in Microbiology

ISSN 1664-8714
ISBN 978-2-8325-5543-9
DOI 10.3389/978-2-8325-5543-9

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Preparation, function and application of postbiotics

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Citation

Li, Z., Peng, C., Deng, L., Sun, Z., Krych, L., eds. (2024). *Preparation, function and application of postbiotics*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5543-9

Topic Editor Zhaojie Li is employed by YuanDa Biotechnology Co., Ltd., China. Topic Editor Chuantao Peng is the founder and Chairman of YuanDa Biotechnology Co., Ltd., China. Topic Editor Lukasz Krych is employed by GenXone S.A., Poland. The other Topic Editors declare no competing interests with regard to the Research Topic subject.

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frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 30 September 2024
DOI 10.3389/fmicb.2024.1491550

OPEN ACCESS

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RECEIVED 05 September 2024
ACCEPTED 18 September 2024
PUBLISHED 30 September 2024

CITATION
Li Z, Peng C, Sun Z, Deng L and Lukasz K (2024) Editorial: Preparation, function and application of postbiotics.
Front. Microbiol. 15:1491550.
doi: 10.3389/fmicb.2024.1491550

Editorial: Preparation, function and application of postbiotics

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KEYWORDS

postbiotics, metabolites, vesicles, sphingolipids, colorectal cancer, rheumatoid arthritis, ulcerative colitis, yogurt

Editorial on the Research Topic
Preparation, function and application of postbiotics

The use of probiotics in healthcare areas is prevalent and has a long history. However, given the existed issues that viable probiotics face in terms of stability (intolerance to high temperature, oxygen, etc.), stress resistance (sensitivity to complicated food matrixes or *in vivo* gastrointestinal tract) (Li et al., 2019; Aponte et al., 2020), and safety (the use of probiotics in certain groups such as neonates and vulnerable populations, and the possible risk of transmitting antibiotic resistance genes) (Ohishi et al., 2010; Goldenberg et al., 2017; Imperial and Ibana, 2016), in recent years, more and more attentions have been payed to the inactive bacterial cells or their components, termed as postbiotics, making them a prospective alternative to active probiotics. And now, postbiotics have become a new frontier and hotspot in food and pharmaceutical research.

In 2021, the International Scientific Association of Probiotics and Prebiotics (ISAPP) defined postbiotic as "a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). Increasing evidence suggests that postbiotics are becoming valuable tools in combating human diseases. Animal and clinical trials have consistently demonstrated that postbiotics can positively impact immunity, support oral health, prevent osteoporosis, and fight against allergies, and so on (Dong et al., 2024). Certainly, the functions of postbiotics extend well beyond these. Xie et al. reviewed the resisting colorectal cancer (CRC) activity of postbiotics and the mechanisms of its action. It is suggested that intestinal microbial species are strongly associated with the development of CRC, showing increased abundance of pro-inflammatory opportunistic bacteria and decreased abundance of beneficial bacteria. This disorder would destroy the intestinal mucosal barrier, regulate the cell cycle of CRC tumor cells, promote CRC proliferation and metabolism, reprogram the tumor immune microenvironment, cause DNA damage, trigger inflammatory responses, induce gene mutations and alter the resistance to tumor chemotherapy, and so on. The mechanisms of anti-CRC of postbiotics are multifaceted, including regulating intestinal microbiota, enhancing intestinal mucosal barrier function, regulating immune response and regulating systemic metabolism. Ying et al. provided an overview of the self-limiting autoimmune disease, rheumatoid arthritis (RA), summarizing its etiological factors, and reviewing

the beneficial effects of postbiotics on RA. One significant cause of RA is intestinal dysbiosis, an imbalance that can lead to inflammation and ultimately speed up the progression of the disease. The involvement of intestinal microbiota in the development of RA is primarily reflected in mucosal immunity and is linked to the differentiation of T cells, particularly regulatory T cells (Treg) and helper T (Th) cells. Therefore, the intestinal microbiota is an important target for treating rheumatoid arthritis. Postbiotics have been considered as a novel supplement for managing RA. Administering postbiotics orally can boost immunity, regulate the intestinal microbiota, and strengthen the intestinal mucosal barrier function. Moreover, metabolites produced by the intestinal microbiota can enhance the integrity of the intestinal barrier and regulate the Treg/Th17 cell balance, leading to reduced serum IL-17 levels and accelerated bone repair. Diabetic retinopathy (DR), a prevalent microvascular complication in diabetic patients, is commonly associated with gut dysbiosis. As an emerging strategy for treating DR, the beneficial effects of postbiotics on DR were reviewd by Chen et al. Numerous animal studies have shown that postbiotic intervention lowers hyperglycemia, reduces damage to retinal peripapillary and endothelial cells, improves retinal microcirculatory dysfunction, and thereby slows the progression of DR. Postbiotics function through systemic reactions *in vivo* and local reactions in the intestinal lumen, such as balancing the intestinal microbiota, boosting human immunity, and regulating physiological functions by crossing the intestinal barrier.

Like probiotics, the health benefits of postbiotics are also strain-specific. Therefore, animal and clinical trials have been used to screen for new postbiotic products. Moreover, it is important to compare the health benefits of active probiotics with those of their corresponding postbiotics. Bu et al. investigated the effects of *Lacticaseibacillus rhamnosus* 2016SWU.05.0601 (Lr-0601) and its postbiotics on male Kunming mice with dextran sulfate sodium salt (DSS)-induced ulcerative colitis (UC). The results showed that supplementation with both Lr-0601 and its postbiotics can effectively alleviate DSS-induced UC in mice by reducing colonic mucosal damage, down-regulating the levels of pro-inflammatory cytokines, up-regulating tight junction proteins. More importantly, postbiotics offer several advantages, including greater stability and enhanced safety compared to viable probiotics. The results of this study support that postbiotics can be a promising alternative to probiotics to be applied in the prevention and treatment of UC. A prospective, double-blind, placebo-controlled, randomized, parallel study was conducted on females by Motei et al.. The study assessed the impact of dietary supplementation with a postbiotic extract derived from *Bifidobacterium breve* BB091109 on levels of pro-inflammatory cytokines (CRP, IL-6, IL-10, TNF-α, and IFN-γ) and markers of endocrine function (DHEA, estradiol, estriol, progesterone, cortisol, and human growth hormone). The findings indicated that supplementation with this *B. breve*-derived postbiotic could improve endocrine function in women over 40 and promote beneficial changes in inflammatory markers.

As we know, postbiotics possess various functional activities, which might be due to their source bacterial strains, different composition and preparation methods. The active components responsible for the functions of postbiotics include inactivated bacteria, and bacterial fractions (cytosolic polypeptides, phosphoglycolic acids, peptidoglycans, teichoic acids, and surface proteins), etc. What needs to be highlighted is that metabolites can certainly be part of a postbiotic preparation, but they are not essential components of a postbiotic product (Vinderola et al., 2024). Recently, researchers have found some new active components in postbiotics. Bleibel et al. reviewed the role of bacterial extracellular vesicles in exerting the benefits of psychobiotics for neuropsychiatric treatment. It has been found that extracellular vesicles from psychobiotics can be absorbed from the gastrointestinal tract, reach the brain, and deliver intracellular contents to exert beneficial effects in multiple directions. These vesicles seem to enhance the expression of neurotrophic molecules, improve serotonergic neurotransmission, and possibly provide astrocytes with glycolytic enzymes. Sphingolipids, present in both higher animals and prokaryotes, are a class of lipids distinguished by their long-chain bases, which serve as the backbone, and feature an amine group along with two or three hydroxy groups at one end of their structure. The impact of microbial sphingolipids on host health was reviewed by Bai et al. Intestinal microbial sphingolipids can migrate from the gut to various host organs, positively affecting the immune system and metabolism. The administration of these sphingolipids to mice has been shown to produce an anti-inflammatory effect and decrease the number of colonic NKT cells. It has been shown that sphingolipids within outer membrane vesicles (OMVs) of *Bacteroidetes* act as agonists for TLR2 signaling in macrophages, thereby playing a key role in mitigating inflammatory signaling. On the other hand, the preparation process of postbiotics, and fermentation conditions determine the composition of their active ingredients, which in turn define their specific functionality. Nealon et al. investigated and compared metabolite profiles of postbiotics prepared with three lactic acid bacteria strains (*Limosilactobacillus fermentum*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*) cultured with and without rice bran. It was found that postbiotics prepared from rice bran fermented by *L. fermentum* and *L. paracasei* exihibited stronger antibacterial activity against *S. Typhimurium* than their respective probiotic-alone postbiotics. Non-targeted metabolomics analysis revealed that many antibacterial metabolites in *L. fermentum* and *L. paracasei* rice bran postbiotics increased significantly. Sadighbathi et al. investigated the impact of supplementing postbiotics derived from *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) in cheese whey (CW) and skim milk (SM) on the antioxidant activity, viability of yogurt starter cultures, and quality parameters of low-fat yogurt during storage. The results showed that The LB-CW (*Lactobacillus bulgaricus* postbiotic-containing cheese whey) sample exhibited the highest antioxidant activity, and the LB-CW and LB-SM yogurt samples exhibited significantly higher body and texture scores. Tong et al. developed a solid-state fermentation preparation method for postbiotics with increased antimicrobial, antioxidant, and anti-inflammatory activities.

There is a broad consensus that the metabolic changes in microorganisms are regulated by genes. By analyzing the whole genome of the source strain and optimizing fermentation conditions to regulate the expression of certain functional genes, it may be a good strategy for preparing a postbiotic with

specific function. Wu et al. analyzed the whole genome of *Bacillus subtilis* BS21 derived from pig feces and identified seven gene clusters involved in antimicrobial biosynthesis of secondary metabolites. They utilized response surface methodology (RSM) to optimize the medium components and fermentation parameters for antimicrobial secondary metabolite production by strain BS21. As a result, the production of antimicrobial secondary metabolites by BS21 was increased by 43.4%. Fang et al. investigated the changes and effects of fermented milk metabolites in mutant strains of *L. paracasei* following the knockout of the *ldh* gene. The results indicated that all differential metabolites in the mutant strain were upregulated ($P < 0.05$), including amino acids and their precursors, acetyl coenzyme A, and other metabolites involved in amino acid and fatty acid synthesis, all of which are linked to the formation of fermented milk flavor. The data established a connection between the *ldh* genes and strain growth and metabolism, offering potential targets for regulating flavor compounds in fermented milk.

With this Research Topic, we have gathered a collection of 12 research articles that offer fresh perspectives on various aspects of postbiotic research. These studies explored the multifunctional roles of postbiotics and underscored their promising potential for applications in healthcare.

Author contributions

ZL: Conceptualization, Funding acquisition, Writing – original draft. CP: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. ZS: Conceptualization, Formal analysis, Writing – review & editing. LD: Formal analysis, Investigation, Validation, Writing – review & editing. KL: Investigation, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Natural Science Foundation of Shandong Province (ZR2020MC217).

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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TYPE Original Research
PUBLISHED 09 June 2023
DOI 10.3389/fmicb.2023.1195360

OPEN ACCESS

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RECEIVED 28 March 2023
ACCEPTED 15 May 2023
PUBLISHED 09 June 2023

CITATION
Fang S, Song X, Cui L, Bai J, Lu H and Wang S (2023) The lactate dehydrogenase gene is involved in the growth and metabolism of *Lacticaseibacillus paracasei* and the production of fermented milk flavor substances. *Front. Microbiol.* 14:1195360. doi: 10.3389/fmicb.2023.1195360

The lactate dehydrogenase gene is involved in the growth and metabolism of *Lacticaseibacillus paracasei* and the production of fermented milk flavor substances

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Objective: Lactate dehydrogenase (ldh) in lactic acid bacteria is an important enzyme that is involved in the process of milk fermentation. This study aimed to explore the changes and effects of fermented milk metabolites in mutant strains after knocking out the *ldh* gene of *Lacticaseibacillus paracasei*.

Methods: The *ldh* mutant Δ*AF91_07315* was obtained from *L. paracasei* using clustered regularly interspaced short palindromic repeats technology, and we determined fermented milk pH, titratable acidity, viable count, and differential metabolites in the different stages of milk fermentation that were identified using metabolomic analysis.

Results: The results showed that the growth rate and acidification ability of the mutant strain were lower than those of the wild-type strain before the end of fermentation, and analysis of the differential metabolites showed that lactate, L-cysteine, proline, and intermediate metabolites of phenylalanine, tryptophan, and methionine were downregulated ($P < 0.05$), which affected the growth initiation rate and acidification ability of the strain. At the end of fermentation (pH 4.5), the fermentation time of the mutant strain was prolonged and all differential metabolites were upregulated ($P < 0.05$), including amino acids and precursors, acetyl coenzyme A, and other metabolites involved in amino acid and fatty acid synthesis, which are associated with the regulation of fermented milk flavors. In addition, riboflavin was upregulated to promote the growth of the strain and compensate for the growth defects caused by the mutation.

Conclusion: Our data established a link between the *AF91_07315* gene and strain growth and metabolism and provided a target for the regulation of fermented milk flavor substances.

KEYWORDS

***Lacticaseibacillus paracasei*, lactate dehydrogenase, CRISPR, fermented milk, metabolomics**

1. Introduction

Fermented milk is a nutrient product made from milk co-cultured with a starter culture and is widely accepted for its unique taste and flavor (Wang et al., 2021c). Lactic acid bacteria (LAB) are a traditional starter culture that uses milk as a means to produce nutrients and flavored substances. These substances include organic acid, peptides, amino

acids, and short-chain fatty acids, which improve the digestion and bioavailability of milk components, inhibit harmful bacteria in the gastrointestinal tract, prevent cancer, reduce lactose intolerance, and lower cholesterol levels (Shiby and Mishra, 2013). Organic acid is the main component of fermented milk, and lactic acid enhances the flavor of fermented milk and maintains probiotics in the human gastrointestinal gut, which is beneficial against septic and bacterial infections, stimulates intestinal motility, and enhances the immune response to antigenic invasion (Shukla et al., 2008; Vieco-Saiz et al., 2019; Mathur et al., 2020). However, the mechanism of metabolic regulation by LAB in fermented milk is unclear, and understanding LAB metabolic pathways can help regulate the production of flavored substances in fermented milk.

Lactate dehydrogenase, a key enzyme in the glycolytic pathway for lactate production, uses pyruvate as an electron acceptor, accompanied by the oxidation of nicotinamide adenine dinucleotide, to catalyze the pyruvate reaction to produce lactate (Kandler, 1983). To date, genetic engineering has been applied to knockout and overexpress *ldh* to change the energy flow and achieve high industrial lactate production (Singhvi et al., 2018; Bleckwedel et al., 2020). Furthermore, changes in the flavor of fermented milk caused by *ldh* gene deletion have been investigated. This study showed that lactic acid production was eliminated by knocking out the *ldh* gene in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, thereby increasing acetic acid and ethanol production (Desai et al., 2004). In addition, the elimination of *ldhD* using the double cross strategy increased the production of acetaldehyde, diacetyl, and acetoin in *Lactobacillus johnsonii* (Lapierre et al., 1999). These flavored substances have only been characterized in the medium; however, few studies have investigated the role of lactate dehydrogenase in fermented milk, and effective characterization of its metabolic components during fermentation is important for improving its flavor.

Mass spectrometry-based untargeted metabolomics is a highly specific and sensitive technology for monitoring small molecular substrates produced by microorganisms, and it screens samples for biomarkers or differential substances and helps identify differential metabolites that modulate phenotypes (Johnson et al., 2016; Guijas et al., 2018). Research on milk metabolomics has mainly focused on the effect of *Streptococcus thermophilus* (*S. thermophilus*), co-cultured with lactobacilli on the fermentation process and quality (Tomassini et al., 2019; Li et al., 2021), and there are few metabolic studies on single-strain fermented milk. We used *L. paracasei* CGMCC4691 as a test bacterium owing to its beneficial characteristics. As a co-fermenter of fermented milk, it can enhance immunity, improve intestinal metabolites, and regulate intestinal flora. In addition, the inactivated strain can improve the brain–intestinal axis and reduce continuous damage to brain nerves (Miao et al., 2022; Zhang et al., 2022). In this study, the mutant strain Δ*AF91_07315* was generated from *L. paracasei* using the clustered regularly interspaced short palindromic repeats (CRISPR) technology, and pH, titratable acidity (TA), and bacterial counts were compared between the wild-type (WT) and Δ*AF91_07315* strains. In addition, differential metabolites were identified using non-targeted metabolomics to assess the metabolic role of Δ*AF91_07315* and provide targets for the modulation of flavored substances.

TABLE 1 Primer sequences used in this study.

Primer	Sequence(5′-3′)
Up-F	TTTTCTAAACTAGGGCCCCACTACTGGCCGCCTAC
Up-R	TGGAGGGGAAGGGTTTTCTTATGCCTATCCACTCGA CATTGAC
Down-F	TCGAGTGGATAGGCATAAGAAAACCCTTCCCCTCCACT
Down-R	GTCGGTGCTTTTTTTGAGACACGATTATGGGCACGG
Sgrna-F	CCGTGCCCATAATCGTGTCTCAAAAAAAGCACCGACTCG
Sgrna-R	CATGAGGAGGAATTTGAGTCTAGA-GACGCATCTGATGGATGTAG GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
deleteYZ-R	CCATCAAAAGCTTTGATCAACGC
deleteYZ-F	CAATGATCGCAATGTTGCGAATAT
Ldhyz-F	CTATCAACACACTCTTAAGTTTGCTTCTAAG
Ldhyz-R	AGTTTTGAGGCAAAATTTTTGAGTGACA

2. Materials and methods

2.1. Strains, culture media, and growth conditions

L. paracasei CGMCC4691 was obtained from our laboratory. The pLL plasmid was provided by the University of Shanghai for Science and Technology. *E. coli* Top10 competent cell was purchased from TianGen Biotech. *L. paracasei* CGMCC4691 and Δ*AF91_07315* mutants were cultured in De Man Rogosa Sharpe (MRS) medium at 37 °C for 14–16 h for skim milk fermentation. *E. coli* Top10 was used for cloning and was grown on Luria Bertani medium at 30 °C for 12–14 h. When needed, a suitable antibiotic was supplemented at 10 μg/ml for Δ*AF91_07315* and 50 μg/ml for *E. coli* Top10.

2.2. Construction of *L. paracasei* CGMCC4691 Δ*AF91_07315*

2.2.1. Construction of knockout plasmids

The knockout plasmid was constructed using polymerase chain reaction (PCR) and seamless cloning technology. The primers used in this study are presented in Table 1. First, the vector skeleton was generated using an *Apa*I-*Xba*I (Vazyme Biotech Co., Ltd.)-digested pLL plasmid. Three fragments, 1,300 bp homologous arms upstream and downstream (obtained by PCR using primers up/down-F/R with *L. paracasei* CGMCC4691 genome as a template), and sgRNA (obtained by PCR using primers sgRNA-F/R with pLL as a template) were generated and purified using a gel purification kit (Axygen, SV, USA), all of which were connected to generate up–down-sgRNA fragments. The resulting fragment was ligated into the vector skeleton to produce pLL-07315 using a seamless cloning kit (Vazyme Biotech Co., Ltd.) and verified using ldhYZ-F/R following transformation into *E. coli* Top10 competent cells.

2.2.2. Deletion of the AF91_07315 gene

A competent cell of *L. paracasei* CGMCC4691 was prepared as described by Song et al. (2017). The constructed plasmid was electroporated into cells using a GenePulser Xcell (Bio-Rad) and a 2 mm cuvette (BTX) at 2.0 KV, 200Ω, and 25 μF. After recovery for 3 h in the MRS medium, the cells were separated on agar plates and grown for 72 h. A single strain was selected and verified by PCR and named pLL: 07315. Precise sequencing was also performed using BGI.

2.2.3. Fermented milk preparation

Skim milk (10% w/v) was prepared from skim milk powder (Shijiazhuang Junlebao Dairy Co., Ltd.). Skim milk powder was dissolved in 50°C sterile water and autoclaved at 115 °C for 15 min. After cooling to 37 °C, 400 ml of milk was transferred into six different sterile flasks. Pre-cultured *L. paracasei* CGMCC4691 and its mutants were inoculated in triplicate at a 5% inoculum size (2×10^7 CFU). The mixed milk was fermented until the pH reached an end-point of 4.5. Subsequently, a sample from each flask was collected before (4 h) and after (8 h) the fermentation periods and end-points. Before analysis, these samples were stored at −80 °C.

2.3. TA and bacterial count determination

2.3.1. Determination of pH and TA

pH was measured using a pH meter (METTLER TOLEDO, China). TA was determined using 0.1 mol/L NaOH with phenolphthalein as a color indicator, and the result was expressed as °T.

2.3.2. Determination of viable count

The viable counts of different periods during milk fermentation were determined as follows: 1 ml of the sample was diluted in 9 ml of normal saline (0.85%) by vortex oscillation, and the dilution was performed continuously. The diluted liquid (100 μl) was cultured using the MRS agar plate pouring method. The viable counts were calculated after incubation at 37 °C for 48 h and expressed as CFU/ml.

2.4. UPLC-Q-TOF MS^E analysis

2.4.1. Sample pretreatment

The fermented milk samples stored at −80 °C were thawed. We transferred 50 mg of each sample into a fresh centrifuge tube, and 400 μl methanol solution (containing 5 μg/ml L-2-chloro-phenylalanine as the internal standard) was added and vortexed for 1 min. The sample was then mixed two times at 60 Hz for 3 min and centrifuged at 13,000 rpm at 4°C for 10 min using a cryogenic centrifuge (Thermofisher FRESCO21, USA). Supernatants were collected for analysis. Equal volumes of all samples were mixed as quality control samples. All reagents were analytically pure and purchased from Shanghai.

2.4.2. Ultra performance liquid chromatography analysis

The samples were analyzed on an Agilent 1290 infinity (USA), incorporating a HSS T3 chromatographic column (water, 2.5 μm, 100*2.1 mm). The mobile phases A and B consisted of water and acetonitrile (mixed with 0.1% formic acid). The flow rate of the mobile phase was 0.4 ml/min at a column temperature of 40°C. The injection volume was 4 μl. The optimized gradient elution was 0–3 min, 20% B; 3–9 min, 20–95% B; 9–13 min, 95% B; 13–13.1 min, 95–5% B; and 13.1–16 min, 5% B.

2.4.3. Mass spectrometry (MS) analysis

Mass spectrometry (MS) analysis was performed using a Q-TOF-MS^E (Agilent 6545 UHD, USA), coupled with positive (ESI+) and negative (ESI–) modes. The capillary voltage was 4.5 kV and 3.5 kV in ESI+ and ESI–, respectively, and the drying gas flow was 8 L/min and 10 L/min at a temperature of 325°C, respectively. The nebulizer voltage was set at 20 psi. The fragmentation voltage was set to 120 V. The MS scans were performed over an entire information range of 50–1,500 m/z.

2.5. Statistical analysis

Fermentation was performed in triplicate for the starter culture. Metabolic data were collected using Agilent MassHunter Qualitative Analysis B.0 8.0 software. A series of procedures were performed using the R software XCMS package, including peak identification, retention time correction, and automatic integration. The chromatographic peak values were normalized by dividing the original peak area by the internal standard peak. Differences in metabolic profiles and visual differences were achieved using R-script, such as principal component analysis and orthogonal projections to latent structures discriminant analysis (OPLS-DA).

Differential metabolites were identified using the OPLS-DA model, variable importance in the projection (VIP $\geq$1), independent sample *t*-test (P <0.05), and fold change (FC $\geq$1.1 or $\leq$0.9). The differential metabolites of interest were identified by matching accurate molecular weights against the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp) online database using the following qualitative methods: the adduct ions in ESI+ mode included [M+H]+ and [M+Na+]+ and those in ESI– mode included [M–H] –; the quality error value was selected as 30 ppm. Thereafter, the metabolic pathway was matched against KEGG using MetaboAnalyst software, and the metabolic effects were assessed by metabolic enrichment analysis.

3. Results

3.1. Construction of *L. paracasei* CGMCC4691Δ*AF91_07315*

To produce mutant strains, cells were transformed with pLL-07315. After obtaining a single-clone strain, specific primers flanking the homologous arms were designed for verification

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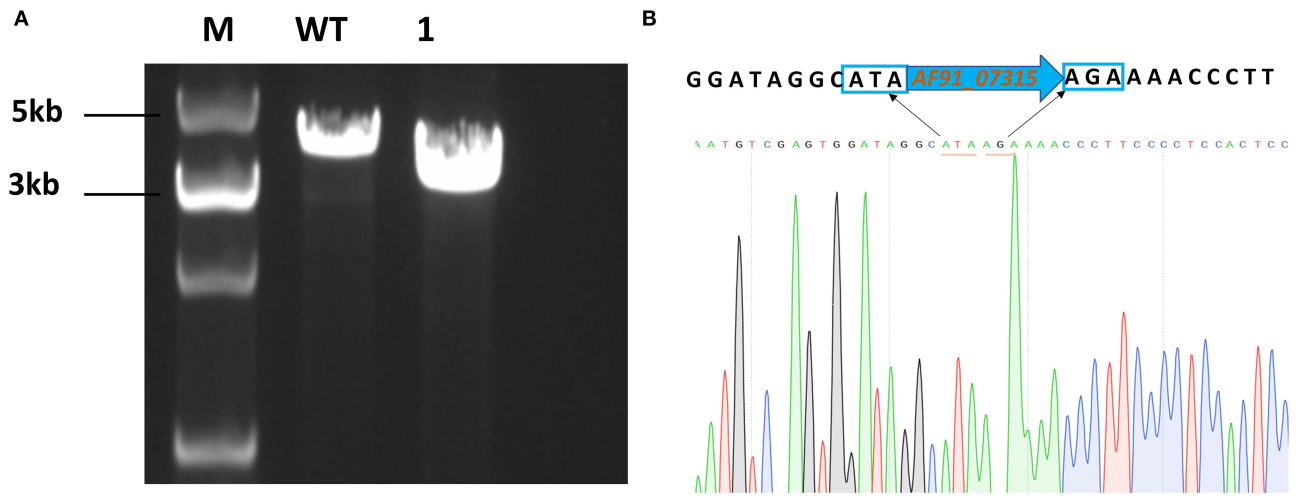

FIGURE 1
Chromosomal deletion of *ldh* (*AF91_07315*). **(A)** PCR identification of Δ*AF91_07315* mutants. M:5000 bp DNA marker; WT: wild-type strain was used as control (3735bp); lane 1: a mutant strain of *AF91_07315* (2844bp). **(B)** Sequencing of Δ*AF91_07315* mutant.

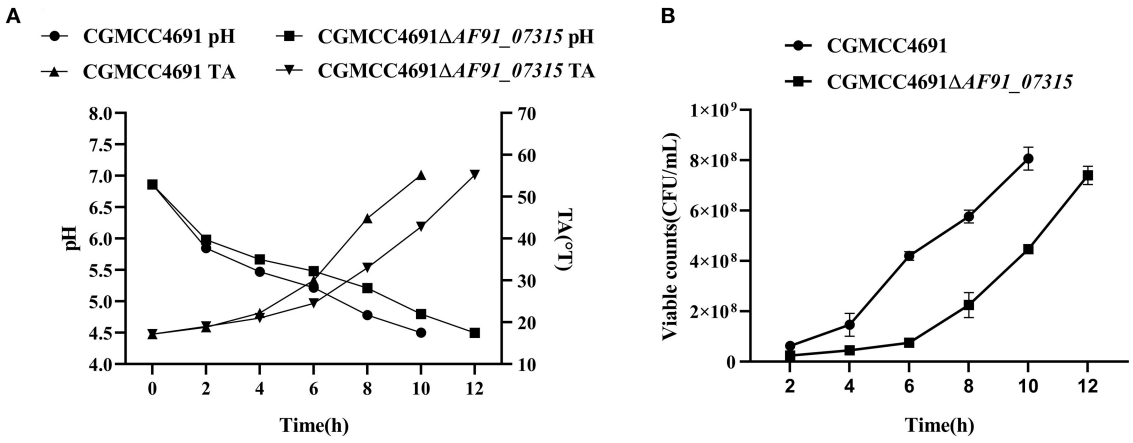

FIGURE 2
Summary of fermentation characteristics; pH, titration acidity **(A)**, and viable counts **(B)** of *L. paracasei* CGMCC4691 and *L. paracasei* CGMCC4691 Δ*AF91_07315*. The average was calculated from three independent biological replication. The error bars represent the standard deviation.

using PCR (Figure 1A). The results are shown in Figure 1. The sequencing results further confirmed the gene deletion (Figure 1B).

3.2. Fermented milk characteristics of *L. paracasei* CGMCC4691 and mutants

The pH, TA, and viable counts in the fermented milk were measured every 2 h until the pH reached 4.5. The results are shown in Figure 2. The pH and TA showed opposite trends, and differences were observed as pH decreased (Figure 2A). At 8 h of fermentation, the pH of the mutant strain was 0.4 points higher than the WT strain. Simultaneously, the TA of the mutant strain increased to 33 °T, which was 12 °T higher than that of the WT strain. The fermentation times of the WT and mutant strains were 10 h and 12 h, respectively, at the fermentation end-point. The viable counts consistently decreased during fermentation, and the growth of the WT and mutant strains reached the exponential phase at 4 h (6.4×10^7 CFU/ml) and 6 h (7.5×10^7 CFU/ml), respectively. Next, samples were collected for metabolomic analysis.

3.3. Untargeted metabolic analysis of *L. paracasei* CGMCC4691 and *L. paracasei* CGMCC4691Δ*AF91_07315*

3.3.1. Differential metabolic analysis of fermented milk at pre-fermentation

OPLS-DA, a common classification method, was performed to identify the differences between the two groups, which exploits dimension reduction by combining regression models; the

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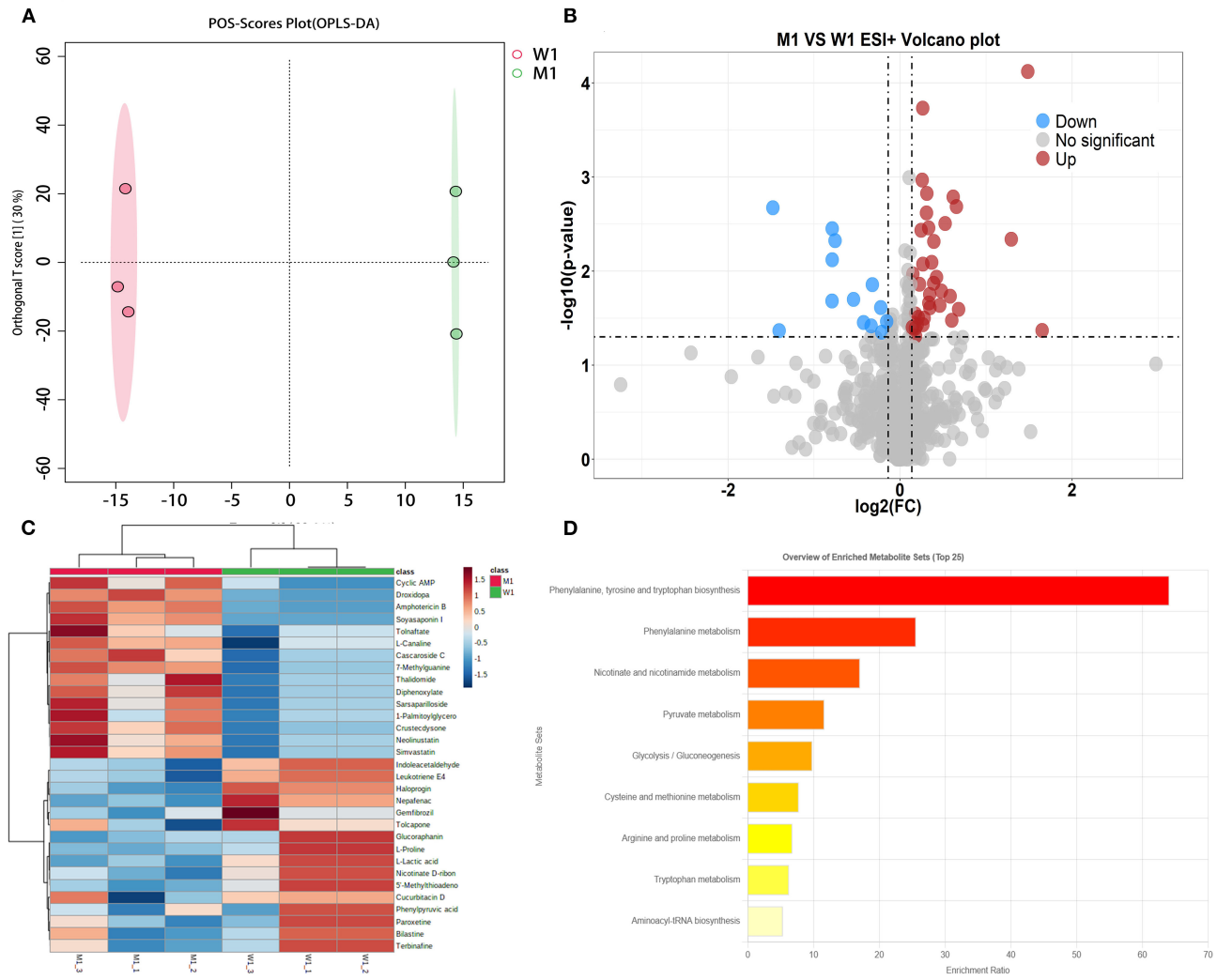

FIGURE 3
Fermented milk metabolomic analysis of wild-type strain CGMCC4691 (W1) and its mutant strain *ΔAF91_07315* (M1) at 4 h. **(A)** Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA). **(B)** Volcano plot showing differential metabolites in comparison with W1 in the positive ion (ESI+) model, where each dot represents a substance. The abscissa and ordinate indicate fold change (FC) and *p*-value, respectively, after data processing. **(C)** Heatmap indicates the relative abundance of differential metabolites between the two sample groups. **(D)** Pathway enrichment analysis of differential metabolites. The x-axis indicates metabolic pathway impact values obtained from topological analysis and the color scale represents the contribution of metabolites in the pathway. Red indicates the highest and yellow indicates the lowest.

regression results were analyzed using a discriminant threshold. A total of 1,096 and 927 substances were generated from the fermented milk in the ESI+ and ESI− modes, respectively. The OPLS-DA of fermented milk is shown in Figure 3. The abscissa represents the principal component score during the quadrature signal correlation; the farther the location of the two milk samples, the greater the difference between the two groups. The ordinate represents the quadrature component score value; the farther the parallel samples are, the greater the difference within the group. The milk fermented by the WT strain at 4 h (W1) was located away from the milk fermented by the mutant strain (M1), indicating that the two groups had significant differences, and a total of 52.4% was explained by the dataset. The critical parameters used to evaluate the OPLS-DA model are presented in Table 2. In the ESI+ model, the R^2X, R^2Y, and Q^2 values were 0.524, 1, and 0.654, respectively. In the ESI− model, the R^2X, R^2Y, and Q^2 values were 0.632, 0.999, and 0.762, respectively, where R^2 and Q^2 represent the explanatory ratio and predictability, respectively. The closer the value is to 1, the higher the model accuracy. Generally, when R^2 and Q^2 >0.5, the model fits well.

To select the differential metabolites, a volcano plot was generated from the OPLA-DA filtering results (Figure 3B). In ESI+, the red dots indicate that the metabolites were upregulated, with FC >1.1 and $P \leq 0.05$, and the blue dots indicate that the metabolites were downregulated, with FC <0.9 and $P \leq 0.05$. The farther the dot is from the origin, the greater the difference between the two groups. A total of 51 metabolites were identified. Next, these potentially differential compounds were further used for enrichment analysis by (1) KEGG ID conversion using the MBRole online database and (2) importing the ID into the

TABLE 2 Critical parameters for evaluating the OPLS-DA model in the positive and negative modes.

Mode and Sample pair	Statistics analysis mode	R2X(cum) (cumulative)	R2Y(cum) (cumulative)	Q2(cum) (cumulative)
Positive ion mode				
M1VSW1	OPLS-DA	0.524	1	0.654
M2VSW2	OPLS-DA	0.806	1	0.896
M3VSW3	OPLS-DA	0.592	0.997	0.759
Negative ion mode				
M1VSW1	OPLS-DA	0.632	0.999	0.762
M2VSW2	OPLS-DA	0.541	0.994	0.837
M3VSW3	OPLS-DA	0.584	0.996	0.73

TABLE 3 Differential metabolites identified at the initial stage of fermentation compared with the wild-type strain.

Name	FC	vipV	*p*-value	Formula	m/z	RT	KEGG id
Cucurbitacin D	0.83507	1.879249	0.004019	C30H44O7	515.2997	10.12569	C08796
Cyclic AMP	1.8493	1.651685	0.045625	C10H12N5O6P	328.0485	1.135302	C00575
Indoleacetaldehyde	0.64864	1.779966	0.017819	C10H9NO	158.0612	4.754723	C00637
L-Lactic acid	0.78275	1.905832	0.002613	C3H6O3	89.0249	0.980151	C00186
Leukotriene E4	0.72455	1.714153	0.02395	C23H37NO5S	438.2444	7.451532	C05952
Nicotinate D-ribonucleoside	0.80047	1.711194	0.025026	C11H14NO6	255.0804	14.01767	C05841
Phenylpyruvic acid	0.82577	1.658416	0.044803	C9H8O3	163.0411	2.629356	C00166
5′-Methylthioadenosine	0.59283	2.001216	0.004753	C11H15N5O3S	298.0974	2.392717	C00170
L-Proline	0.8568	1.847439	0.024406	C5H9NO2	116.0708	0.731764	C00148

4,691 KEGG metabolic pathways for pathway enrichment analysis. This resulted in 31 compounds, and a heatmap was generated to show their relative abundances (Figure 3C). Enrichment analysis of the two groups was performed using L. paracasei CGMCC4691 (lpq), and nine differential metabolites were enriched in the pathways. The results are shown in Figure 3D, and the metabolites are presented in Table 3. The key pathways include phenylalanine, tyrosine, and tryptophan biosynthesis, as well as phenylalanine metabolism, pyruvate metabolism, and nicotinate and nicotinamide metabolism.

3.3.2. Differential metabolic analysis of fermented milk at mid-fermentation

A method similar to that described above was used to identify differential metabolites between the two sample groups (M2 and W2) at 8 h. In total, 1,117 differential metabolites were identified as potential candidates (VIP >1) in the OPLS-DA plot, and significant separation was observed in the positive mode (Figure 4A). The model evaluation parameters (R^2X, R^2Y, and Q^2 were 0.806, 1, and 0.896 for ESI+; and 0.541, 0.994, and 0.837 for ESI−) indicated that the model was both reliable and valid. In ESI+, the volcano plot showing the FC of these candidates is presented in Figure 4B, and the criteria were FC >1.1 or ≤0.9 and $P \leq 0.05$. We obtained 207 differential substances from the positive model. The dot represents a variable, and the farther the variable is from the origin, the greater the contribution to the difference between the two groups. Subsequently, the KEGG ID of 80 variables was obtained from the MBRole database, and a heatmap was generated to visualize these substances (Figure 4C). Pathway enrichment analysis was performed using these metabolites (Table 4). A total of 18 signaling pathways are shown in Figure 4D, and the top signaling pathways were phenylalanine metabolism, cysteine and methionine metabolism, biotin metabolism, aminoacyl-tRNA biosynthesis, and pyruvate metabolism.

3.3.3. Differential metabolic analysis of fermented milk at the end of fermentation

The differential metabolites in fermented milk at pH 4.5 were compared using the multiple analyses described above (Figure 5). A total of 1,132 were identified, and OPLS-DA showed significant separation in the positive mode (Figure 5A). The model evaluation parameters (R^2X, R^2Y, and $Q^2 = 0.592$, 0.997, and 0.759, respectively, in ESI+; and 0.584, 0.996, and 0.73, respectively, in ESI−) are presented in Table 2, which indicates that the model has good stability and predictability. In ESI+, the volcano plot showed 123 differential metabolites according

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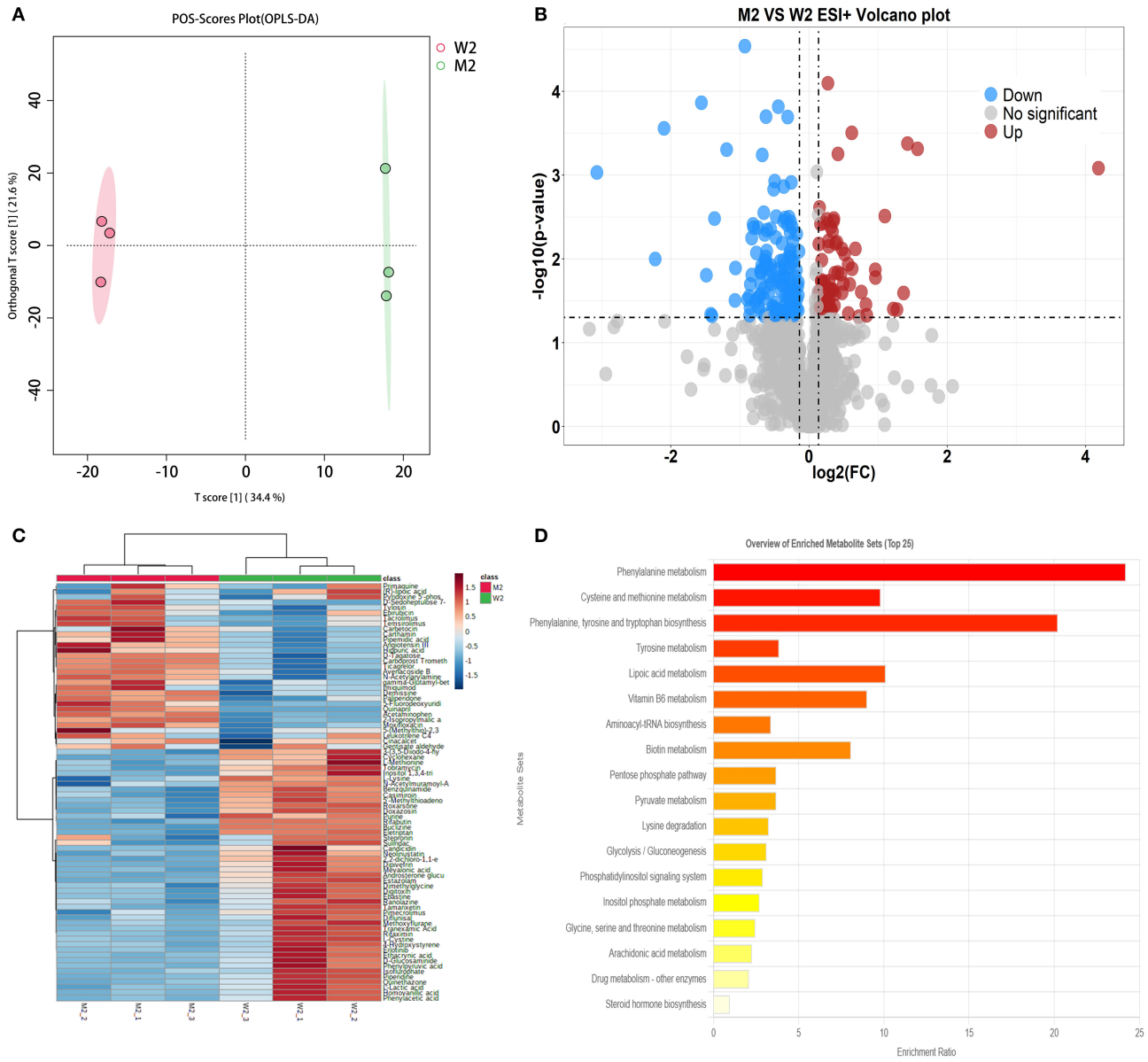

FIGURE 4
Fermented milk metabolomic analysis of wild-type strain CGMCC4691 (W2) and its mutant strain Δ*ldh* (M2) at 8 h. **(A)** Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA). **(B)** Volcano plot showing differential metabolites in comparison with W1 in the positive ion (ESI+) model, where each dot represents a substance. The abscissa and ordinate indicate fold change (FC) and p-value, respectively, after data processing. **(C)** Heatmap represents the relative abundance of differential metabolites between the two sample groups. **(D)** Pathway enrichment analysis of differential metabolites. The x-axis represents the metabolic pathway impact values obtained from topological analysis, and the color scale represents the contribution of metabolites in the pathway. Red indicates the highest and yellow indicates the lowest.

to the criteria mentioned above, where the red and blue dots represent significantly differential metabolites (Figure 5B). Next, **two** statistical analysis methods were performed to further select featured substances: 49 KEGG IDs were obtained from the MBRole database, and a heatmap was used to visualize their relative abundance (Figure 5C). Pathway enrichment analysis was performed using these metabolites, and the details are presented in Table 5. A total of 20 signaling pathways are presented in Figure 5D, and phenylalanine, tyrosine, and tryptophan biosynthesis, as well as biotin metabolism and pyruvate metabolism were involved in key metabolic pathways.

4. Discussion

4.1. Fermented milk serves as a vector for studying genetic functions

Lactic acid bacteria (LAB) mutants can change the flavor and texture of fermented milk, and the potential functions of these genes can be inferred from changes in the properties of fermented milk (Sorensen et al., 2016). Courtin et al. (2002) developed *prtS* and *prtB* mutants of *S. thermophilus* and *L. bulgaricus* and confirmed the importance of both genes for strain

TABLE 4 Differential metabolites identified at the middle stage of fermentation compared with the wild-type strain.

Name	FC	vipV	*P*-value	Formula	RT	KEGG id
5′-Methylthioadenosine	0.78193	1.551246	0.012062	C11H15N5O3S	2.392717	C00170
Androsterone glucuronide	0.6413	1.603995	0.005122	C25H38O8	4.40443	C11135
Cyclohexane	0.84815	1.419903	0.038262	C6H12	7.580534	C11249
D-Sedoheptulose 7-phosphate	1.1823	1.406733	0.040502	C7H15O10P	0.961874	C05382
gamma-Glutamyl-beta-aminopropiononitrile	1.2721	1.484472	0.023982	C8H13N3O3	1.022341	C06114
L-Lysine	0.6544	1.580373	0.008054	C6H14N2O2	0.557608	C00047
L-Methionine	0.87224	1.393502	0.044758	C5H11NO2S	0.97255	C00073
Leukotriene C4	1.2231	1.50636	0.018387	C30H47N3O9S	2.463467	C02166
Mevalonic acid	0.68071	1.552892	0.011932	C6H12O4	1.145254	C00418
Primaquine	1.1073	1.433174	0.039787	C15H21N3O	4.431745	C07627
Tobramycin	0.63517	1.627186	0.002817	C18H37N5O9	4.399161	C00397
Tylosin	1.2211	1.488243	0.023878	C46H77NO17	5.1029	C01457
(R)-lipoic acid	1.148	1.425164	0.043946	C8H14O2S2	1.495122	C16241
4-Hydroxystyrene	0.74018	1.401187	0.038492	C8H8O	4.483189	C05627
5-(Methylthio)-2,3-dioxopentyl phosphate	1.1983	1.428032	0.044147	C6H11O6PS	3.779332	C15650
Gentisate aldehyde	1.255	1.515439	0.018559	C7H6O3	3.677932	C05585
Hippuric acid	2.3633	1.414017	0.043724	C9H9NO3	3.423774	C01586
Inositol 1,3,4-trisphosphate	0.68773	1.589626	0.002927	C6H15O15P3	0.530975	C01243
L-Cystine	0.64625	1.450834	0.025939	C6H12N2O4S2	2.63662	C00491
L-Lactic acid	0.8042	1.483528	0.022785	C3H6O3	0.980151	C00186
Moxifloxacin	1.5457	1.663139	0.000941	C21H24FN3O4	10.50109	C07663
Phenylacetic acid	0.62279	1.541756	0.009869	C8H8O2	2.629517	C07086
Phenylpyruvic acid	0.65179	1.473979	0.02142	C9H8O3	2.629356	C00166
Pyridoxine 5′-phosphate	1.2979	1.496891	0.035118	C8H12NO6P	1.824106	C00627

growth and acidification. Yamauchi et al. (2019) developed a urease-deficient mutant Δ*ureC* of *S. thermophilus* and confirmed that urease is the essential factor for yogurt acidification. In addition, Xu et al. (2021) inactivated the glutathione transport protein (*Ptrp*) gene and measured the glutathione content on the cell surface to determine the transport function of *Ptrp-2* and *Ptrp-4*. Therefore, it is important to study the fermentation characteristics of Δ*AF91_07315* strains using milk as a carrier. Furthermore, mutant strains affected the production of fermented milk metabolites. Although the *AF91_07315* gene mutants obtained using CRISPR technology cannot be applied to food production, the mutant strains in this study can enable us to understand the key metabolic pathways during dairy product development and provide targets for flavor substance interventions.

4.2. Advantages of CRISPR technology

The methods used to generate gene mutants of LAB are classified into natural mutagenesis and genetic engineering. Natural mutagenesis has been applied to *L. bulgaricus and L. helveticus* to improve the quality and taste of production (Moller et al., 2007; Guan et al., 2021), which mainly depends on random mutagenesis and large-scale labor-intensive screening. Although the double-crossover homologous recombination strategy compensates for this deficiency and has been used to produce highly purified lactate by cloning the *ldh* gene (Jin et al., 2016; Okano et al., 2018), this method requires the screening of markers. Furthermore, CRISPR gene editing technology has the advantages of high specificity and ease of operation. Tian et al. (2021) used the CRISPR system to knockout and overexpress the *ldh* gene in *L. paracasei* NCBIO01-M2 and screened L-lactate strains with a yield of 221 g/L at 45°C and 99% optical and chemical purity. Our previous study optimized the CRISPR system for *L. paracasei* CGMCC4691; thus, this is the optimal choice to knockout *ldh* using this technique.

4.3. Viable count differences between the Δ*AF91_07315* and WT strains

Yogurt is considered beneficial when the bacterial counts reach 10^7 CFU/ml (Corcoran et al., 2006). TA and pH directly affect

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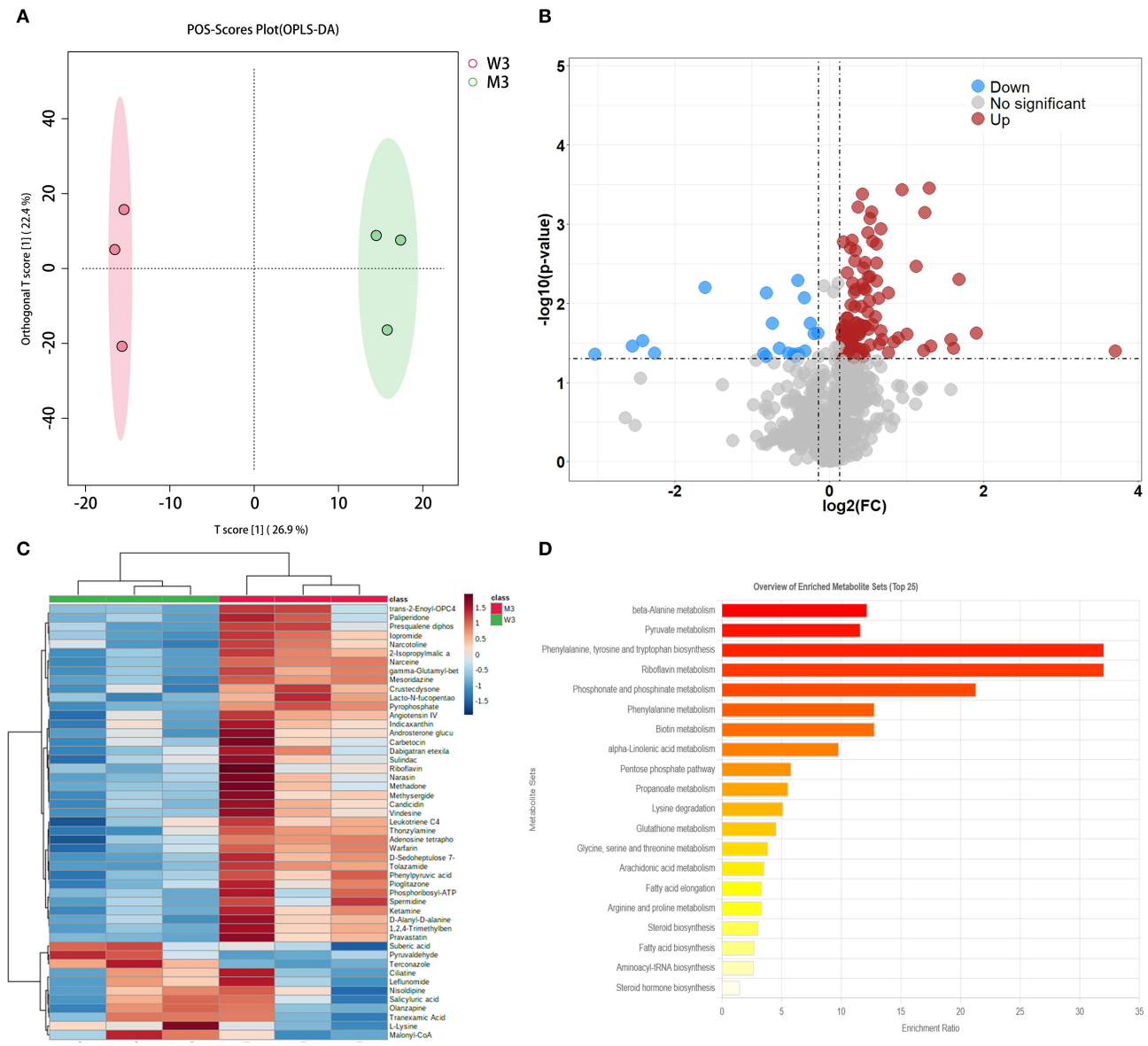

FIGURE 5
Fermented milk metabolomic analysis of wild-type strain CGMCC4691 (W3) and its mutant strain Δ*ldh* (M3) at the end of the fermentation. **(A)** Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA). **(B)** Volcano plot showing differential metabolites in comparison with W1 in the positive ion (ESI+) model, where each dot represents a substance. The abscissa and ordinate indicate fold change (FC) and *p*-value, respectively, after data processing. **(C)** Heatmap represents the relative abundance of differential metabolites between the two sample groups. **(D)** Pathway enrichment analysis of differential metabolites. The x-axis indicates the metabolic pathway impact values obtained from topological analysis, and the color scale represents the contribution of metabolites in the pathway. Red indicates the highest and yellow indicates the lowest.

the taste. Therefore, fermentation was completed at a pH of 4.5 to ensure that the fermentation process met commercial criteria. The result showed that the Δ*AF91_07315* strain has a lag in both acidity and viability, which is in agreement with Bleckwedel et al., where the *ldh1* gene (mainly responsible for lactate production) of *Fructobacillus tropaeoli CRL 2034* was inactivated, and cell counts and acidity were lagging (Bleckwedel et al., 2020). Viana et al. (2005) reported that the knockout of *ldh* affected the growth rate of mutants in different sugars, showing a higher final pH (5.2) than the WT strain (4.5). Even though lag growth was observed, the viable counts at the end of fermentation were consistent with the WT strain; hence, it had no effect on the fundamental metabolism throughout life.

4.4. Differential metabolite screening of fermented milk at different time periods

Non-volatile metabolite profiles were analyzed using liquid chromatography-MS metabolomics during the fermentation process of *L. paracasei* CGMCC4691, and most compounds were

TABLE 5 Differential metabolites identified at the end of fermentation compared with the wild-type strain.

Name	Formula	m/z	FC	VIP	*p*-value	Output
Androsterone glucuronide	C25H38O8	489.2373	1.669046	1.669046	0.024102	C11135
D-Alanyl-D-alanine	C6H12N2O3	161.0924	1.839027	1.839027	0.004139	C00993
D-Sedoheptulose 7-phosphate	C7H15O10P	291.0487	1.890057	1.890057	0.000617	C05382
gamma-Glutamyl-beta-aminopropiononitrile	C8H13N3O3	200.1035	1.898383	1.898383	0.00071	C06114
L-Lysine	C6H14N2O2	147.1131	1.634573	1.634573	0.040164	C00047
Leukotriene C4	C30H47N3O9S	626.3108	1.567769	1.567769	0.049884	C02166
Malonyl-CoA	C24H38N7O19P3S	854.1408	1.661685	1.661685	0.024537	C00083
Narcotoline	C21H21NO7	400.1322	1.610517	1.610517	0.03951	C09593
Phenylpyruvic acid	C9H8O3	165.0564	1.702135	1.702135	0.027714	C00166
Phosphoribosyl-ATP	C15H26N5O20P4	743.0135	1.622855	1.622855	0.037154	C02739
Pyruvaldehyde	C3H4O2	73.02846	1.536173	1.536173	0.044004	C00546
Riboflavin	C17H20N4O6	377.1469	1.524313	1.524313	0.048974	C00255
Spermidine	C7H19N3	146.1652	1.664869	1.664869	0.03077	C00315
trans-2-Enoyl-OPC4-CoA	C35H54N7O18P3S	986.2695	1.640686	1.640686	0.024748	C16336
2-Isopropylmalic acid	C7H12O5	175.0614	2.1562	1.834587	0.000927	C02504
Ciliatine	C2H8NO3P	124.0161	0.89907	1.792065	0.006253	C03557
Presqualene diphosphate	C30H52O7P2	585.318	2.2076	1.594077	0.033686	C03428
Pyrophosphate	H4O7P2	176.9372	1.3437	1.654038	0.027718	C00013

upregulated or downregulated compared to those in the WT strain. Specifically, 31, 80, and 49 differential metabolites were identified in the pre, mid, and end stages of fermentation, respectively. The heatmap shows the relative abundance of differential metabolites, where the mid-term metabolites were higher than the pre-term and end stages of fermentation, suggesting the main role of lactate dehydrogenase in mid-fermentation. After enrichment analysis of the metabolic pathway, 9, 24, and 18 substances were identified; however, other substances were not identified, which could be explained by the selection of a model strain for ID conversion. We used *L. paracasei* ATCC334 as a model strain for metabolic pathway analysis, and some metabolites were not involved in metabolic pathway conversion as separate substances in *L. paracasei* CGMCC4691.

4.4.1. Differential metabolites in pre-fermentation

In total, nine differential metabolites were detected in the mutant strains during pre-fermentation when compared to the WT strain. Figure 3D shows the results of the enrichment analysis. The metabolites involved in pyruvate, amino acid, and pyrimidine metabolism included (S)-lactate, phenylpyruvate, L-proline, 5′-methylthioadenosine, and indole-3-acetaldehyde, all of which were downregulated. (S)-lactate, which is catalyzed by lactate dehydrogenase in the last step of glycolysis, provides energy to cells and is the main substance that adds flavor to fermented milk. The Δ*AF91_07315* strain failed to reach the exponential growth phase during pre-fermentation, which may be related to reduced lactate metabolism, insufficient energy supply, and delayed growth of the strain (Rico et al., 2008; Jingjing et al., 2021). In addition, proline, phenylpyruvate, and indole-3-acetaldehyde were downregulated, and the growth-promoting effect of proline on *Lactobacillus* has been demonstrated (Wang et al., 2021a). Sun et al. (2019) added amino acids, uracil, and other substances during the fermentation of *L. rhamnosus*, which improved the start-up speed. In this study, phenylpyruvic acid, indole-3-acetaldehyde, and 5′-methylthioadenosine as the precursors of phenylalanine, tryptophan, and methionine were less synthesized, which affected the growth of the strain (Shapiro, 1962; Yonezawa et al., 2010; Meng et al., 2021).

4.4.2. Differential metabolites in mid-fermentation

During mid-fermentation, the largest gap in pH and viability was observed when compared to the WT strain, and the metabolites detected at this time were mainly L-methionine, 5′-methylthioadenosine, L-cysteine, phenylpyruvate, phenylacetic acid, L-lysine, and L-lactic acid. In particular, the gap in lactic acid content increased compared to the previous stage, which indicates the major role of lactic acid production in regulating the pH of fermented milk (Morelli et al., 1986; Gaspar et al., 2013), and the knockout of *ldh* had a significant effect on lactic acid production. Nevertheless, lactic acid was still detected throughout the fermentation phase in the mutant strain, probably owing to the synergistic effects of other genes encoding acid-producing enzymes (Viana et al., 2005). The synthesis of nutrients, such as amino acids, in LAB is limited to fermented milk; therefore, exogenous nitrogen

sources are used to promote their growth, such as the breakdown of amino acids and peptides. In this study, the L-cysteine content was reduced in the Δ*AF91_07315* strain except for methionine, phenylalanine, and its precursors. Wang et al. (2021b) reported different pathways of amino acid biosynthesis at different stages of fermentation, and the synthesis of L-cysteine may be due to different protein hydrolase activities.

4.4.3. Differential metabolites at the end of fermentation

Fermentation was stopped at pH 4.5 to identify the various differential metabolite strains and the differential metabolites detected were all upregulated in the Δ*AF91_07315* strain, including malonyl-CoA, L-lysine, phenylpyruvate, riboflavin, alpha-isopropylmalate, and spermidine, which may be due to the prolonged fermentation time of the mutant strain. In addition, alpha-isopropylmalate and spermidine were involved in pyruvate and arginine metabolism, respectively, providing energy and improving the nutritional quality of fermented milk. Malonyl-CoA is involved in fatty acid synthesis and carbon metabolism, which is related to yogurt flavor (Foster, 2012). Riboflavin is a trace organic substance required for growth and metabolism that stimulates the growth of LAB (Sun et al., 2022). The fermented milk of the Δ*AF91_07315* strain did not complete fermentation when the WT strain was fermented at a pH of 4.5, after which the viable counts of the Δ*AF91_07315* strain continued fermentation and reached the same pH as the WT strain, probably because riboflavin, L-lysine, alpha-isopropylmalate, and spermidine compensated for the growth defect in the Δ*AF91_07315* strain. Further investigation is required because the metabolites of the mutants were upregulated at the end of fermentation, some of which may be toxic, and it is unclear whether there are biosafety issues with fermented milk.

In this study, the *ldh* gene of *L. paracasei* CGMCC4691 was knocked out using CRISPR gene editing technology. The fermented milk results showed that the Δ*AF91_07315* strain had slower growth and acidification capacities than the WT strain. At the initial stage of fermentation, the WT strain entered the exponential growth phase, and metabolites, including (S)-lactate, phenylpyruvate, L-proline, and indole-3-acetaldehyde, were downregulated compared to the WT strain, which affected the start-up speed of the Δ*AF91_07315* strain. At the mid-stage of fermentation, pH and viable counts were the most disparate, with (S)-lactate being the main cause of the differences in pH. In addition, L-cysteine and precursor metabolites involved in amino acid metabolism were reduced because of the influence of protease activity. Although the fermentation time of the mutant strain was prolonged at the end of fermentation, malonyl-CoA, L-lysine, the amino acid intermediate metabolites, phenylpyruvate, and spermidine were upregulated, contributing to the formation of flavored substances. Upregulation of metabolites, such as riboflavin, compensated for the growth defects of the mutant strain. In conclusion, lactate dehydrogenase plays an important role in the growth and acidification of *L. paracasei* and affects the production of flavored milk substances.

Data availability statement

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

Author contributions

XS and SW designed this project and revised the article. SF and LC processed the samples and performed the experiments. SF wrote the article. SF and JB collected the data. HL analyzed the data. SW guided the experiment and provided funding for the research. All the authors read and approved the final submission.

Acknowledgments

We would like to acknowledge the financial support from the National Natural Science Foundation of China [grant number 32272361] and 2022-Research and Development-14.

Conflict of interest

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

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frontiers | Frontiers in Microbiology

TYPE Mini Review
PUBLISHED 15 June 2023
DOI 10.3389/fmicb.2023.1211447

OPEN ACCESS

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RECEIVED 24 April 2023
ACCEPTED 29 May 2023
PUBLISHED 15 June 2023

CITATION
Bleibel L, Dziomba S, Waleron KF, Kowalczyk E and Karbownik MS (2023) Deciphering psychobiotics' mechanism of action: bacterial extracellular vesicles in the spotlight.
Front. Microbiol. 14:1211447.
doi: 10.3389/fmicb.2023.1211447

Deciphering psychobiotics' mechanism of action: bacterial extracellular vesicles in the spotlight

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The intake of psychobiotic bacteria appears to be a promising adjunct to neuropsychiatric treatment, and their consumption may even be beneficial for healthy people in terms of mental functioning. The psychobiotics' mechanism of action is largely outlined by the gut-brain axis; however, it is not fully understood. Based on very recent studies, we provide compelling evidence to suggest a novel understanding of this mechanism: bacterial extracellular vesicles appear to mediate many known effects that psychobiotic bacteria exert on the brain. In this mini-review paper, we characterize the extracellular vesicles derived from psychobiotic bacteria to demonstrate that they can be absorbed from the gastrointestinal tract, penetrate to the brain, and carry the intracellular content to exert beneficial multidirectional action. Specifically, by regulating epigenetic factors, extracellular vesicles from psychobiotics appear to enhance expression of neurotrophic molecules, improve serotonergic neurotransmission, and likely supply astrocytes with glycolytic enzymes to favor neuroprotective mechanisms. As a result, some data suggest an antidepressant action of extracellular vesicles that originate even from taxonomically remote psychobiotic bacteria. As such, these extracellular vesicles may be regarded as postbiotics of potentially therapeutic application. The mini-review is enriched with illustrations to better introduce the complex nature of brain signaling mediated by bacterial extracellular vesicles and indicates knowledge gaps that require scientific exploration before further progress is made. In conclusion, bacterial extracellular vesicles appear to represent the missing piece of the puzzle in the mechanism of action of psychobiotics.

KEYWORDS

probiotics, psychobiotics microorganisms, postbiotic, neuropsychiatric disorder, mental health, extracellular vesicles, gut brain axis, mechanism of action

1. Introduction

During the last few decades, studies have revealed the fascinating connection between human gut microorganisms and neuropsychological functioning. The gut is home to a unique and diverse community of microbes, collectively known as the gut microbiota. This composed microecosystem plays a crucial role in our overall condition, including mental health (Cuesta et al., 2021). Probiotics—being defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014)—appear to be effective

in advantageous modulation of the gut microbiota composition or function (Hemarajata and Versalovic, 2013; Morelli and Patrone, 2015; Oliphant and Claud, 2022). More specifically, the term psychobiotics (Dinan et al., 2013) has been coined to differentiate the gut microbiota modulating preparations that influence the brain function and mental health. Although currently psychobiotics encompass both probiotics and prebiotics (nutritional factors for the intestinal microbiota; Sarkar et al., 2016), in this review article, we refer to the original definition of psychobiotics as mental health-benefiting live microorganisms (Sarkar et al., 2016), such as *Lactobacillus helveticus* and *Bifidobacterium longum* (Messaoudi et al., 2011), or *Akkermansia muciniphila* (Ding et al., 2021), among others.

Recent research shed light on the role of psychobiotics on the gut microbiome and the diverse range of neuropsychiatric disorders. Individuals suffering from depression and anxiety tend to have lower abundance of beneficial gut bacteria with their functional impairment (Jiang et al., 2015; Aizawa et al., 2016; Kelly et al., 2016; Peter et al., 2018; Kazemi et al., 2019), while taking probiotics has been found to reduce depressive and anxiety symptoms (Huang et al., 2017; Ng et al., 2018; Reis et al., 2018; Goh et al., 2019; Liu et al., 2019; Chao et al., 2020; Cohen Kadosh et al., 2021; El Dib et al., 2021). Also, healthy people may benefit from the use of psychobiotics in terms of mental functioning (Messaoudi et al., 2011; Huang et al., 2017; Chao et al., 2020). Moreover, psychobiotics appear to reverse the neurocognitive deterioration in Alzheimer's disease and mild cognitive impairment (Den et al., 2020; Liu et al., 2023). These findings provide compelling evidence that bacterial psychobiotics may play a vital role in neuropsychiatric treatment and general well-being.

The gut-brain axis (GBA) has been outlined as a framework for mechanism of action of psychobiotics. GBA is a bidirectional communication network between the intestine (together with the residing microbiota) and the brain. This communication is mediated by multiple pathways and messengers, such as the vagus nerve (Breit et al., 2018), the hypothalamic–pituitary–adrenal axis (Frankiensztajn et al., 2020), microbiota-derived neurotransmitters and their precursors (Tillmann et al., 2018; Chen et al., 2021), neurotrophic factors (Ait-Belgnaoui et al., 2014; Mohammadi et al., 2019; Heidarzadeh-Rad et al., 2020), specific bacterial metabolites such as short chain fatty acids (O'Riordan et al., 2022; Karbownik et al., 2023), and immune system, including the modulation of cytokine release (Mohammadi et al., 2019; Rutsch et al., 2020; Partrick et al., 2021). However, the psychobiotics' mechanism of action is still a mystery to the scientists. Recently, another factor has emerged as potentially shaping the GBA function following the use of psychiobiotics. This factor includes bacterial extracellular vesicles (EVs). It appears that EVs represent the missing piece of the puzzle in GBA and the mechanism of action of psychobiotics.

Psychobiotic bacteria within the gut—as all the living cells—produce and release nanosized EVs carrying bacterial cellular components (Cuesta et al., 2021). Bacterial EVs contribute to communication not only between the microbes, but also at the inter-kingdom level to affect the host cells (Haas-Neill and Forsythe, 2020; Cuesta et al., 2021; Pirolli et al., 2021). Bacterial EVs are small enough to be absorbed from the gastrointestinal tract and penetrate to the brain. In this way, EVs cargo a range of foreign bioactive compounds to affect the central nervous system (CNS) function. In this review, we decipher the role of bacterial EVs in the mechanism of action of psychobiotics by characterizing the nature of probiotic EVs, suggesting the way of their distribution to the CNS, and providing evidence for their action therein. We argue that bacterial EVs represent the novel mechanism of action of psychobiotics and highlight the future directions in relevant research.

2. Biogenesis and characteristics of bacteria-derived EVs

Bacteria have been reported to secrete several types of vesicles. While their biogenesis has not been completely explained, the structure and cargo of the bacterial EVs is dependent on the parental cell and is likely reflected in pharmacological effect under exposition (Toyofuku et al., 2019). In this section, we present only the outline of the current state of knowledge on this topic (Figure 1).

Outer membrane vesicles (OMVs) are the main type of EVs released by Gram-negative bacteria. OMVs are formed from the outer membrane of the cell and are packed with periplasmic material. Some works have also reported the presence of cytosolic proteins as well as genetic material [deoxyribonucleic (DNA) and ribonucleic acids (RNA)] in OMVs isolates (Schwechheimer and Kuehn, 2015; Lee et al., 2016). The secretion of OMVs might be initiated by the local polarization of the membrane (Mashburn-Warren et al., 2008) or intercalation of chemicals into outer membrane (Kobayashi et al., 2000; Florez et al., 2017). Both triggers are assumed to disturb the curvature of membrane which leads to OMVs formation. Increased turgor pressure in periplasmic space, due to the accumulation of biochemicals like misfolded proteins or phospholipids, have also been associated with outer membrane vesiculation (McBroom and Kuehn, 2007; Roier et al., 2016). Small quorum-sensing molecules and proteins additionally stimulate vesicle production (McMillan and Kuehn, 2021).

The OIMVs are double bilayered vesicles, enveloping a fragment of cytosol with a coat composed of inner (cellular) and outer membrane. Thus, OIMVs are considered to contain all functional components of parental cell including lipopolysaccharide (LPS) and the fragments of chromosomal DNA (Baeza et al., 2021). OIMVs might be formed similarly to OMVs through blebbing as a result of autolysin activity that transiently breaks down peptidoglycan to allow OIMV release (Pérez-Cruz et al., 2013; McMillan and Kuehn, 2021). Alternatively, OIMVs might be secreted in "explosive cell lysis" process (Turnbull et al., 2016; Baeza et al., 2021). OIMVs are less abundant fraction than OMVs (their content does not typically exceed few percent of total EVs population during the logarithmic growth phase; Pérez-Cruz et al., 2013; Baeza et al., 2021). Although the size of both types of vesicles is dependent on bacterial species and strain, the diameter of OIMVs typically exceeds 100 nm, and OMVs are usually smaller than OIMVs (Pérez-Cruz et al., 2013).

In the case of Gram-positive bacteria, the mechanism of cytoplasmic membrane vesicles (CMVs) generation is based on selective degradation of thick peptidoglycan cell wall layer. It might lead to the death of the cell which is termed "bubbling cell lysis" (Liu et al., 2018; McMillan and Kuehn, 2021). It is assumed to be analogous to explosive cell lysis occurring in Gram-negative bacteria. Both are initiated by phages or stress conditions like DNA damage (SOS

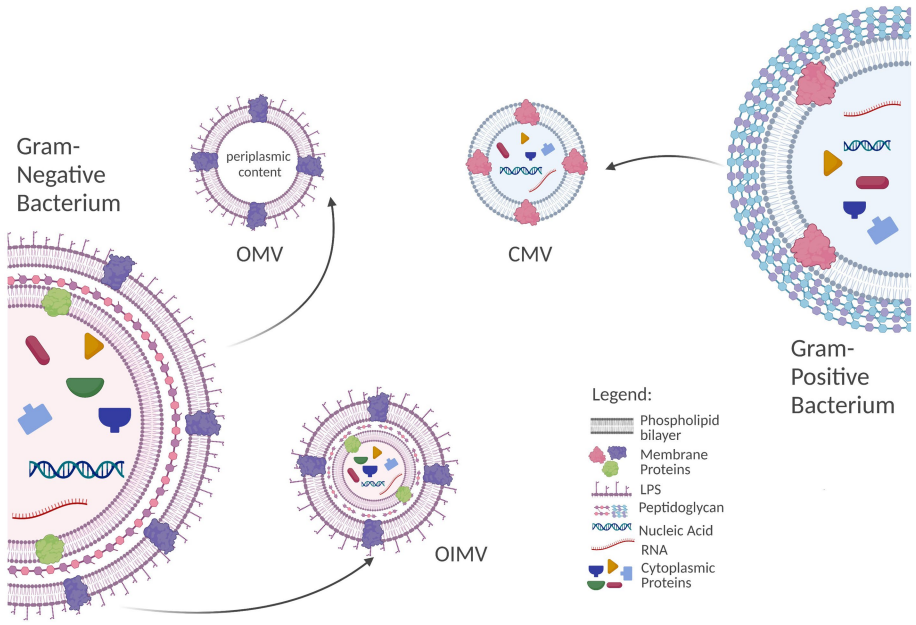

FIGURE 1
Biogenesis and characteristics of bacteria-derived extracellular vesicles (EVs). Outer-inner membrane vesicles (OIMVs) are formed from Gram-negative bacteria through blebbing or in "explosive cell lysis" process. OIMVs are considered to contain all functional components of parental cells including lipopolysaccharide (LPS) and the fragments of chromosomal deoxyribonucleic acid. Outer membrane vesicles (OMVs) are the most abundant vesicle type secreted by Gram-negative bacteria. OMVs are formed through blebbing and predominantly contain periplasmic content of the parental cell. In the case of Gram-positive bacteria, the mechanism of cytoplasmic membrane vesicles (CMVs) generation is based on selective degradation of peptidoglycan cell wall layer. It might lead to the cell death which is termed "bubbling cell lysis" Created with BioRender.com.

response) or perforation of peptidoglycan by exogenous factors (e.g., enzymes or antibiotics). In response, the expression of endolysins and fragmentation of peptidoglycan occurs. Perforation of thick peptidoglycan in Gram-positive bacteria leads to the "leakage" of cell interior (through the pores) in a form of vesicles ("bubbles") while in Gram-negative the process is more rapid (cell "explodes"). Nevertheless, CMVs released in this process typically feature 20–200 nm in size and contain all cellular components of parental cell, and reflects the cell condition at the time of death (Toyofuku et al., 2019).

3. Signaling and distribution of psychobiotic EVs to the brain

Extracellular vesicles may represent the efficient nanostructure for transport of bacterial bioactive compounds to the human brain. After being absorbed from the gastrointestinal tract (Stentz et al., 2018), the most likely mechanism of EVs distribution to the CNS is by (1) crossing the blood–brain barrier (BBB; Matsumoto et al., 2017). Other potential ways include (2) vagal nerve transport and (3) activated leukocyte trafficking to the brain (Figure 2).

A set of *in vitro* experiments have been performed to document the EVs transport through the intact BBB. In the study by Morad et al., an *in vitro* static BBB model has been constructed. This was a two-channel microfluidic culture device that contained a vascular channel lined by induced pluripotent stem-derived human microvascular endothelial cells, which were separated by a porous extracellular matrix-coated membrane from an abluminal channel containing primary human astrocytes and pericytes (Morad et al., 2019). In this way, the model could replicate the barrier function of *in vivo* intact human BBB (Park et al., 2019). By labeling tumor-derived 150 nm size EVs and flowing them through the vascular channel, a fluorescent signal was then detected in the abluminal chamber and increased significantly over time. Fluorescence microscopy analysis confirmed the presence of EVs that were taken up by astrocytes in the abluminal chamber. These findings demonstrate that EVs can interact with endothelial cells under flow conditions and continuously cross the endothelial layer through transcytosis (Morad et al., 2019).

Furthermore, a zebrafish model was used for *in vivo* studies to explore transcytosis of EVs through the BBB. Zebrafish develop a mature BBB at 3 days postfertilization and are a suitable model for BBB studies. Intracardiac injection of the brain-seeking EVs into zebrafish was performed and their distribution in the brain was monitored through live imaging. The results of this study showed that EVs were taken up by multiple cells within the brain parenchyma, proving their ability to cross the BBB *in vivo*. Furthermore, transport of EV-containing endocytic vesicles within endothelial cells could be observed using time-lapse imaging. As in transcytosis, these vesicles moved toward the plasma membrane and fused with the membrane. Most importantly, the BBB remained intact throughout the duration of these experiments, highlighting the absence of an inflammatory process (Morad et al., 2019).

Although several other papers similarly documented the passage of EVs of various origin through the intact BBB (Banks et al., 2020; Jakubec et al., 2020; Morales-Prieto et al., 2022; Zhou

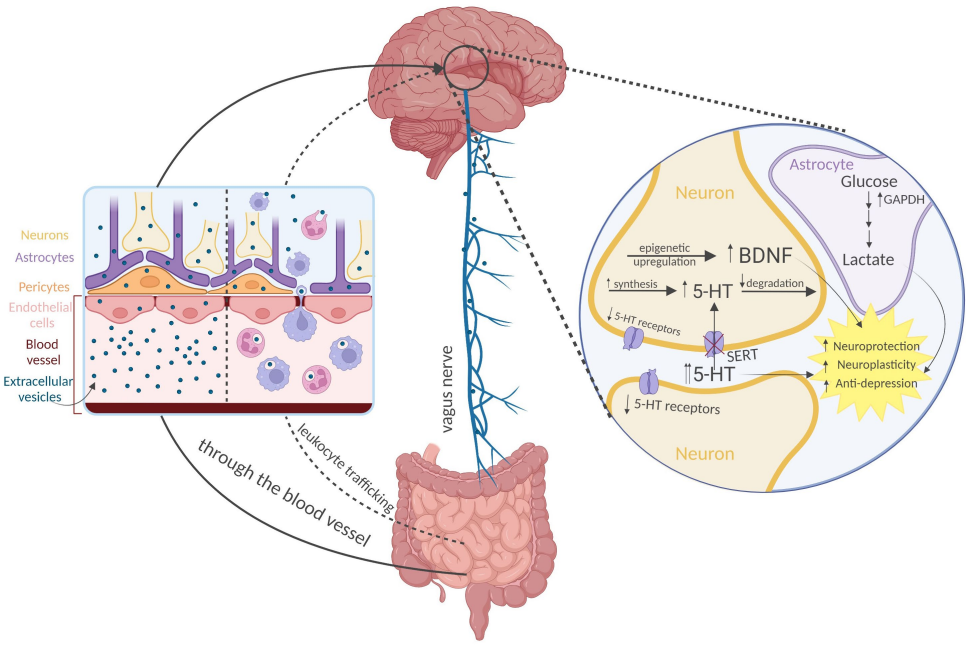

FIGURE 2
Distribution and action of the psychobiotic bacteria-derived extracellular vesicles (EVs) on the brain. Direct crossing of the blood–brain barrier appears to be the predominant way of EVs transport to the brain. Other routes include vagal nerve transport and activated leukocyte trafficking. According to the current evidence, EVs from psychobiotic bacteria produce antidepressant-like effect mediated by epigenetic upregulation of neurotrophic factors (e.g., brain-derived neurotrophic factor, BDNF), modulation of serotonergic system expression, and possibly by supplementation the astrocytes with glycolytic enzymes (glyceraldehyde 3-phosphate dehydrogenase, GAPDH). 5-HT—serotonin, and SERT—5HT transporter Created with BioRender.com

et al., 2022), the other mechanisms appear to exist as well. A second pathway that bacterial EVs may penetrate to the brain is through the vagal nerve. *Paenalcaligenes hominis* isolated from the feces of elderly people and aged mice were transplanted into young mice, and bacterial EVs-mediated cognitive impairment and colonic inflammation was noted (Lee et al., 2020). Specifically, Lee et al. (2020) orally gavaged conjugated *P. hominis* EVs or LPS in mice. The results showed that conjugated EVs and LPS were detectable in microglial cells and in the pyramidal region of the hippocampus. Furthermore, conjugated EVs were more abundant than LPS. However, following vagotomy, conjugated EVs abundance in this region was significantly reduced, unlike the conjugated LPS, which was not affected. Additionally, bacterial 16S ribosomal DNA levels in the hippocampus were increased after oral gavage of *P. hominis* or its EVs, whereas the process was inhibited by vagotomy. These findings suggest that EVs of *P. hominis* are orally absorbed and may be able to exert their actions on the brain at least partially through transport via the vagus nerve (Lee et al., 2020).

Other putative mechanisms that have not been extensively studied include EVs crossing the BBB via activated leukocytes trafficking to the brain. Bacterial peptidoglycans, which are a component of EVs, have been identified in the brains of patients with multiple sclerosis, which are heavily infiltrated with blood derived leukocytes. This suggests that these immune cells could be a source of EVs entry into the CNS, although it was not directly investigated (Schrijver et al., 2001). This mechanism of action is also common for viruses' (size 20–200 nm) ability to reach and infect the brain (Wurdinger et al., 2012; Branton et al., 2013).

4. Effects of EVs derived from psychobiotics on the CNS

Mounting evidence confirms that pathogenic bacteria EVs produce deleterious effects on the CNS function, whereas probiotic bacteria EVs exert beneficial effects on peripheral tissues (Cuesta et al., 2021; Pirolli et al., 2021). This provides indirect evidence for advantageous CNS action of EVs from psychobiotics. Depending on the species of origin, bacterial EVs may have both beneficial and detrimental effects on the CNS, with the latter being more extensively studied (Cuesta et al., 2021). Post-mortem brain samples from patients with Alzheimer's disease have shown the presence of bacterial nucleic acids that could have been transported in EVs (Emery et al., 2017; Cuesta et al., 2021; Vandendriessche et al., 2021). EVs from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* were shown to cross the BBB after intracardiac injection to mice, carrying bacterial extracellular nucleic acids, which increased expression of tumor necrosis factor alpha, a known pro-inflammatory cytokine, in the brain cortex (Han et al., 2019). On the other hand, probiotic bacteria EVs from lactobacilli (Al-Nedawi et al., 2015; Behzadi et al., 2017; Li et al., 2017; Seo et al., 2018; Choi et al., 2020; Kim et al., 2020), bifidobacteria (Kim et al., 2016), as well as other probiotic genera (de Rodovalho et al., 2020), were found to exert beneficial anti-inflammatory action through various mechanisms as tested in human peripheral cells or animal models relevant to non-CNS disorders.

However, very recent literature provides direct evidence for CNS-relevant advantageous action of EVs from psychobiotics (Figure 2). Choi et al. (2019) investigated the therapeutic potential of EVs derived from *Lactiplantibacillus plantarum* on depression model.

The authors induced depressive-like behavior in stressed mice and administered intraperitoneal injections of *L. plantarum* EVs. The results showed a reduction in depressive-like behavior, an increase in brain-derived neurotrophic factor (BDNF) gene expression in the hippocampi, and an additional increase in BDNF expression in a glucocorticoid-suppressed mouse neuronal cell line culture following *L. plantarum*-derived EVs treatment. This evidence was the first to suggest that EVs from psychobiotics may be beneficial at both molecular and functional levels. This indicated the potential for psychobiotic EVs as a therapeutic option for depression, and supported the notion that EVs play a significant role in the psychobiotics' mechanism of action (Choi et al., 2019).

A follow-up study conducted by the same research team has suggested that the effect is not limited to EVs from just one bacterial psychobiotic strain. Choi et al. (2022) induced chronic stress-like conditions in mice and administered EVs isolated from taxonomically different potential psychobiotic bacteria *L. plantarum*, *Bacillus subtilis*, or *Akkermansia muciniphila*. The authors noted that all the EVs produced an anti-depressive-like effect and restored diminished hippocampal BDNF as well as neurotrophin-3 and -4/5 expression; however, the behavioral effect was the most pronounced in case of EVs from *L. plantarum*, and EVs from *A. muciniphila* affected partially different epigenetic regulators of the expression of tested neurotrophic factors than that of *L. plantarum* and *B. subtilis*. The superior effect of *L. plantarum* EVs was not surprising as the authors found particularly remarkable stress-induced decrease in lactobacilli abundance in the gut microbiota in the tested animal model, which produced a gap for therapeutic response from the relevant EVs (Choi et al., 2022).

It becomes clearer that EVs from psychobiotic bacteria produce more versatile CNS-relevant effects than that mediated by neurotrophic factors. A study by Yaghoubfar et al. (2020) has shed light on the effects of EVs from potential psychobiotic *A. muciniphila* (Ding et al., 2021) on expression of serotonergic system in mice hippocampi. Oral treatment with the EVs led to an increase in the messenger RNA expression of the gene encoding rate limiting serotonin (5-HT) synthesizing enzyme tryptophan hydroxylase, whereas a decrease in degrading enzyme monoamine oxidase was noted. Also, 5-HT level was found to be increased, possibly extracellularly, as the expression of 5-HT transporter reduced. At the same time, the expression of some 5-HT receptors was decreased. As the treatment with *Akkermansia* EVs in the experiment lasted for 4 weeks (Yaghoubfar et al., 2020), the resulted serotonergic regulation may be comparable to that following chronic and therapeutically relevant treatment with antidepressants (Artigas, 2013; Gray et al., 2013; Bowman and Daws, 2019).

Another proposed mechanism for the beneficial effects of EVs from psychobiotics involves glycolytic enzymes. As a byproduct of glycolysis, astrocytes produce lactate, and it happens regardless of sufficient oxygen availability (Takahashi, 2021). Astrocyte-derived lactate is believed to fuel neurons, support synaptic plasticity processes (Suzuki et al., 2011) and its transport may prevent depression (Yao et al., 2023). Thus, high glycolytic activity in astrocytes may be beneficial (Takahashi, 2021). In a study by Bajic et al. (2020), EVs released by the dairy isolate *L. plantarum* were found to be enriched in enzymes involved in central metabolic pathways, including glycolysis, in particular, glyceraldehyde 3-phosphate dehydrogenase. This suggests that glycolytic enzymes may be supplemented by psychobiotic EVs to astrocytes, thus contributing to neuroprotection, however, a direct causation needs to be examined.

A study by Yang et al. (2022) explored the potential of EVs derived from *L. plantarum* against ischemic brain injury. Although this condition represents an acute clinical event, post-stroke complications typically include depression and cognitive deterioration (Wijeratne and Sales, 2021). The results showed that EVs from *Lactiplantibacillus* significantly reduced brain damage and improved neurological function in mice following a stroke. Moreover, they reduced infarct size and decreased neurological deficits. The mechanism of this protective effect involved the regulation of a specific microRNA, miR-101a-3p, and its downstream targets, c-Fos and transforming growth factor-β, leading to the inhibition of neuron apoptosis. Importantly, miR-101a-3p could also serve as a marker for neurological recovery in ischemic stroke patients. This finding may further shed light on the EVs-mediated mechanism of CNS-relevant action of psychobiotic bacteria (Yang et al., 2022).

5. Discussion

Gut-brain axis outlines complex mechanisms in which psychobiotics exert action on the brain. Until recently, it has not been appreciated that the GBA-mediated action may be additionally evoked through bacterial secreted EVs; this topic has been neglected in majority of recent review papers in the area of psychobiotics' mechanism of action (Del Toro-Barbosa et al., 2020; Dey and Mookherjee, 2021; Johnson et al., 2021; Tremblay et al., 2021; Yang et al., 2021; Magalhães-Guedes, 2022; Suda and Matsuda, 2022). Nevertheless, here we provide substantial evidence supporting such supposition. EVs from psychobiotic bacteria are small enough to be absorbed from the gastrointestinal tract and transported to the brain, where they can interact with the CNS components, affecting various range of brain processes. EVs from psychobiotics—even originated from taxonomically remote bacteria—may produce antidepressant effects. This may be through modulation of the expression of neurotrophic factors (Choi et al., 2019, 2022), neurotransmitter regulation (Yaghoubfar et al., 2020), or possible supplementation of the astrocytes with glycolytic enzymes (Bajic et al., 2020), among the investigated mechanisms. There is also a large body of evidence supporting anti-inflammatory action of probiotic EVs (Al-Nedawi et al., 2015; Kim et al., 2016, 2020; Behzadi et al., 2017; Li et al., 2017; Seo et al., 2018; Choi et al., 2020; de Rodovalho et al., 2020), however, none was performed in the CNS-relevant model. Collectively, EVs appear to intermediate a great deal of known mechanisms within the GBA. Moreover, psychobiotic bacteria-derived EVs may represent the "concentrated" messenger to the brain, as some of their effects were multiplied in comparison to administration of parental psychobiotic bacteria (Yaghoubfar et al., 2020). In this light, EVs may represent the missing piece of the puzzle in mechanism of action of psychobiotics.

Psychobiotics encompass Gram-positive and Gram-negative bacteria. Despite their differences, numerous bacteria from both these groups were shown to secrete EVs and to feature similar pharmacological effect in the GBA (Choi et al., 2022). On the other hand, EVs even from the same bacteria species (e.g., *Escherichia coli*) may have the opposite strain-dependent pro- (Imamiya et al., 2023) or anti-inflammatory properties (Güttsches et al., 2012), and this

phenomenon results from the altered form of LPS expressed by the parental bacteria (Güttsches et al., 2012). Little is known about specific ingredients and markers of bacterial EVs [e.g., fatty (Schroeder and Bäckhed, 2016; Champagne-Jorgensen et al., 2021) or ribonucleic acids (Lee et al., 2020), kinases (Gradowski et al., 2020), and other enzymes (Bajic et al., 2020)] that translate to the properties of psychobiotics. Scientific efforts toward their identification are urgently needed to understand EVs-mediated mechanism of action.

Limited knowledge on the biogenesis of bacterial EVs and their still evolving classification make it difficult to unequivocally identify the type of vesicles responsible for certain CNS-relevant effects. Since 2018, the updated recommendation of International Society of Extracellular Vesicles considering, *inter alia*, isolates characterization has increased the reporting standards (Théry et al., 2018), with the hope for deciphering relationship between probiotic EVs quality and their pharmacological effects. Still, the employment of analytical techniques enabling subpopulations differentiation is highly desirable (Steć et al., 2022). In addition, the quantity of bacterial EVs that could represent their CNS-effective but safe dose requires elucidation.

Numerous works have proved intensive secretion of EVs by bacteria under unfavorable stress conditions, e.g., misfolded proteins accumulation (McBroom and Kuehn, 2007; Roier et al., 2016), DNA damage or perforation of peptidoglycan, and reflecting cell condition at the death (Toyofuku et al., 2019). It is intriguing how such stress-induced EVs produced by psychobiotics may exert any beneficial effect to the human. The phenomenon may be partially explained by the nature of parental cells (Güttsches et al., 2012; Ashrafian et al., 2019). However, the bacterial EVs may be produced also under physiological conditions during their logarithmic growth (Baeza et al., 2021; Laurin et al., 2022). Moreover, the reason for bacteria to produce EVs remains a major paradox (McMillan and Kuehn, 2021), and these issues require further studies.

Also, the EVs from psychobiotic bacteria tested in the CNS-relevant models have been obtained in precisely defined conditions (Choi et al., 2019; Yaghoubfar et al., 2020; Choi et al., 2022). Nevertheless, EVs composition and size can change drastically, depending on environment and growth conditions (Ñahui Palomino et al., 2021). The potential effect of variable host physiological status on probiotic bacteria EVs generation and action requires further attention. Moreover, psychobiotic bacteria EVs may change the properties of other bacterial vesicles (McMillan and Kuehn, 2021) or host-derived EVs (Imamiya et al., 2023), further entangling their mechanism of action.

Although convincing evidence supports the role of bacterial EVs in the psychobiotics' mechanism of action, many questions need to be addressed, with some being raised above. Once solved, psychobiotic bacteria-derived EVs have potential to become a new generation postbiotics.

Author contributions

MK: conceptualization, supervision, and project administration. LB and MK: methodology and visualization. LB, SD, KW, and MK: literature search and writing—original draft. EK: funding acquisition. LB, SD, KW, EK, and MK: writing—review and editing. All authors contributed to the article and approved the submitted version.

Funding

The research was supported by Medical University of Lodz (Łódź, Poland; grant number 503/5-108-03/503-51-001-19-00). The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish it.

Conflict of interest

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

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 07 September 2023
DOI 10.3389/fmicb.2023.1229952

OPEN ACCESS

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RECEIVED 27 May 2023
ACCEPTED 16 August 2023
PUBLISHED 07 September 2023

CITATION
Tong Y, Guo H, Abbas Z, Zhang J, Wang J, Cheng Q, Peng S, Yang T, Bai T, Zhou Y, Li J, Wei X, Si D and Zhang R (2023) Optimizing postbiotic production through solid-state fermentation with *Bacillus amyloliquefaciens* J and *Lactiplantibacillus plantarum* SN4 enhances antibacterial, antioxidant, and anti-inflammatory activities.
Front. Microbiol. 14:1229952.
doi: 10.3389/fmicb.2023.1229952

Optimizing postbiotic production through solid-state fermentation with *Bacillus amyloliquefaciens* J and *Lactiplantibacillus plantarum* SN4 enhances antibacterial, antioxidant, and anti-inflammatory activities

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Background: Postbiotics are an emerging research interest in recent years and are fairly advanced compared to prebiotics and probiotics. The composition and function of postbiotics are closely related to fermentation conditions.

Methods: In this study, we developed a solid-state fermentation preparation method for postbiotics with antimicrobial, antioxidant, and anti-inflammatory activities. The antibacterial activity was improved 3.62 times compared to initial fermentation conditions by using optimization techniques such as single factor experiments, Plackett–Burman design (PBD), steepest ascent method (SAM), and central composite design (CCD) methods. The optimized conditions were carried out with an initial water content of 50% for 8 days at 37°C and fermentation strains of *Bacillus amyloliquefaciens* J and *Lactiplantibacillus plantarum* SN4 at a ratio of 1:1 with a total inoculum size of 8%. The optimized SSF medium content ratios of peptide powder, wheat bran, corn flour, and soybean meal were 4, 37.4, 30, and 28.6%, respectively.

Results: Under these optimized conditions, postbiotics with a concentration of 25 mg/mL showed significant broad-spectrum antibacterial capabilities against *Escherichia coli*, *Salmonella, and Staphylococcus aureus* and strong antioxidant activity against ABTS, DPPH, and OH radicals. Moreover, the optimized postbiotics exhibited good anti-inflammatory ability for reducing nitric oxide (NO) secretion in RAW 264.7 macrophage cells in response to LPS-induced inflammation. Furthermore, the postbiotics significantly improved intestinal epithelial wound healing capabilities after mechanical injury, such as cell scratches in IPEC-J2 cells ($p < 0.05$).

Conclusion: In brief, we developed postbiotics through optimized solid-state fermentation with potential benefits for gut health. Therefore, our findings suggested that the novel postbiotics could be used as potential functional food products for improving body health.

KEYWORDS

solid-state fermentation, postbiotics, antibacterial, antioxidant, anti-inflammatory

1. Introduction

The gastrointestinal (GI) tract is a complex ecosystem teeming with microorganisms such as bacteria, fungi, archaea, protozoa, and viruses that form the intestinal microbiota. The intestinal microbiota has adopted a remarkable spectrum of vital functions for the host including digestion, nutrient absorption, fermentation of dietary fibers, energy generation, and pathogen defense, due to its close relation and evolution with the intestinal environment (Goto, 2019; Lavelle and Sokol, 2020). Food-borne pathogens and subsequent secondary infections cause GI microbial dysbiosis and its metabolites thus causing severe damage to the intestinal mucosa, leading to recurrent intestinal inflammation and simultaneously leading to a variety of diseases such as inflammatory bowel diseases (IBDs), ulcerative colitis, Crohn's disease, and colorectal cancer (Gut et al., 2018; Cheung et al., 2021; Bai et al., 2022). Recent studies revealed that the gut microbiota and its metabolites play a significant role in promoting gut health by preserving the intestinal barrier and the immunity of the host. These metabolites were named postbiotics and defined as "inanimate microorganisms and/or their components that confer a health benefit on the host" in 2021 by "The International Scientific Association of Probiotics and Prebiotics" (ISAPP) (Salminen et al., 2021). The concept of postbiotics is fairly advanced in comparison with prebiotics and probiotics. Postbiotics are differentiated by their elemental composition such as organic acids, bacteriocins, fatty acids, bioactive peptides, phenolic acid, polysaccharides, or bioactive effects including antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory (Rad et al., 2021b; Blazheva et al., 2022). Postbiotic supplements are not as extensively available yet, but they are superior to probiotics because of their purity, definitive chemical structure, safety profile, long shelf life, mass production capability, precise action, and more targeted responses by specific ligand–receptor interactions (Nataraj et al., 2020; Rad et al., 2021a). Therefore, researchers are using postbiotics to modulate microbial signatures of health, nutrition, and disease status.

Oxidative stress and inflammation are two major factors involved in the progression of a wide variety of chronic human diseases (Kruidenier and Verspaget, 2002; Piechota-Polanczyk and Fichna, 2014). Free radicals, especially oxygen radicals, not only induce oxidative stress but can also interact with pro-inflammatory cytokines in a complex manner. Overproduced pro-inflammatory cytokines may cause excessive inflammation and the deterioration of cardiac and/or renal dysfunction (Rapa et al., 2019). Free radicals such as nitrogen and oxygen radicals are generated by various endogenous systems, exposure to different physiochemical conditions, or pathological states in the body (Silpak et al., 2017). Examples of free radicals include hydroxyl free radicals (OH·), superoxide free radical anion (O_2^-), nitric oxide (NO·), and peroxyl (RO_2·). The host body possesses antioxidant protection and repair systems to defend against oxidative damage; these mechanisms are not always effective in preventing all harm (Osseni et al., 2000). Furthermore, antioxidants present in diets may support gut health through the direct involvement in inhibiting oxidative stress in the gut environment and by protecting the microbiota against challenging environmental conditions (Wadanambi et al., 2023). Recent *in vivo* and *in vitro* studies reported that postbiotics have antibacterial, antioxidant, anti-inflammatory, immune-modulatory, and intestinal barrier-regulating capabilities (Aguilar-Toalá et al., 2018; Vallejo-Cordoba et al., 2020). These compounds offer a promising approach to combating food-borne pathogens, reducing oxidative stress and inflammatory response, strengthening the gut barrier, and/or regulating the host immune system (Aggarwal et al., 2022). *Lactiplantibacillus* spp.-derived postbiotics (cell-free supernatants) showed strong antioxidant activity and anti-*Staphylococcus* properties, which were mainly associated with lactic acid and other antibacterial components (Khani et al., 2023). Lactic acid bacteria (LAB) postbiotics also exhibited high antioxidant capacity and total phenolic content; combinations with natural preservatives such as EDTA could reduce food-borne pathogens (Incili et al., 2022). Some postbiotics were even incorporated into bacterial nanocellulose for preparing antibacterial films and were expected to extend food shelf life (Mohammadi et al., 2022).

Functional characteristics and compositions of postbiotics are correlated with the strains, culture medium, and condition under preparation (Chang et al., 2021). Kareem et al. (2014) highlight the importance of fermentation media in influencing the secretion of metabolic products and the biological activities of postbiotics. Solid-state fermentation (SSF) is a traditional fermentation technique used to enhance the nutritional values and functional properties of food processing by-products, with high-end-concentration products that are stable and cost-effective (Hölker et al., 2004; Yafetto, 2022). It can also reduce mutual inhibition between metabolites and increase the yield of active substances, making it a promising method for preparing bioactive postbiotics (Pacularu-Burada et al., 2022). In addition, it is more efficient to enrich the postbiotics with targeted bacteria and optimum conditions (Amiri et al., 2021; Melini et al., 2023). Ooi et al. (2015) reported that the optimization of fermentation media can enhance the inhibitory activity of postbiotics, such as bacteriocins produced by *Lactiplantibacillus plantarum* I-UL4. Furthermore, the antimicrobial activity of postbiotic RS5 was enhanced by 108%, reducing the cost by 85% through their refined medium (Ooi et al., 2021). However, optimizing the fermentation conditions and culture media components usually using the one-factor-at-a-time (OFAT) method is not adequate for investigating the interaction between different variables. This limitation can be overcome using statistical approaches such as Plackett–Burman design (PBD) and response surface methodology (RSM). Pacularu-Burada et al. (2021), using the statistical tools of PBD and RSM, enhanced the postbiotic properties and evidenced differences in the metabolite profiles, thus highlighting its functional potential. Postbiotics are commonly prepared by culturing the LAB in culture media [mainly de Man, Rogosa, and Sharpe (MRS) broth], followed by an extraction step (centrifugation or dialysis and filtration) (Gomez-Sala et al., 2016; Dunand et al., 2019; Barros et al., 2020; Moradi et al., 2021), and other fermentation alternatives such as milk and milk-related media also successfully produced remarkable antifungal activity (Gamier et al., 2019). Most studies revealed the antibacterial or antioxidant functions of postbiotics from submerged fermentation (Rossoni et al., 2020; Kienesberger et al., 2022; Banakar et al., 2023; Khani et al., 2023); however, they barely illustrated the SSF or developed multifunctional postbiotics. Moreover, studies on the preparation of functional postbiotics by solid-state fermentation with LAB and *Bacillus* strains or

their combination for the production of potential antibacterial, antioxidant, and anti-inflammatory postbiotics are lacking.

Therefore, this study was designed to obtain postbiotics through SSF using statistically optimized approaches to enhance its antibacterial, antioxidant, and anti-inflammatory potentials, thus improving gut health. Furthermore, this study will highlight the pharmacological capabilities of postbiotics and their safer use in nutrition research in future.

2. Materials and methods

2.1. Materials, microorganisms, and culture conditions

Peptide powder, wheat bran, soybean meal (not genetically modified), and corn were obtained from Beijing Longgang Biotechnology Research Center (Zhuozhou, Hebei Province, China). For the formulation of the SSF fermentation medium, the corn flour, soybean meal, peptide powder, and wheat bran powder consisted of the basal solid medium, totaling 100% (w/w), together with other ingredients ranging from 0.5 to 1% (w/w) and 2 to 4% (w/w) according to PBD or RSM runs and sterilized at 121°C for 15 min (LS-B100L, Binjiang Medical, China).

Bacillus amyloliquefaciens J and *Lactiplantibacillus plantarum* SN4 were selected based on specific functional properties, such as antibacterial activity (Supplementary Figure S1). These strains were possessed by the Laboratory of Feed Biotechnology at China Agriculture University and were deposited at the China General Microbiological Culture Collection Center (CGMCC), Beijing, China. The stock cultures of the *L. plantarum* strain and *Bacillus* strains were reactivated in de Man, Rogosa, and Sharpe (MRS) broth and beef peptone yeast (BPY) broth at 37°C aerobiosis. The cell viability counts of fermentation strains were determined using saline (0.9%, w/v) serial dilutions inoculated in MRS and BPY agar plates. For the established optical density and incubation conditions (12 h, 37°C, aerobiosis), an inoculum size of ca 1×10^8 colony-forming units per milliliter (CFU/mL) was obtained for all fermentation strains.

The pathogen indicator bacteria (*Staphylococcus aureus* ATCC 43300, *S. aureus* ATCC 6385, *Staphylococcus aureus* ATCC 1882, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, *Salmonella pullorum* CVCC 519, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 25325, Escherichia coli O157:H7 ATCC 43889, *Clostridium perfringens* CVCC 2030, *Pseudomonas aeruginosa* ATCC 27853, and *Pseudomonas aeruginosa* ATCC 9027) used in this study were maintained in our laboratory. All pathogenic bacteria were cultured overnight in Luria–Bertani (LB) broth at 37°C under aerobic conditions.

2.2. Determination of the antibacterial activity

The postbiotics were extracted by adding three to five times the weight of sterile water after fermentation, depending on the experiment design, optimization stage, and value of the antibacterial rate. According to the OFAT and PBD experiment designs, postbiotics were diluted three times, and for the RSM experiment design, the dilution was five times for acquiring and distinguishing the difference between runs. Then soaked in an oscillatory for 1 h and centrifuged at 6,000 rpm for 10 min at 4°C, the supernatant was collected and filtered through 0.22 μm filter (Millex-GP, Merck Millipore Ltd., Germany). The microtiter plate-based antibacterial assay was performed according to the method reported by Casey et al. (2004) and Matsue et al. (2019), with slight modifications. To improve screening efficiency in our experiments, the indicator microorganism *E. coli* ATCC 25922 was cultured to the logarithmic growth stage, followed by centrifugation, washing, dilution, and resuspension. Then, 10 μL of bacterial solution with a final concentration of 5×10^6 CFU/mL, sterilized LB medium (140 μL), and test samples (50 μL) were added to the 96-well plates. The wells with only bacterial suspension were used as positive control, and only LB medium was used as test quality control. The plates were incubated for several hours at 37°C in an incubator shaker at 200 rpm (LYZ-D2403, Longyue, China). At the indicated time, the turbidity at OD_{600nm} was measured using a multi-well photometric microplate reader (Spectra Max 190, Molecular Devices, USA). Having established the turbidity values prior to and immediately following incubation, antibacterial activity was calculated using the following Equation (1):

$$\text{Inhibition}\% = \frac{(P_{12} - P_0) - (T_{12} - T_0)}{(P_{12} - P_0)} \times 100 \quad (1)$$

where inhibition is the antibacterial ratio (%) of test samples against *E. coli* ATCC 25922, $P_{12} - P_0$ is the turbidity change in positive control wells after incubation for 12 h at 37°C, and $T_{12} - T_0$ is the turbidity change in the test sample wells under the same condition.

2.3. Analytical approach for optimizing culture conditions

2.3.1. One-factor-at-a-time design

One-factor-at-a-time experiments were conducted to explore the influence of different factors, including fermentation strains, temperature, time, inoculum sizes, and water content on antibacterial activity. The values of the main factors in this study were as following: fermentation strains involving single strains, and its combination; temperature set was 28, 31, 34, 37 and 40°C; time was 2, 4, 6, 8, and 10 d; inoculum size was 4, 6, 8, 10, and 12%; and water content was 35, 40, 45, 50 and 60%.

2.3.2. Determination of significant factors using Plackett–Burman design

The PBD was used to identify the key factors of solid medium components that contribute to the antibacterial properties while assuming that the interactions among these factors can be negligible. The corn flour, soybean meal, peptide powder, and wheat bran powder consisted of the basal solid medium, totaling 100%; other components were added extra based on the solid medium. A nine-factor orthogonal matrix was created, with each factor having a high-level (+1) and a low-level (−1) representation. The candidate influent factors were: corn flour (20, 40%) (w/w), soybean

meal (15, 30%) (w/w), glucose (4, 8%) (w/w), molasses (2.5, 5%) (w/w), growth factor (0.5, 1%) (w/w), peptide powder (2, 4%) (w/w), $CaCO_3$ (0.5, 1%) (w/w), yeast extract, and KH_2PO_4 contents (0.2, 0.4%) (w/w), respectively. Correspondingly, (−1, +1) levels were selected for *E. coli* ATCC 25922 inhibition, each experiment reported in the PBD was performed in triplicate (Table 1).

2.3.3. Steepest ascent method

The path of the SAM was achieved to set up basal concentrations of media components selected from the PBD to be used in a central composite design (CCD). It permitted rapid movement toward the most favorable of variable concentrations. Increments of X_1 (glucose), X_2 (corn flour), and X_3 (soybean meal) were 2, 5, and 5, respectively. Experiments were performed along with the SAM until the response did not increase anymore, and each experiment was conducted in triplicate. The medium level of factors is represented in Table 2.

2.3.4. Central composite design and response surface

Once the critical factors were identified via screening and the experimental design space was approached by SAM, the CCD was used to define the level of the significant parameters and the interactions between them, which significantly influence the antibacterial activity. Each parameter was analyzed at five levels coded as ($\pm\alpha$, ±1, 0), as shown in Table 3.

2.3.5. Regression models and statistical analysis

The experimental data were fitted using Design-Expert 8.0.6 (Stat-Ease, Inc., Minneapolis, MN, USA) and SPSS 26.0 (SPSS Corp., Chicago, IL, USA) software. To determine the inhibition capabilities in relation to input factors according to Table 3, a polynomial regression model was used as follows:

$$Y_1 = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i<j=2}^{3} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{ii} X_i^2 \quad (2)$$

where Y_1 is the antibacterial activity, X_i are input variables (three variables retained), and β_0, β_i, β_{ij}, and β_{ii} are the regression coefficients for the intercept, linear, interaction, and quadratic effects.

In addition, ANOVA was used to assess the statistical variables for the optimization of the fermentation medium, with an F-test used to verify the statistical significance. The coefficient of determination (R^2) was employed for the quality of the polynomial model equation.

2.4. Preparation of postbiotic extracts

After the fermentation with the aforementioned optimized culture conditions, postbiotics were extracted with sterile water and soaked in an oscillatory for 1 h. The extracts were then centrifuged at 6,000 rpm for 10 min, 4°C, and the supernatant collected and filtered through 0.22 μm filter (Millex-GP, Merck Millipore Ltd., Germany). The filtered solutions were freeze-dried

TABLE 1 Plackett–Burman experiments design matrix with factors given in coded levels and inhibition values.

Run	A: Corn flour %	B: Soybean meal %	C: Glucose %	D: Molasses %	E: Growth factor %	F: Peptide powder %	G: $CaCO_3$ %	H: Yeast extract %	J: KH_2PO_4 %	Inhibition %
1	40 (+1)	30 (+1)	4 (−1)	5.0 (+1)	1.0 (+1)	4 (+1)	0.5 (−1)	0.2 (−1)	0.2 (−1)	91.45
2	20 (−1)	30 (+1)	8 (+1)	2.5 (−1)	1.0 (+1)	4 (+1)	1.0 (+1)	0.2 (−1)	0.2 (−1)	53.56
3	40 (+1)	15 (−1)	8 (+1)	5.0 (+1)	0.5 (−1)	4 (+1)	1.0 (+1)	0.4 (+1)	0.2 (−1)	98.14
4	20 (−1)	30 (+1)	4 (−1)	5.0 (+1)	1.0 (+1)	2 (−1)	1.0 (+1)	0.4 (+1)	0.4 (+1)	0
5	20 (−1)	15 (−1)	8 (+1)	2.5 (−1)	1.0 (+1)	4 (+1)	0.5 (−1)	0.4 (+1)	0.4 (+1)	21.69
6	20 (−1)	15 (−1)	4 (−1)	5.0 (+1)	0.5 (−1)	4 (+1)	1.0 (+1)	0.2 (−1)	0.4 (+1)	0
7	40 (+1)	15 (−1)	4 (−1)	2.5 (−1)	1.0 (+1)	2 (−1)	1.0 (+1)	0.4 (+1)	0.2 (−1)	0
8	40 (+1)	30 (+1)	4 (−1)	2.5 (−1)	0.5 (−1)	4 (+1)	0.5 (−1)	0.4 (+1)	0.4 (+1)	55.11
9	40 (+1)	30 (+1)	8 (+1)	2.5 (−1)	0.5 (−1)	2 (−1)	1.0 (+1)	0.2 (−1)	0.4 (+1)	97.77
10	20 (−1)	30 (+1)	8 (+1)	5.0 (+1)	0.5 (−1)	2 (−1)	0.5 (−1)	0.4 (+1)	0.2 (−1)	97.91
11	40 (+1)	15 (−1)	8 (+1)	5.0 (+1)	1.0 (+1)	2 (−1)	0.5 (−1)	0.2 (−1)	0.4 (+1)	97.96
12	20 (−1)	15 (−1)	4 (−1)	2.5 (−1)	0.5 (−1)	2 (−1)	0.5 (−1)	0.2 (−1)	0.2 (−1)	0

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TABLE 2 Experimental design and response of the SAM experiments.

Run	X_1: Glucose (%)	X_2: Corn flour (%)	X_3: Soybean meal (%)	Inhibition (%)
1	4	20	15	0
2	6	25	20	9.79
3	8	30	25	50.6
4	10	35	30	64.21
5	12	40	35	64.09
6	14	45	40	58.34

TABLE 3 Response surface of CCD and inhibition of *Escherichia coli* ATCC 25922.

Run	Glucose		Corn Flour		Soybean meal		Inhibition (%)
	$\overline{X_1}$	X_1 (%)	$\overline{X_2}$	X_2 (%)	$\overline{X_3}$	X_3 (%)	
1	−1	8	−1	30	−1	25	68.37
2	1	12	−1	30	−1	25	47.57
3	−1	8	1	40	−1	25	30.48
4	1	12	1	40	−1	25	26.13
5	−1	8	−1	30	1	35	50.65
6	1	12	−1	30	1	35	30.42
7	−1	8	1	40	1	35	35.36
8	1	12	1	40	1	35	42.95
9	−α	6.64	0	35	0	30	51.88
10	α	13.36	0	35	0	30	41.14
11	0	10	−α	26.59	0	30	68.28
12	0	10	α	43.41	0	30	55.18
13	0	10	0	35	−α	21.59	26.07
14	0	10	0	35	α	38.41	24.55
15	0	10	0	35	0	30	71.19
16	0	10	0	35	0	30	66.37
17	0	10	0	35	0	30	70.13
18	0	10	0	35	0	30	68.79
19	0	10	0	35	0	30	71.46
20	0	10	0	35	0	30	70.32

by using a lyophilizer (Beijing Fourring Scientific Instrument Co. Ltd., Beijing, China) and stored in a sterile container at −20°C for further research.

2.5. Bioactive profile of postbiotics

The profile of postbiotic compositions was detected by various colorimetric and modern instrument analytical methods. The 3-phenyllactic acid (Wu et al., 2020), lactic acid (Russo et al., 2017), and ferulic acid (Kaur et al., 2013) were determined by high-performance liquid chromatography (HPLC), and the content of short-chain fatty acids (SCFAs) including acetic acid, propionic acid, butyric acid, and pentanoic acid was detected by gas chromatography (GC) (Beards et al., 2010). The total protein was measured with BCA Protein Assay Kit (Solarbio) according to the manufacturer's recommendation. Phenolic acid was measured by the Folin–Ciocalteu's phenol reagent (Tan et al., 2021). Total soluble sugar was determined by using the phenol–sulfuric acid method (Nielsen, 2017).

2.6. Determination of minimal inhibitory concentrations and minimal bactericidal concentrations

The broth microdilution assay was used to assess the MICs and MBCs, similar to our previous research (Guo et al., 2021),

with slight modifications. Sterilized Mueller–Hinton broth (MHB) medium (150 μL) and postbiotic solution (50 μL) were added to sterile 96-well plates at final postbiotic concentrations of 0.4–50 mg/mL. The bacteria were cultured to the logarithmic growth stage, followed by centrifugation, washing, dilution, and resuspension. Then, 2 μL of bacterial solution with a concentration of 5×10^6 CFU/mL was added to the 96-well plates. The wells with only bacterial suspension or MHB medium were used as controls. The minimum postbiotic concentration at which bacterial growth was invisible in the 96-well plate was defined as the MIC of the postbiotics.

The MBCs were tested after determining the MICs. A 20 μL aliquot of culture medium in the wells without visible bacterial growth was spread on MHB agar plates and cultured overnight at 37°C to determine the MBCs based on the absence of bacterial colony growth. Each trial was repeated three times.

2.7. *In vitro* antioxidant activity assays

1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich. 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) was obtained from Biotopped life sciences Co., LTD, Beijing, China. Ascorbic acid and salicylic acid were obtained from Solarbio, and other reagents were obtained from Sinopharm Chemical Reagent. The antioxidant activity of hydroxyl radical, DPPH, and ABTS radical scavenging activities were calculated by the flowing formula:

$$\%\text{Scavenging} = 1 - \frac{A1 - A}{A0} \times 100 \quad (3)$$

where A_1 refers to the sample mixed with the working solution, A refers to the sample without the work solution, and A0 is the absorbance of the working solution without the sample as a blank control. Ascorbic acid was used as a positive control.

2.7.1. Hydroxyl radical scavenging activity

The scavenging activity of hydroxyl radicals was detected based on the Fenton reaction of Fe^{2+}/H_2O_2 (Kaur and Sud, 2023). A 200 μL of the aqueous solutions of different concentrations (0–10 mg/mL) of postbiotics and 300 μL of 1.8 mM $FeSO_4$ solution were mixed with 90 μL of 1.6 mM salicylic acid and ethanol solution, and then, 210 μL of 3% (v/v) H_2O_2 solution was added. After shaking well, the sample solution was added, and the absorbance was measured at 510 nm.

2.7.2. DPPH radical scavenging activity

The DPPH radical scavenging activity was determined according to the previous method of Wei et al. (2023). In brief, an aliquot of 200 μL of different concentrations (0–10 mg/mL) of postbiotics was added to 800 μL of DPPH solution (100 μM DPPH in absolute methanol). After incubation for 30 min, the absorbance of the incubated samples was measured at 517 nm. Methanol was used as a blank.

2.7.3. ABTS radical scavenging activity

The ABTS radical scavenging was measured according to the method of Sah et al. (2014). A 200 μL of sample solution was added to 600 μL of ABTS radical working ($OD_{734nm} = 0.7$) solution and incubated in the dark for 30 min, and the absorbance of the mixture at 734 nm was measured. A measure of 200 μL of dd water instead of the sample was used as a blank.

2.7.4. Reducing power

To determine the reducing power, the methods were the same as Kou et al. (2013). The test samples with different concentrations (0–10 mg/mL) of postbiotics (200 μL), 500 μL of sodium phosphate buffer (0.01 M, pH = 6.6), and 500 μL of potassium ferricyanide solutions (1%, w/v) were mixed and incubated the mixtures for 30 min at 50°C. Then, 500 μL of trichloroacetic acid (10%, w/v) was added and centrifuged for 10 min at 3,000 rpm. The upper layer (200 μL) was added with 100 μL of ferric chloride (0.1%, w/v), and the absorbance at 700 nm was measured.

2.8. Cell viability assay

The viability of the postbiotic-treated IPEC-J2 cells and RAW264.7 cells was determined using a Cell Counting Kit-8 (CCK-8) assay kit (Solarbio). The IPEC-J2 cells and RAW264.7 cells (3×10^4 cells/mL) were cultured overnight in 100 μL of DMEM in 96-well plates. Postbiotic final concentrations of 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 mg/mL with cell culture medium were added to IPEC-J2 cells, and final concentrations of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 mg/mL were added in RAW264.7 cells, followed by incubation for 6 h. The medium was replaced with 100 μL of fresh DMEM and 10 μL of CCK-8 solution. After incubation at 37°C for 2–4 h, the absorbance of the plates at 450 nm was determined using a microplate reader. The cell viability was calculated using the following formula:

$$\%\text{Cell viability} = \frac{\text{Abs of samples}}{\text{Abs of control}} \times 100 \quad (4)$$

2.9. Anti-inflammatory assay

NO is an important inflammatory biomarker responsible for inflammation. We emulated the NO concentration in the RAW 264.7 cell culture supernatants using the Griess reagent according to Han and Hyun (2023). Cultured RAW 264.7 cells (3×10^4 cells/well) were retreated with different concentrations of postbiotics (0–4 mg/mL) and then LPS (100 ng/mL) in 96-well plates and incubated for 24 h. Cell culture supernatants were mixed with an equal volume (50 μL) of the Griess reagent and incubated in 96-well plates for 10 min at room temperature. Absorbance was measured at 540 nm using a microplate reader.

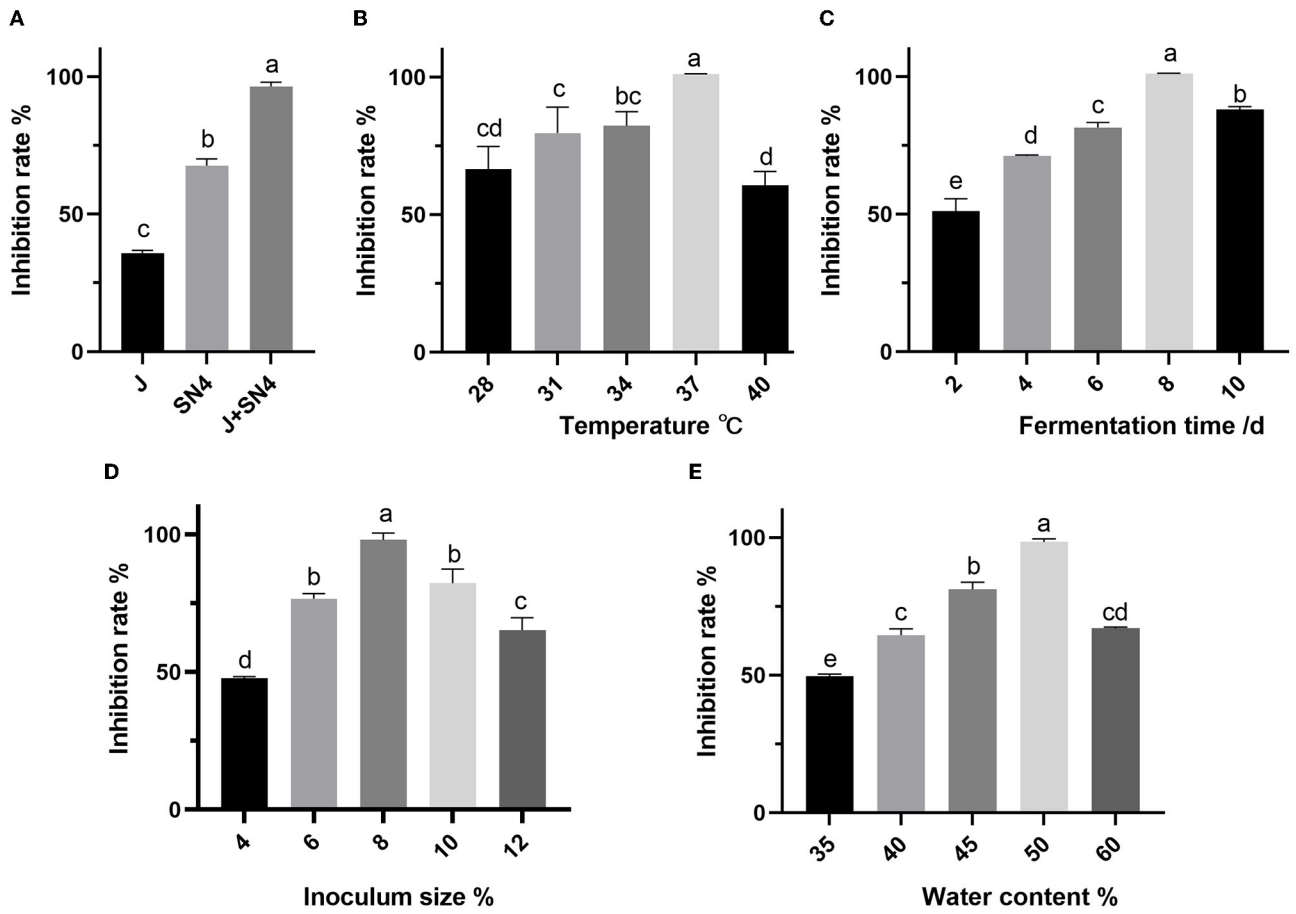

FIGURE 1
Effect of different fermentation strains **(A)**, temperature **(B)**, time **(C)**, inoculum size **(D)**, and water content **(E)** on the inhibition rate of *Escherichia coli* ATCC 25922. a–e: different lowercase letters represent significance ($p < 0.05$), and p-values were expressed as mean ± SEM ($n = 3$).

2.10. Wound healing assay

To assess wound healing capabilities, IPEC-J2 cells were seeded in 6-well imaging plates at 4×10^5 cells per well and grown overnight to confluency. Selected samples were pretreated with the postbiotics for 6 h, and a straight scratch was generated with constant pressure, speed, and angle using a 100 μL plastic pipette tip and washed by PBS to remove excess debris. For scanning of the scratch-wounded area, images were taken every 12 h by a microscope (DSZ2000). Cell scratch area image analysis was carried out using Image J software.

$$\%\ \text{Cell healing rate} = \frac{0\ \text{h cell scratch area} - n\ \text{h cell scratch area}}{0\ \text{h cell scratch area}} \times 100\% \quad (5)$$

2.11. Statistical analysis

The results were described as mean ± standard error of the mean (SEM). The data were analyzed by one-way ANOVA using an SPSS system. Student Newman–Keuls multiple comparison test was used to determine the difference between treatments. All data were subjected to one-way analysis of variance (ANOVA) with the general linear model, and the significant differences in all of the figures were tested by multiple comparisons and visualized using GraphPad Prism 9 software (Graphpad Software Inc., San Diego, CA). Statistical significance was expressed using p-value of <0.05.

3. Results

3.1. Effect of fermentation strains, temperature, time, inoculum size, and water content on antibacterial activity of postbiotics

The antibacterial activity of postbiotics varied depending on the number and combination of fermentation strains, with one to two strains producing different levels of activity as shown in Figure 1. Fermenting with two strains (1:1, v/v) simultaneously was found to be preferable for achieving higher inhibition rates against *E. coli* ATCC 25922 (Figure 1A). Other fermentation conditions with mixed strains fermentation affected by antimicrobial properties are presented in Figure 1. The temperature and water content of fermentation showed a significant influence on inhibition rate,

when the temperature was 28–40°C and water content was 35–60%, the antibacterial activity first increased and then decreased as temperature value or water content increased. The inhibition rate achieved the maximum value when the temperature was 37°C and the water content was 50% (Figures 1B, E). The inhibition rate increased significantly from 50 to 100% as fermentation time was extended from 2 to 8 days. With the extended fermentation time to 10 days, the inhibition rate decreased significantly ($p < 0.05$) (Figure 1C). The optimum time was recorded 8 days. Furthermore, a significantly higher inhibition rate ($p < 0.05$) was observed as the inoculum size increased from 4 to 8%, while decreased with the inoculum size of 10–12% ($p < 0.05$). So, the optimized inoculum size was 8%.

3.2. Corn flour, soybean meal, and glucose as key factors affecting antibacterial activity

The PBD was used to screen the effect of different factors of corn flour, soybean meal, glucose, molasses, growth factor, peptide powder addition, $CaCO_3$, yeast extract, and KH_2PO_4 on antibacterial activity of postbiotics (Table 1). After analyzing the inhibition rate of postbiotics, corn flour (A), soybean meal (B), and glucose (C) were the main factors that significantly affected the antibacterial activity ($p < 0.05$) (Table 4). According to the three significant factors on antibacterial activity, corn flour (A), soybean meal (B), glucose (C), molasses (D), and peptide powder (F) showed a positive impact, whereas the growth factor (E), $CaCO_3$(G), yeast extract (H), and KH_2PO_4 (J) had a negative impact (Figure 2). The obtained polynomial model was expressed in terms of coded factors:

$$Y_1\% = -102.38 + 2.23^*A + 1.978^*B + 13.35^*C + 13.11^*D - 7.02^*E + 2.19^*F - 9.55^*G - 5.66^*H - 5.71^*J \quad (6)$$

3.3. Optimization by steepest ascent method

The direction of the steepest ascent method was determined by the results of PBD. X_1 (Glucose), X_2 (Corn Flour) and X_3 (Soybean Meal) by incremental steps of 2, 5, and 5, respectively, were added to study the medium component and locate the region of significance, and the results are as shown in Table 2. Glucose, Corn Flour, and Soybean Meal were fixed at 10, 35, and 30%, respectively, and exhibited the highest inhibition against *E. coli* ATCC 25922. This point was chosen as a clue to set up basal concentrations for further optimization by CCD (the center point for optimization by CCD).

3.4. Optimization by using CCD

The CCD was used to define the optimum levels of the significant factors and study their interactions. X_1 (Glucose), X_2 (Corn Flour), and X_3 (Soybean Meal) were studied at five levels ($-\alpha$, -1, 0, 1, α). The experimental design and the experimental responses of inhibitions are reported in Table 3. Based on these data analyzed by Design-Expert 8.0.6, the polynomial regression of predicted responses Y_1 for inhibition in terms of coded factors is expressed as follows:

$$Y_1 = 69.76 - 4.09X_1 - 6.16X_2 - 1.15X_3 + 5.53X_1X_2 + 1.56X_1X_3 + 7.07X_2X_3 - 8.52X_1^2 - 3.14X_2^2 - 16.01X_3^2 \quad (7)$$

where Y_1 is the inhibition, and X_1, X_2, X_3 are independent variables in coded units. The regression model was designed using the F-test, and ANOVA was utilized to evaluate the significance and adequacy of the model (Raza et al., 2011).

As shown in Table 5, the *p*-values obtained using ANOVA and the F-test were <0.01, demonstrating that the model terms were significant ($p < 0.01$). The coefficients for X_1 and X_2 were highly significant ($p < 0.01$), meaning that glucose and corn flour had a remarkable impact on the inhibition ability of postbiotics. The model was highly significant with a very low *p*-value < 0.01 (*p*-value Probability > F). The R^2 and adjusted R^2 values for inhibition were 0.9826 and 0.9670, respectively, which demonstrated the high accuracy of the polynomial regression model (Zhang et al., 2020).

The contour plots and 3D response surfaces to visually demonstrate the polynomial regression are shown in Figure 3. These findings also demonstrated the response across a range of independent variables and the association between the experimental levels of each factor. Figures 3A, D displays the impact of glucose and corn flour on antibacterial ability, and the maximum inhibition rate was observed when the addition of glucose and corn flour was at 8.82 and 30.02%, respectively. Subsequently, the inhibition rate of postbiotics decreased as the addition of corn flour and glucose increased, as presented in Figures 3B, E, C, F. With the increase of soybean meal by more than 30%, a decline in the inhibition rate was observed (Figures 3C, F). According to the model, the maximal value of the antimicrobial activity of 76.80% is achieved for $X_1 = 8.82$, $X_2 = 30$, and $X_3 = 28.57$ (%). Finally, according to the optimized contents addition obtained from the CCD model, the experiment was repeated three times in fermentation bags to verify the real reliability of the predicted values. The inhibition rate was measured to be 78.56%, which was close to the predicted value. Compared with the initial antimicrobial activity (21.69%) of the fermentation condition, the

TABLE 4 Statistical analysis of factors using Plackett–Burman design.

Coefficient	Value	*p*-value
A	3,897.53	3×10^{-4}
B	1,729.14	6×10^{-4}
C	5,604.00	2×10^{-4}
D	1,350.45	7×10^{-4}
E	387.47	2.6×10^{-3}
F	37.75	2.55×10^{-2}
G	717.18	1.40×10^{-3}
H	251.54	4.00×10^{-3}
J	256.22	3.90×10^{-3}

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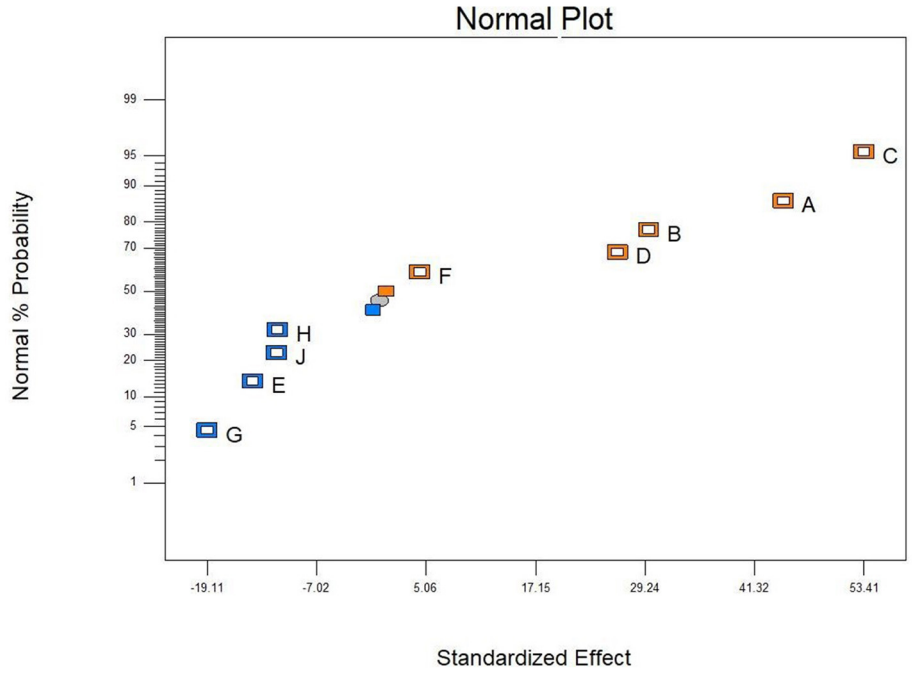

FIGURE 2
Influence of the factors on the antibacterial activity by PB design. Corn flour **(A)**, soybean meal **(B)**, glucose **(C)**, molasses **(D)**, growth factor **(E)**, peptide powder **(F)**, $CaCO_3$ **(G)**, yeast extract **(H)**, and KH_2PO_4 **(J)**. Orange block represented positive effect and blue block means negative effect, all factors analyzed by Design-Expert 8.0.6.

optimized condition improved the inhibition against *E. coli* ATCC 25922 by 3.62 times.

3.5. Bioactive profiling of postbiotics

The profiles of bioactive contents of postbiotics are presented in Table 6, and the phenyllactic acid, lactic acid, acetic acid, butyric acid, pentanoic acid, total soluble sugar, and total phenolic acid including ferulic acid significantly increased ($p < 0.05$) by optimization process except for the total protein and propionic acid.

3.6. Determination of minimal inhibitory concentrations and minimal bactericidal concentrations

The MICs of postbiotics against all tested strains, including *S. aureus, S. typhimurium, S. pullorum, Escherichia coli, C. perfringens,* and *P. aeruginosa*, were within the range of 12.5–25 mg/mL, while the MBCs ranged from 12.5 to 50 mg/mL (Table 7). Postbiotics showed effective antibacterial activity (the lowest values of MICs with 12.5 mg/mL) against *S. aureus* ATCC 1882, *C. perfringens* CVCC 2030, *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 9027 compared with other tested strains. As for the MASA strain, *S. aureus* ATCC 43300, postbiotics represented antibacterial ability with MIC of 18.75 mg/mL and MBC of 25 mg/mL, which showed the same results in *S. aureus* ATCC 6385 and *S. aureus* ATCC 25923. The MICs values of postbiotics recorded 25 mg/mL for *E. coli* ATCC 25922 and *E. coli* O157: H7 ATCC 43889 and the MBCs values displayed 50 and 43.75 mg/mL, respectively. *Escherichia coli* strains of ATCC 25922, ATCC 25325, and *E. coli* O157: H7 ATCC

TABLE 5 Analysis of CCD test results for inhibition.

Source	df	Adj SS	Adj MS	*F*-value	*p*-value
Model	9	5,835.98	648.44	62.86	<0.0001
X_1	1	228.42	228.42	22.14	0.0008
X_2	1	518.16	518.16	50.23	<0.0001
X_3	1	18.11	18.11	1.76	0.2147
X_1X_2	1	244.98	244.98	23.75	0.0006
X_1X_3	1	19.56	19.56	1.90	0.1985
X_2X_3	1	400.02	400.02	38.78	<0.0001
X_1^2	1	1,045.72	1,045.72	101.37	<0.0001
X_2^2	1	141.85	141.85	13.75	0.0041
X_3^2	1	3,695.62	3,695.62	358.26	<0.0001
Residual	10	103.16	10.32		
Lack of fit	5	85.35	17.07	4.79	0.0552
Pure error	5	17.80	3.56		
Cor total	19	5,939.13			

$R^2 = 98.26\%$; adj $R^2 = 96.70\%$, df: degree of freedom; Adj SS: adjust the sum of squares; Adj MS: adjust mean square.

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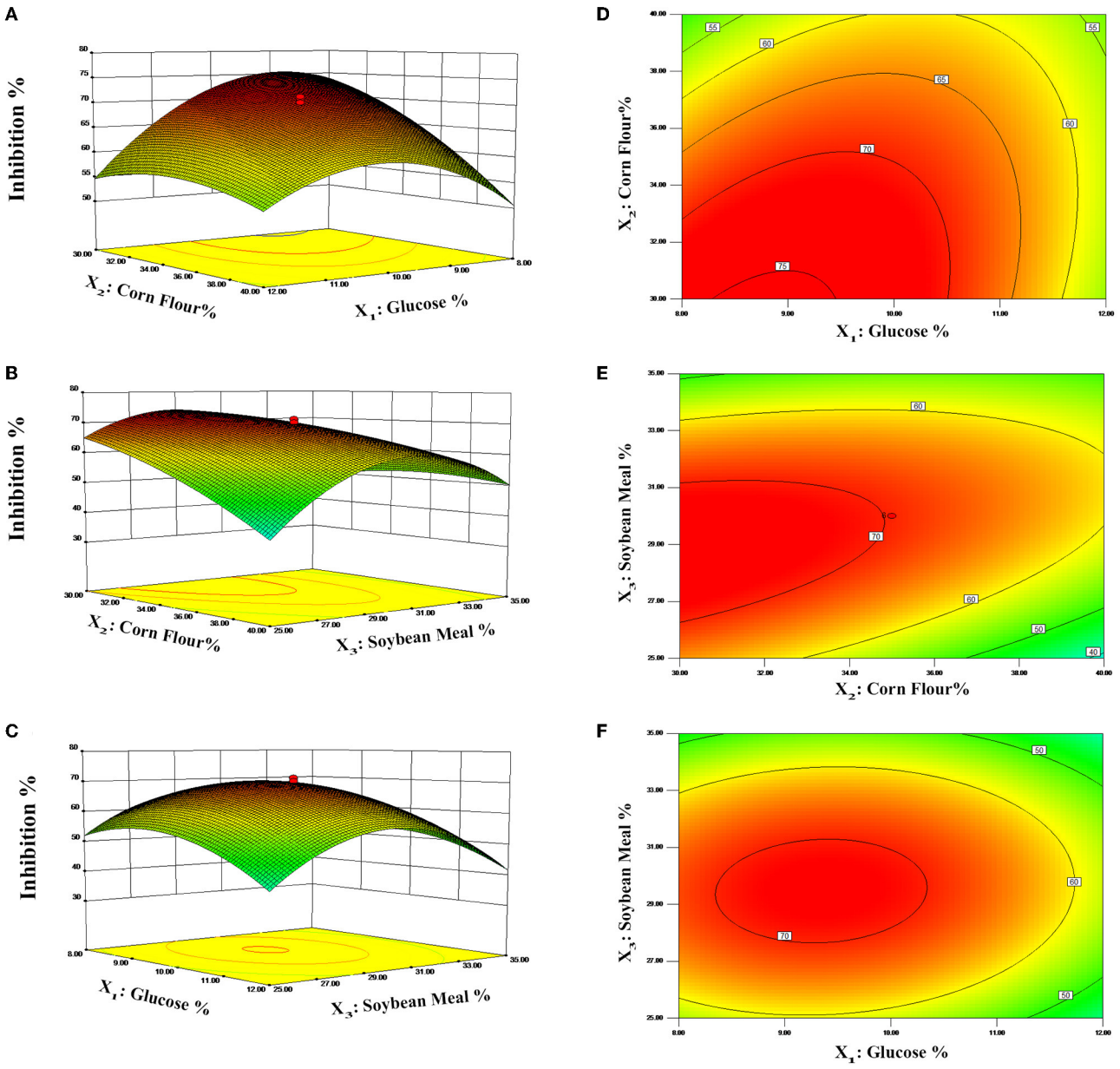

FIGURE 3
Response surface curves **(A–C)** and contour plots **(D–F)** predicted by the retained polynomial model.

43889 showed higher concentrations of MBCs compared to other indicator bacteria.

3.7. *In vivo* antioxidant activity of postbiotics

In this study, the *in vitro* antioxidant activity of postbiotics was evaluated by hydroxyl radical, DPPH, and ABTS radical scavenging and reducing capacity. The results are shown in Figure 4. The IC_{50} value of postbiotics for hydroxyl radical, DPPH, and ABTS radical was ~0.5 mg/mL. However, the scavenging ability was lower than 50% even though the postbiotic concentration was higher before optimization. Optimized postbiotics presented superior activity to non-optimized postbiotics against hydroxyl radical, DPPH, and ABTS radical and better reducing capacity as shown in Figure 4. Optimized postbiotics showed similar activity to ascorbic acid in terms of hydroxyl, DPPH, and ABTS radical scavenging ability at a same concentration of 1.5–2 mg/mL. The reducing ability of postbiotics depended on the concentration.

3.8. Mammals' cell viability

The cell viability of the intestinal porcine epithelial cell line (IPEC-J2) and the mouse macrophage cell line (RAW 264.7) was

TABLE 6 Contents in postbiotics analyzed by colorimetric and modern instrument analytical methods.

Contents	After optimization (mg/g)	Before optimization (mg/g)	*p*-value
Total protein	1.528 ± 0.0519	1.599 ± 0.0598	0.19271
Phenyllactic acid	0.178 ± 0.0275	0.07 ± 0.02	0.00528
Lactic acid	52.273 ± 0.5768	10.926 ± 0.1148	<0.0001
Acetic acid	4.565 ± 0.0815	0.761 ± 0.0979	<0.0001
Butyric acid	2.601 ± 0.3145	0.489 ± 0.0836	0.00036
Propionic acid	0.075 ± 0.0175	0.085 ± 0.0275	0.60858
Pentanoic acid	0.543 ± 0.0178	0.737 ± 0.0315	0.00075
Total Phenolic acid	4.951 ± 0.0377	2.332 ± 0.0057	<0.0001
Ferulic acid	0.187 ± 0.0002	0.004 ± 0.0001	<0.0001
Total soluble sugar	86.855 ± 3.3178	67.8791 ± 2.7364	0.00146

Data were given as the mean value ± SD from three biological replicates.

TABLE 7 MICs and MBCs of CTPs on different pathogens.

Strains	MIC (mg/mL)	MBC (mg/mL)
S. aureus ATCC 43300	18.75	25
S. aureus ATCC 6385	18.75	25
S. aureus ATCC 1882	12.5	25
S. aureus ATCC 25923	18.75	25
S. typhimurium ATCC 14028	18.75	31.25
S. pullorum CVCC 519	18.75	31.25
E. coli ATCC 25922	25	50
E. coli ATCC 25325	18.75	50
E. coli O157: H7 ATCC 43889	25	43.75
C. perfringens CVCC 2030	12.5	12.5
P. aeruginosa ATCC 27853	12.5	25
P. aeruginosa ATCC 9027	12.5	25

assessed after treatment with varying concentrations of postbiotics (Figures 5A, B). The results of the present study demonstrated that postbiotics did not affect the viability of IPEC-J2 cells when treated with concentrations up to 12 mg/mL. Concentrations of postbiotics ranging from 2 to 10 mg/mL even promoted the growth of IPEC-J2 cells (Figure 5A). On the other hand, RAW 264.7 macrophage cells exhibited lower tolerance to postbiotics, with cell viability being preserved at ∼75% when treated with concentrations up to 5 mg/mL (Figure 5B).

3.9. Effect of postbiotics on NO production during the inflammatory response

As shown in Figure 6, NO production is significantly induced by lipopolysaccharide (LPS) at a concentration of 15.8 μM ($p < 0.05$). Postbiotics significantly inhibited NO production in LPS-activated RAW 264.7 macrophages starting at 0.5 mg/mL ($p < 0.05$), and 0.5–1.5 mg/mL of postbiotics reduced the same extent of NO content secreted by mouse macrophagocytes ($p > 0.05$). When the concentration of postbiotics was 2.0 mg/mL, the content of NO was decreased to the same level as the control group without LPS co-incubation. With the increase in the number of postbiotics to 4 mg/mL, the secretion of NO from RAW 264.7 cells also declined. Therefore, the minimum effective concentration of postbiotics to lessen NO production was 2 mg/mL. These results also reflected that the postbiotics showed anti-inflammatory ability against LPS-induced inflammation.

3.10. Wound healing capabilities of postbiotics *in vitro*

Treatment of IPEC-J2 cells with postbiotics (2.8 mg/mL) after scratching led to a significant increase in wound healing when compared to the control (Figures 7A, B). In the first 12 h, treatment with 2.8 mg/mL of postbiotics did not provide any enhancement of the wound closure speed. In fact, the enhancement of the wound closure area in the postbiotic treatment was more than controlled after 24 h. Furthermore, complete healing was observed after 36 h of postbiotic treatment. As time extended to 48 h (images not shown), the control treatment also could not achieve complete healing.

4. Discussion

Scientific evidence has demonstrated that postbiotics exhibit diverse functional properties, which include, but are not limited to, antimicrobial, antioxidant, and immunomodulatory activities (Aguilar-Toalá et al., 2018; Cuevas-Gonzalez et al., 2020). Furthermore, postbiotics have great potential in the food industry as functional supplements, food quality improvers, and food preservatives (Zhong et al., 2022). In the present study, the antibacterial activity was increased by 3.62 times after the optimization of preparation conditions and fermentation medium. The profile of bioactive substances in postbiotics was also detected by colorimetric determination and modern instrument analytical methods. The optimized postbiotics showed a broad-spectrum antibacterial effect, antioxidant ability, and anti-inflammatory properties *in vitro*. We also evaluated the cell safety of postbiotics, which exhibited absolute safety at concentrations within the effective range. Furthermore, postbiotics had improved intestinal cell wound healing and gut health benefit potential.

The strain combinations with *B. amyloliquefaciens* J and *L. plantarum* SN4 tended to yield higher antibacterial ability compared to single fermentations of the J strain which was 35%, and SN4 was 67%, under the same conditions. *B. amyloliquefaciens* was found to secrete abundant amylase, protease, and cellulase (González Pereyra et al., 2020; Ngalimat et al., 2021), while *B. amyloliquefaciens* J exhibited strong abilities on amylase and protease, as shown in Supplementary Figure S2. On the other hand, *L. plantarum* SN4 exhibited weak activity of amylase and protease but could produce lactic acid and other bioactive substances from degradable carbon sources, which suggested that

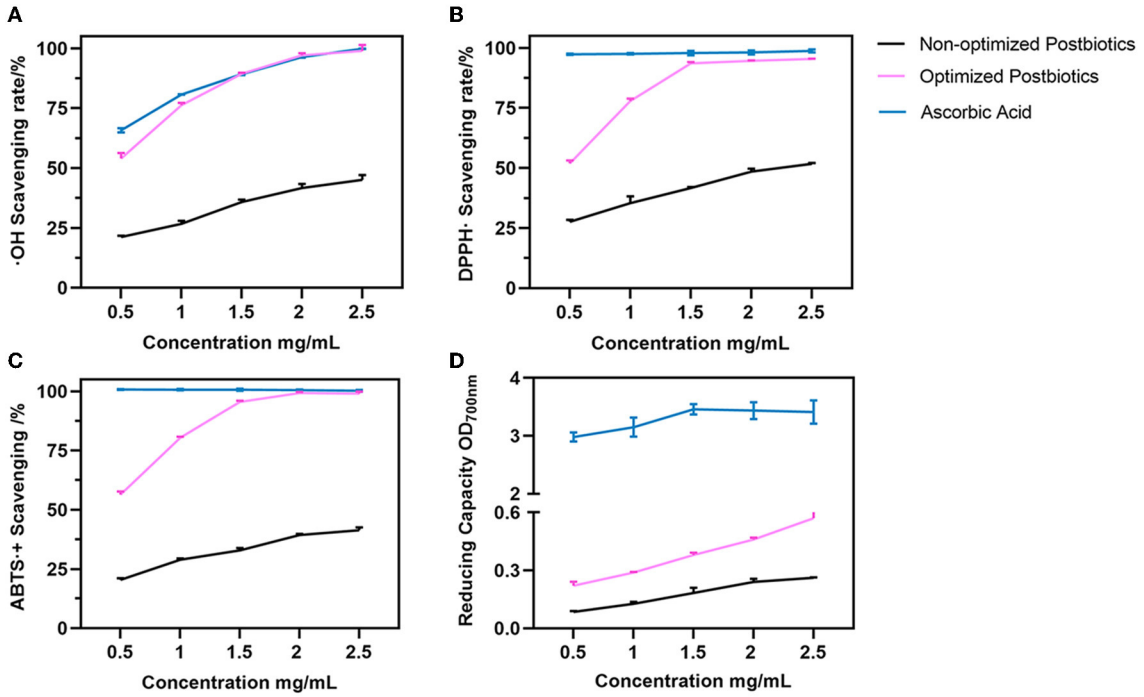

FIGURE 4
Hydroxyl radical **(A)**, DPPH **(B)**, ABTS **(C)**, and radical scavenging and reducing capacity **(D)** between the non-optimized and optimized postbiotics. Ascorbic acid as a positive control. Data were given as the mean value ± SD from three biological replicates.

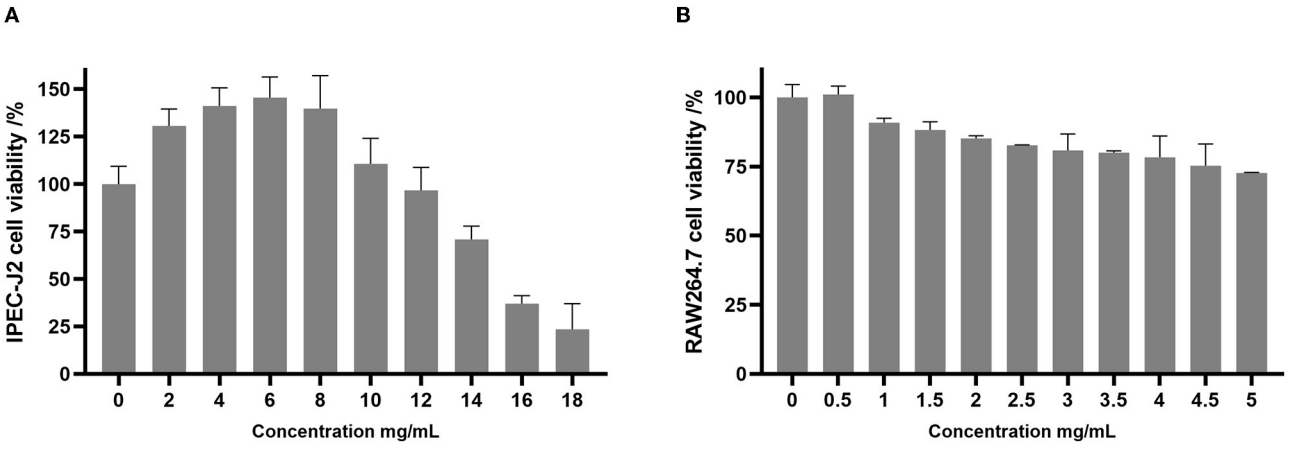

FIGURE 5
Cell viability of IPEC-J2 **(A)** and RAW264.7 **(B)** upon exposure to postbiotics. Data were given as the mean value ± SD from three biological replicates.

strain combination fermentation in SSF compensated for the incomplete enzyme systems of individual strains (Jia et al., 2019; Huang et al., 2023). The optimum temperature, water content, and inoculum size were 37°C, 50, and 8%, respectively, indicating that antimicrobial properties could be compromised by excessively high temperatures, too much water in fermentation progress, and living bacteria. It has been reported that appropriate temperature, water, and inoculum in solid media are crucial for creating an ideal environment for microorganisms' growth and metabolism (Guo et al., 2019). The fermentation time is determined by various factors such as the growth stage of bacteria strains, the production of inhibitors, and nutrient availability (Kanagasabai et al., 2019). Based on the findings of the one-factor-at-a-time experiments in this study, it was determined that a fermentation time of 8 days was appropriate. The optimized fermentation conditions of postbiotic preparation greatly enhanced the inhibition rate against *E. coli* ATCC 25922 in the initial stage of optimization.

To maximize the antimicrobial ability of postbiotics, Plackett–Burman design (PBD) and central composite design (CCD) of response surface methodology (RSM) were applied to optimize the

fermentation medium composition. Wang et al. (2017) reported an increase of 3.8 times in antimicrobial activity with optimal medium composition compared with the initial medium. Ghribi et al. (2012) proved that a central composite design and a response surface methodology successfully increased the antimicrobial activity of biosurfactants. PBD and RSM had become common strategies to increase the production of bactericidal substances in SSF but were barely used in postbiotic preparation (Yun et al., 2018; Chen et al., 2021). In this study, corn flour, soybean meal, and glucose had a significant positive effect on antibacterial ability ($p < 0.05$). These three significant factors, according to the PBD results were, adjacently optimized by the steepest ascent method (SAM), and the optimal point was further used as the center point in the CCD. Based on the solution of polynomial regression of predicted responses and validation experiments, the inhibition rate for *E. coli* ATCC 25922 was found to have increased by 3.62 times compared to the original medium. By combining the results of fermentation conditions optimization, we have developed postbiotics with significantly higher bioactivities using agriculture by-products such as corn flour, soybean meal, and bran powder. Moreover, 3-phenyllactic acid, lactic acid, acetic acid, butyric acid, pentanoic acid, total phenolic acid, and total soluble sugar were significantly increased compared to unoptimized conditions ($p < 0.05$).

The postbiotics prepared in this study demonstrated broad-spectrum antibacterial properties against both gram-positive and gram-negative bacteria. It was revealed that 3-phenyllactic acid, lactic acid, and acetic acid might be the key points. The 3-phenyllactic acid was reported to possess a broad-spectrum antibacterial activity linked to severely affecting the structure of biomembranes (Kleinwachter et al., 2021; Jiang et al., 2022). The organic acid was early reported to be used in controlling microbial contamination of carcass meat, attributing its antibacterial activity to it (Cherrington et al., 1991). In addition to antibacterial activity, postbiotics also demonstrated strong free radical scavenging ability and anti-inflammatory effects in the mouse macrophage cell line (RAW264.7).

Hydroxyl free radicals (OH·) and nitric oxide (NO·) are common examples of free radicals. The optimized postbiotics showed an IC_{50} value of 0.5 mg/mL to scavenge them, indicating that postbiotics had excellent antioxidant activity. The results obtained in this study were consistent with previous studies that reported the antioxidant properties of postbiotics derived from *L. plantarum* using liquid fermentation (Yu et al., 2016; Chang et al., 2021). Postbiotic RG14 (cell-free supernatant) produced by *L. plantarum* RG14 showed the highest antioxidant activity against ABTS and DPPH radicals and also enhanced

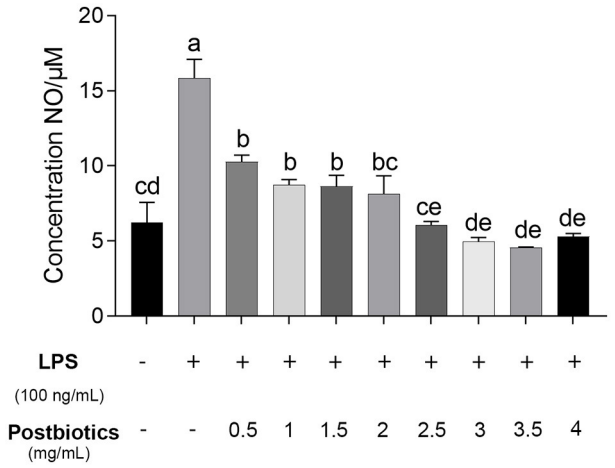

FIGURE 6
Effect of postbiotics on the production of NO in the LPS-induced RAW 264.7 cells. a–e: different lowercase letters show significance among different concentrations, and *p*-values of < 0.05 were expressed as mean ± SEM ($n = 3$).

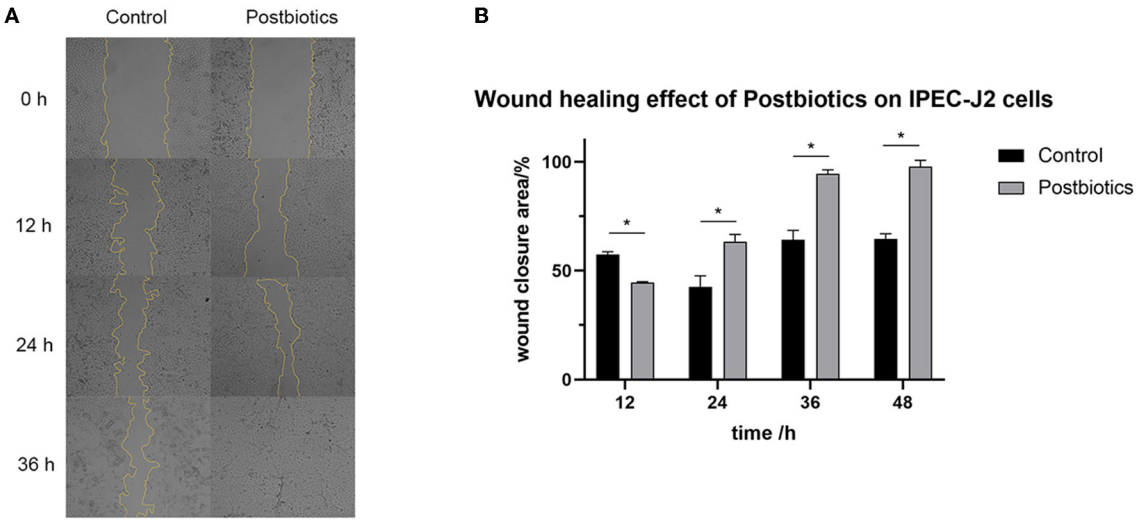

FIGURE 7
Impact of postbiotics (2.8 mg/mL) on the wound healing capacity of IPEC-J2 cells. **(A)** The state of cells in every 12 hours (magnification: 40×). **(B)** The descriptive results of wound closure area. *$p < 0.05$. Data were given as the mean value ± SD from three biological replicates.

the glutathione peroxidase (GPX) enzymes *in vivo* (Izuddin et al., 2020). In this study, optimized postbiotics exhibited an increased free radical scavenging ability against OH·, DPPH, and ABTS and a better reducing capacity compared to non-optimized postbiotics. Our results align with previous studies showing that optimizing fermentation conditions with response surface methodology significantly enhanced antioxidant activities (Miao et al., 2020; Chen et al., 2021; Kuo et al., 2021). Moreover, studies also revealed that the difference in the antioxidant activity of postbiotics depends on mechanisms such as the metal ion chelating ability, the antioxidant enzyme system, and the antioxidant metabolites present in the postbiotic (Yang et al., 2017). Polyphenols, including phenolic acids and fatty aromatic acids, contribute to the antioxidant activity of the postbiotics (Nikmaram et al., 2018). Incili et al. (2022) characterized postbiotics from lactic acid bacteria and found that the concentration and types of phenolics varied greatly, mainly depending on the medium composition. In our study, ferulic acid and total phenolic acid were significantly increased by optimization, which proved the influence of fermentation conditions on postbiotic bioactive components ($p < 0.05$). Additionally, the total soluble sugar in postbiotics was derived from bacterial metabolites, and degraded substrates such as exopolysaccharides and oligosaccharides are also conducive to antioxidant activity. The postbiotic characterization showed that the postbiotics had antioxidant properties that might be correlated with total phenolic acid, including ferulic acid and total soluble sugar in this research. Moreover, the detected bioactive components were also connected with anti-inflammatory capacity.

Lipopolysaccharide (LPS) is a common endotoxin derived from gram-negative bacteria that causes a series of inflammatory reactions in the body. Among these, NO is excessively generated by one of the pro-inflammatory enzymes, iNOS, and consequently results in diverse diseases (Patel et al., 1999). Our study indicated that the postbiotics possessed potent NO inhibitory activity against LPS-induced NO release with an effective concentration of 2 mg/mL in RAW264.7 cells. Meanwhile, to determine whether the inhibitory effects of compounds on NO production were due to specific anti-inflammatory activities, or non-specific cell cytotoxicity leading to false positive results, RAW 264.7 cell viability was determined by using CCK-8 assays. Notably, at a concentration of 5 mg/mL, no significant cytotoxic effects were observed. Normal NO production in the phagocytes is beneficial for host defense against pathogens and cancer cells. Recently, there have been a few studies on the anti-inflammatory activity of postbiotics. Kang et al. (2021) observed that lactic acid bacteria in heat-killed cells had decreased nitric oxide production via the downregulation of inducible nitric oxide synthase. In another study, lyophilized cell-free supernatants decreased the production of nitric oxide and were not cytotoxic to RAW 264.7 cells (Sornsenee et al., 2021).

In this study, we prepared postbiotics through simultaneous solid-state fermentation of *B. amyloliquefaciens* and *L. plantarum* for the first time and obtained postbiotics with antibacterial, antioxidant, and anti-inflammatory properties. More interestingly results in the cell viability test, postbiotics showed high cell safety in the intestinal porcine epithelial cell line (IPEC-J2) and even promoted the growth of the IPEC-J2 cells (Figure 5B). The mechanical barrier of the intestine is an important component of the intestinal barrier and plays a crucial role in maintaining intestinal health. Mechanical intestinal injuries were frequently happening in the body. Continuous production, migration, and apoptosis of epithelial cells provide a dynamic barrier for intestinal health. Previous studies in soluble proteins produced by probiotic bacteria promoted cell growth in human and mouse colon epithelial cells (Yan et al., 2007), and such probiotic-derived factors that bring beneficial effects had been identified as "postbiotic" mediators until 2012 (Tsilingiri et al., 2012). Postbiotics, such as lactobacilli-derived factors, have been proposed and described to enhance innate immunity, promote intestinal epithelial cell survival, and improve barrier function (Cicenia et al., 2014). In this study, we detected that the postbiotics enhance intestinal epithelial wound healing after a mechanical injury such as a cell scratch in IPEC-J2 cells. It was also reported by Lee et al. (2022) that the postbiotics of *L. reuteri* DS0384, prepared with liquid fermentation, promoted intestinal stem cell proliferation and protected intestinal epithelial cells from cytokine-induced injury. These findings indicated that postbiotics played a crucial role in health and nutrition.

5. Conclusion

This study has taken a novel approach for the optimized production of postbiotics through solid-state fermentation techniques, which significantly improved the antibacterial, antioxidant, and anti-inflammatory activities ($p < 0.05$). Furthermore, our findings suggest that the optimized postbiotics produced in our study may promote gut health by reducing inflammation, promoting intestinal epithelial growth, and protecting from mechanical injury. The current study represents postbiotics as a potential remedy for food preservatives and for promoting gut health. Further research is needed to explore the mechanism of action of these postbiotics and evaluate their pharmacological efficacy as drugs and food functional additives.

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

YT conceived and designed the experiments. YT, HG, JZ, TY, and JL performed the experiments. YT, SP, and QC analyzed the data. TB, JW, YZ, and XW finished the visualization of the figures. YT wrote the manuscript. ZA corrected grammar errors. DS and ZA reviewed and edited the manuscript. RZ guided the experiments. All authors have read and agreed to the published version of the manuscript.

All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Beijing Science and Technology Plan with No. Z201100008020018.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1229952/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 25 September 2023
DOI 10.3389/fmicb.2023.1276268

OPEN ACCESS

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RECEIVED 11 August 2023
ACCEPTED 11 September 2023
PUBLISHED 25 September 2023

CITATION
Sadighbathi S, Saris PEJ, Amiri S and Yousefvand A (2023) Development and properties of functional yoghurt enriched with postbiotic produced by yoghurt cultures using cheese whey and skim milk.
Front. Microbiol. 14:1276268.
doi: 10.3389/fmicb.2023.1276268

Development and properties of functional yoghurt enriched with postbiotic produced by yoghurt cultures using cheese whey and skim milk

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This study aimed to examine the effects of supplementation of postbiotics derived from *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) in cheese whey (CW) and skim milk (SM) on antioxidant activity, viability of yoghurt starters, and quality parameters of low-fat yoghurt during 22days of storage. The LB-CW (*L delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey) sample exhibited the highest antioxidant activity, with 18.71% inhibition ($p > 0.05$). This sample also showed the highest water holding capacity (77.93%; $p < 0.05$) and a trend toward receiving the most favorable sensory attributes ($p > 0.05$) compared to the other samples. The LB-CW and LB-SM yoghurt samples exhibited significantly higher body and texture scores compared to the ST-SM-fortified yoghurt ($p < 0.05$). However, there was no significant difference in the overall acceptability of the LB-SM and ST-SM yoghurt samples across both starters ($p > 0.05$). Such findings highlight the potential of postbiotics as functional ingredients to enhance the nutritional and sensory aspects of yoghurt, further contributing to its appeal as a health-promoting product.

KEYWORDS

postbiotics, yoghurt starters, cheese whey, skim milk, syneresis

1. Introduction

The widespread popularity and high consumption of yoghurt make it an appealing choice for incorporating various value-added ingredients, such as probiotic bacteria, prebiotics, plant fibers, and extracts (Fazilah et al., 2018). Postbiotics are another potential supplement derived from beneficial microorganisms, particularly lactic acid bacteria (LAB), that can be generated in culture media, food, or the intestine. While a universally accepted definition is lacking (Aguilar-Toalá et al., 2021; Sabahi et al., 2022; Thorakkattu et al., 2022), postbiotic constituents encompass diverse intracellular and extracellular compounds. However, it is generally acknowledged that the removal of bacterial cells is a necessary step (Wegh et al., 2019; Moradi et al., 2021). The resulting postbiotic solution contains compounds that are safe to consume, and also feature specific chemical structures and a long shelf life, making it suitable for use in food

products (Aguilar-Toalá et al., 2018). Postbiotics are gaining interest due to their inherent stability during processing and storage, making them more suitable for regions lacking reliable cold chains. Unlike probiotics, which often experience die-off during storage, postbiotics maintain stability over time. Probiotic manufacturers use overages to ensure labeled viable cell counts, and the live-to-dead ratio can change, impacting efficacy. Unlike probiotics, postbiotics remain stable at room temperature for years, eliminating viability concerns and allowing fixed microorganism levels at production. This stability makes postbiotics a promising option for areas with storage challenges (Salminen et al., 2021).

Probiotic bacteria produce water-soluble bioactive compounds known as "Postbiotics," which encompass various metabolites such as bioactive lipids like conjugated linoleic acid (CLA), antimicrobial peptides like bacteriocins (BACs), and exopolysaccharides (EPSs) (Aguilar-Toalá et al., 2018). These bioactive compounds offer a multitude of reported advantages, including anti-inflammatory, antimicrobial, anti-diabetic, anti-cancer, immunomodulatory, anti-atherosclerotic, and anti-obesity activities, as documented in recent literature (Dubey et al., 2012; Dahiya and Puniya, 2017; Aguilar-Toalá et al., 2018; Amiri et al., 2020, 2022). Bacterial EPSs, which are polysaccharide molecules, are secreted by certain bacteria into the culture media. EPSs have been extensively studied for their technological applications in the food industry due to their textural and rheological properties. Moreover, they have gained considerable attention recently for their functional properties. For example, emerging research has highlighted their immunomodulatory potential, anti-inflammatory, anti-biofilm, and antioxidant activities (Kumar et al., 2007; Amiri et al., 2021).

Functional foods have the potential to be enriched with postbiotics to enhance the host's immune activity. A mouse model study demonstrated that the cell-free fraction of fermented milk effectively prevented *Salmonella* infection (Dunand et al., 2019). Presently, postbiotics derived from *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 are being utilized in the production of functional foods, especially for cheese products (Sharafi et al., 2022), as well as for modified milk, with their effectiveness assessed in randomized clinical trials. For instance, *Bifidobacterium breve* and *S. thermophilus* postbiotics showed a reduction in the incidence of allergy-related symptoms in infants with a positive history of atopy during their early months of life; this effect persisted even after discontinuation of the preparation (Morisset et al., 2010). Additionally, these postbiotics were associated with a milder course of acute diarrhea in infants (Thibault et al., 2004). Notably, one of the active metabolites of *S. thermophilus* is the aforementioned 3′-GL (Perrin et al., 2000).

S. thermophilus and *L. delbrueckii* ssp. *bulgaricus* are commonly used bacteria in dairy product manufacturing. During the commercial production of probiotics, postbiotics are generated as byproducts and are often considered as waste. Instead of being discarded, the postbiotic solution waste product presents a cost-effective and biologically active alternative source to enhance the nutritional content and shelf life of yoghurt during storage. In many previous studies exploring the use of LAB postbiotics in food, researchers relied on de Man Rogosa and Sharpe (MRS) as a preparation medium for postbiotic solutions. However, there is particular importance in identifying new, inexpensive, and underutilized agro-industrial waste for postbiotic preparation. In this study, postbiotic solutions derived from *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were prepared using cheese whey and skim milk, two innovative growth model media. These postbiotic solutions were subsequently incorporated into yoghurt in the form of powdered nutritional supplements, adding a functional dimension to the yoghurt. The effect of each postbiotic powder on the microbial, chemical, and sensory characteristics of the yoghurt that was enriched with the postbiotic formulations was investigated.

2. Materials and methods

2.1. Microorganisms and inoculums

Freeze-dried cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chr. Hansen, DK-2970 Hørsholm, Denmark) were obtained and individually weighted as recommended by the manufacturer, and grown for 24 h at 37°C in M17 (Neogen, Michigan, United States) and de Man, Rogosa, Sharpe broth (MRS) (Neogen, Michigan, United States), respectively. The cultures were then maintained at 4°C and sub-cultured three times in the same medium before each experiment.

2.2. Preparation of postbiotics solutions

Before the postbiotic preparation, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were cultured at 37°C for 24 h in M17 and MRS, respectively. Following incubation, 50 µL of bacteria culture was separately sub-cultured in plastic tubes containing 50 mL of media, which were incubated at 37°C overnight. Next, the bacteria culture biomass was harvested by centrifugation at 4000× *g* for 10 min at 20°C and washed twice with sterilized standard saline solution. Finally, the harvested cells were resuspended in 10 mL of ultra-high temperature (UHT) milk and used as a bacteria culture to use in the next step. Skim milk (SM) and cheese whey (CW), obtained from Best way, Haulerwijk, Netherlands, were used as cultures media for postbiotic preparation. They were prepared as follows: initially, the pH was adjusted to 4.5 with 5 N hydrochloric acid (Merck, Darmstadt, Germany), then autoclaved at 121°C for 15 min, and the precipitates were separated by centrifugation at 2360× *g* for 5 min. The pH of the media (50 mL) was adjusted to 4.5 and autoclaved at 121°C for 15 min in 100 mL flasks. To optimize the incubation temperature and time of production of the highest postbiotic concentration (Experimental design not included), the method of Amiri et al. (2020) and Amiri et al. (2021) was used for postbiotic preparation in cheese whey and skim milk, with some modifications. Briefly, four different fermentation batches were prepared: ST-SM (*S. thermophilus* postbiotic-containing skim milk solution), ST-CW (*S. thermophilus* postbiotic-containing cheese whey solution), LB-SM (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk solution), and LB-CW (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey solution). The resulting batches of ST-SM, ST-CW, LB-SM, and LB-CW were incubated at 40°C for 68 h, 39.6°C for 68 h, 46°C for 64 h, and 42.1°C for 68 h, respectively. During this time, the advancement of bacterial growth was monitored through the assessment of solution pH, total titratable acidity (TTA), and the turbidity of the solutions visually at 12-h intervals. After production, all fermented batches were

freeze-dried (Martin Christ, Osterode am Harz, Germany) at −60°C with 0.0046 mBar of pressure for 48 h (freeze-drying time). After completing the procedure, these freeze-dried powders of postbiotics produced by bacteria were stored in closed plastic containers in a freezer at −20°C.

2.3. Preparation of postbiotic yoghurts

Low-fat yoghurt was manufactured according to the method of Ghaderi-Ghahfarokhi et al. (2020a) with some modifications. Commercial UHT milk (1.5 g/100 g of fat, 12.8 g/100 g of total solids (TS) content, and pH of 6.67) was used. Five yoghurt formulations, including Control (without postbiotic powder), ST-SM (yoghurt containing 3% *S. thermophilus* postbiotic-containing skim milk powder), ST-CW (yoghurt containing 3% *S. thermophilus* postbiotic-containing cheese whey powder), LB-SM (yoghurt containing 3% *L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk powder), and ST-CW (yoghurt containing 3% *L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey powder) were prepared by the procedure depicted in Figure 1. The experimental batches were inoculated with a yoghurt starter culture, comprising *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* at a concentration of 2% v/v. Following uniform agitation, the resulting yoghurts were packed into 100 mL sterile cups and subsequently incubated at 42°C until they reached a final pH of 4.5. Subsequently, the yoghurt samples were cooled to 4°C and stored for a duration of 22 days. Yoghurt production was performed in triplicate. The analysis encompassed the evaluation of both physicochemical attributes and microbial viability at four specific time points during the storage period: namely, days 1, 8, 15, and 22.

2.4. Physicochemical analysis of yoghurts

pH indexes of the yoghurt were measured using a pH meter (Thermo Orion Model-420A′). In addition, titratable acidity (TTA) of yoghurt samples was measured by the AOAC official method and expressed as % lactic acid (AOAC, 2005).

The syneresis values of yoghurt samples were determined as recommended by Tamime et al. (1996). Briefly, 25 g of each yoghurt batch was weighted on a Whatman paper No. 42 (Whatman) placed on the top of a funnel. Syneresis is expressed as the amount of whey separated from the samples under the force of gravity at 4°C for 2 h of drainage into a flask of known weight divided by the initial yoghurt mass.

The water holding capacity (WHC) of yoghurt samples was determined according to the centrifugation method reported by Sahan et al. (2008). Briefly, each 5 g yoghurt sample was weighted in a falcon tube (M_i) and centrifuged at 3556× g for 30 min at 10°C. The

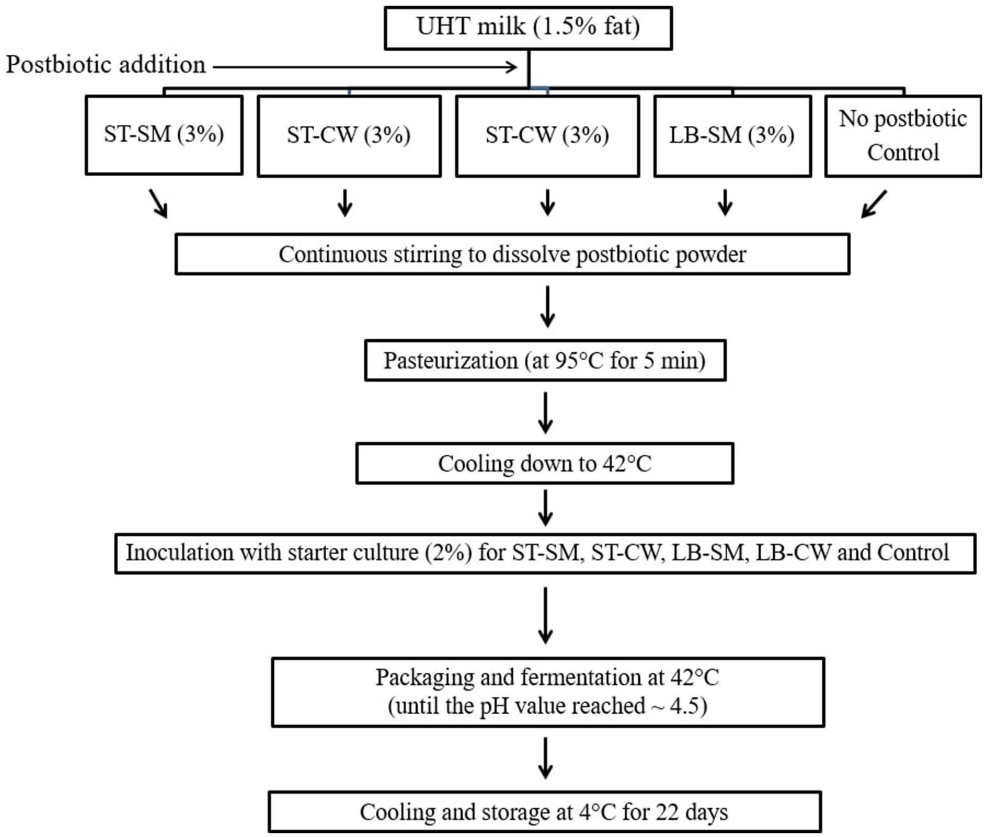

FIGURE 1
Low-fat yoghurt manufacturing flowchart. Control: yoghurt without postbiotic powder; ST-SM: yoghurt containing 3% *S. thermophilus* postbiotic-containing skim milk; ST-CW: yoghurt containing 3% *S. thermophilus* postbiotic-containing cheese whey; LB-SM: yoghurt containing 3% *L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk; LB-CW: yoghurt containing 3% *L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey.

resulting supernatant was discarded, and the expelled precipitate was collected and weighed (M_p). WHC was calculated using the equation:

$$\mathrm{WHC}(\%) = \left[1 - \left(M_p \,/\, M_i\right)\right] \times 100$$

where M_i and M_p were the initial weight of the sample and the final weight of the precipitate, respectively.

2.5. Enumeration of starter cultures

The viability of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* was determined in freshly made yoghurt samples during the storage period as previously described, and expressed as log colony-forming units (CFU) per gram of product (log CFU/g). The yoghurt cup was agitated, and 1 g of each sample was mixed with 9 mL of physiological saline solution using a vortex mixer. Diluted samples were then enumerated using the pour-plate technique. In the count of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, MRS agar and M17 agar were used, respectively. Both bacteria were incubated at 37°C for 72 h under anaerobic (*L. delbrueckii* ssp. *bulgaricus*) and aerobic (*S. thermophilus*) conditions, following Batawy and Khalil (2018).

2.6. Antioxidant activity determination

2.6.1. Yoghurt samples extraction

The extraction method of yoghurt samples was conducted as reported by Demirci et al. (2017). To extract the desired components, 5 g of yoghurt was mixed with an appropriate amount of diluted methanol (80:20, methanol: distilled water) in a ratio of 25 mL. The mixture was then homogenized using an ultra-turrax homogenizer and subsequently centrifuged at 7200 rpm for 10 min at 4°C. The resulting mixture was filtered using Whatman No. 1 filter paper, and the liquid portion obtained after filtration was stored at 4°C for subsequent analysis of antioxidant activity.

2.6.2. DPPH free radical scavenging activity assay

The DPPH radical activity was assessed as described by Yu et al. (2022). Initially, a solution of DPPH (0.01183 g) was prepared by dissolving it in 100 mL of 95% ethanol. Subsequently, 20 mL of the yoghurt sample was thoroughly mixed with 20 mL of the DPPH solution, followed by centrifugation at 10,000× *g* for 10 min. 2 mL of the resulting supernatant were combined with 8 mL of DPPH solution, mixed well, and left undisturbed in darkness for a duration of 30 min. Finally, the absorbance of the mixture was measured at a wavelength of 517 nm, using a blank solution of 95% ethanol, and the results are presented as the percent of DPPH cleared according to the formula:

DPPH clearance rate (%) = $(1 - A_{\mathrm{sample}}/A_{\mathrm{empty}}) \times 100\%$.

2.6.3. ABTS$^+$ free radical scavenging activity assay

The ABTS radical scavenging activity was measured according to the method of Yu et al. (2022). ABTS (7 mM) stock solution was prepared by dissolving ABTS in 2.45 Mm potassium persulfate solution, and stored in the dark at room temperature for 12–16 h. A working solution of ABTS was then created by mixing the stock solution with anhydrous ethanol to achieve a specific absorbance. For the analysis, a small amount of the sample was mixed with the ABTS working solution, shaken, and the absorbance was measured after a short incubation period. The same procedure was followed for the yoghurt samples. The results are expressed in ABTS clearance (%) form according to the following formula:

ABTS clearance rate (%) = $(1 - A_{\mathrm{sample}}/0.700)$ 100%.

2.7. Sensory analysis

The sensory properties of yoghurt samples, including their visual appearance, texture, flavor, and mouth sensation, were assessed by 15 semi-trained panelists (staff, students, and researchers at the University of Helsinki, Helsinki, Finland). Yoghurt samples were served to the evaluators in 100-ml transparent glass cups bearing 3-digit random codes. The 10-point hedonic scale ranging from 1 (dislike very much) to 10 (like very much) was used on day 11 of storage. Yoghurt containers were labeled and the participants were trained to rinse their mouths before starting and between tasting the samples.

2.8. Statistical analysis

All physicochemical analyses and microbial counts were conducted in triplicates. The data obtained for yoghurt's physicochemical, microbial, and sensorial evaluation were analyzed with ANOVA using the General Linear Model procedure, reported as mean ± standard deviations. Tukey's test was used to compare the means; significant differences were estimated based on a $p \leq 0.05$. All statistical analyses were carried out using Minitab 16 program (Minitab Inc., State College, PA, United States).

3. Results and discussion

3.1. pH and TTA of yoghurts

The pH values of the yoghurt samples were measured after 1, 8, 15, and 22 days of storage at 4°C. Our results showed that the postbiotic powder types and storage time had a significant effect on the pH value of the produced yoghurts ($p < 0.05$). On day 1 of the storage period, the index of pH of all yoghurt samples ranged between 4.62 and 4.68 (Table 1). This index decreased throughout the storage period, as also reported in other studies (Karaca et al., 2019; Ghaderi-Ghahfarokhi et al., 2020a). pH values of ST-CW varied from 4.66 to 4.63, and from 4.64 to 4.63 in LB-CW throughout the duration of storage (Table 1). This phenomenon was associated with the occurrence of organic acids present in the postbiotics that were assimilated by the yoghurt. A plausible explanation for this alteration could be attributed to mass exchange. Yoghurt samples containing cheese whey powders (ST-CW and LB-CW) showed a slight decrease in pH compared to skim milk (ST-SM and LB-SM) and Control formulations. The observed effect can also be attributed to the presence of organic acids in the absorbed postbiotics within the yoghurt. These results align with the research conducted by Sharafi et al. (2022), where it was observed that samples containing postbiotics demonstrated a significant reduction in pH values in comparison to

TABLE 1 pH and titratable acidity (TTA; as lactic acid %) of low-fat yoghurts during 22 days of storage at 4°C.

		Storage period (days)			
Yoghurt formulation[1]		1	8	15	22
pH	Control	$4.68 \pm 0.05^{ab,A}$	$4.64 \pm 0.01^{ab,B}$	$4.63 \pm 0.02^{a,BC}$	$4.60 \pm 0.03^{ab,C}$
	ST-CW	$4.66 \pm 0.06^{ab,A}$	$4.65 \pm 0.02^{ab,AB}$	$4.63 \pm 0.01^{aa,AB}$	$4.63 \pm 0.01^{a,B}$
	ST-SM	$4.68 \pm 0.01^{a,A}$	$4.66 \pm 0.01^{a,AB}$	$4.63 \pm 0.02^{a,B}$	$4.59 \pm 0.04^{b,C}$
	LB-CW	$4.64 \pm 0.01^{bc,A}$	$4.64 \pm 0.07^{ab,A}$	$4.64 \pm 0.00^{a,A}$	$4.63 \pm 0.02^{ab,A}$
	LB-SM	$4.62 \pm 0.00^{c,A}$	$4.62 \pm 0.05^{b,A}$	$4.61 \pm 0.01^{a,A}$	$4.60 \pm 0.01^{a,A}$
TTA	Control	$0.96 \pm 0.04^{a,A}$	$0.9 \pm 0.08^{a,A}$	$1.00 \pm 0.08^{a,A}$	$1.20 \pm 0.00^{a,A}$
	ST-CW	$0.76 \pm 0.04^{a,A}$	$0.86 \pm 0.04^{a,A}$	$0.76 \pm 0.04^{a,A}$	$1.03 \pm 0.04^{a,A}$
	ST-SM	$0.83 \pm 0.04^{a,A}$	$0.93 \pm 0.04^{a,A}$	$0.96 \pm 0.04^{a,A}$	$0.96 \pm 0.04^{a,A}$
	LB-CW	$0.96 \pm 0.04^{a,A}$	$0.86 \pm 0.09^{a,A}$	$0.90 \pm 0.08^{a,A}$	$1.00 \pm 0.08^{a,A}$
	LB-SM	$0.96 \pm 0.04^{a,A}$	$0.90 \pm 0.08^{a,A}$	$1.03 \pm 0.012^{a,A}$	$1.06 \pm 0.09^{a,A}$

[a]Values (average ± SD) in the same column with the same superscript letters are not significantly different ($p > 0.05$). [A]Values (average ± SD) in the same row with the same superscript letters are not significantly different ($p > 0.05$) between the storage days of each yoghurt sample.
[1]Abbreviations of different yoghurt formulations: Control (yoghurt without postbiotic); ST-SM (S. thermophilus postbiotic-containing skim milk); ST-CW (S. thermophilus postbiotic-containing cheese whey); LB-CW (L. delbrueckii ssp. bulgaricus postbiotic-containing cheese whey); and LB-SM (L. delbrueckii ssp. bulgaricus postbiotic-containing skim milk) ($n = 3$).

the control samples. In another study, treatment with postbiotic decreased the pH values of the breast fillet samples compared to the control samples (İncili et al., 2021). The post-acidification phenomenon of all yoghurt formulations was seen (Table 1), which is primarily contributed to the continuity of fermentation by starter culture strains throughout the duration of shelf-life (Basiri et al., 2018). This can be observed by the slight decrease of pH in Control samples without any supplementation. At the end of storage, ST-CW, ST-SM, LB-CW, and LB-SM yoghurts displayed a pH drop of ~0.03, 0.09, 0.01, and 0.02 units compared to the first day, respectively, while the Control declined ~0.08 units. These results are in agreement with Elsamani and Ahmed (2014), who reported that pH values of yoghurts produced with or without cheese whey and skim milk were fairly similar, without noticeable difference between them.

The most common acid produced by probiotic bacteria is lactic acid (Ghaderi-Ghahfarokhi et al., 2020b). As seen in Table 1, all yoghurts showed an increase in TTA and a decrease in pH during storage. In our study, the TTA index was distinctly related to the type of media and the strains used to produce postbiotic solutions, giving a higher concentration of lactic acid in LB-CW, LB-SM, and Control formulations as compared to ST-CW and ST-SM (Table 1). On day 8 of storage, the highest concentration of lactic acid was observed in the ST-SM formulation, although this was non-significant compered to others ($p > 0.05$). However, there were some fluctuations in the TTA values of the yoghurt during the storage period, consistent with other studies. For example, Gonzaíez-Martí et al. (2002) also observed a small change in the acid content, with no significant difference in the lactic acid production among the yoghurt samples enriched with different types of cheese whey powder.

3.2. Syneresis and WHC

Yoghurt's coagulum stability is an important quality parameter that should be monitored during storage (Ghaderi-Ghahfarokhi et al., 2020b). As a result of weakening of the gel network, spontaneous syneresis causes the expulsion of whey from the body of yoghurt (Ozcan and Kurtuldu, 2014). The extent of syneresis was significantly influenced by yoghurt formulation and storage time ($p < 0.05$). Accordingly, the addition of CW or SM to reduce syneresis or improve yoghurt texture was noticeably dependent on the type of bacterial culture used. Whey separation varied across yoghurt samples within the range of 23.01–36.2% at the beginning of the experiment ($p > 0.05$). Throughout the cold storage period, all of the samples displayed a reduction in the rate of syneresis. Interestingly, the LB-CW yoghurt samples exhibited a significant decrease in syneresis from 38.58 to 21.60% ($p < 0.05$), while the control sample showed a minor decrease during storage. As depicted in Figure 2A, the LB-SM formulation exhibited the lowest syneresis rate among all samples, decreasing from 23.01% on day 1 of storage to 18.11% on day 22. A possible explanation can be the ability of the postbiotic compound, such as EPSs production by bacteria in LB-SM powder, to retain water in the yoghurt gel structure (Ghaderi-Ghahfarokhi et al., 2020a). In another research, Khider et al. (2022) demonstrated the impact of ESPs on diminishing and lessening syneresis in low-fat yogurt samples containing EPSs, as opposed to the control group. It is likely that the different conditions and strains used in the experiments have a major impact on the syneresis index (Gezginc et al., 2015). Our results accord with the findings of Akalin et al. (2012), who reported that casein-based samples showed firmer gels with less syneresis than yoghurts enriched with cheese whey.

The water holding capacity of a gel structure is an essential factor in yoghurt production, as it is an indicator of their ability to retain serum (whey) (Kpodo et al., 2014). Enriching yoghurt with CW and SM had a major impact on the WHC in yoghurt samples, with values ranging from 48.26 to 65.71% on Day 1 and Day 22 of storage, respectively (Figure 2B). Hence, CW and SM improve the tendency of yoghurts to retain water in comparison with Control samples. While the LB-SM formulation showed the most constant WHC (65.71–70.04%), the percentage of water retention was statistically decreased for other formulations ($p < 0.05$). The yoghurt samples enriched with cheese whey containing postbiotic powder of *S. thermophilus* (ST-CW) and *L. delbrueckii* ssp. *bulgaricus* (LB-CW) exhibited the highest WHC values of 77.93 and 75.47%, respectively. These findings are consistent

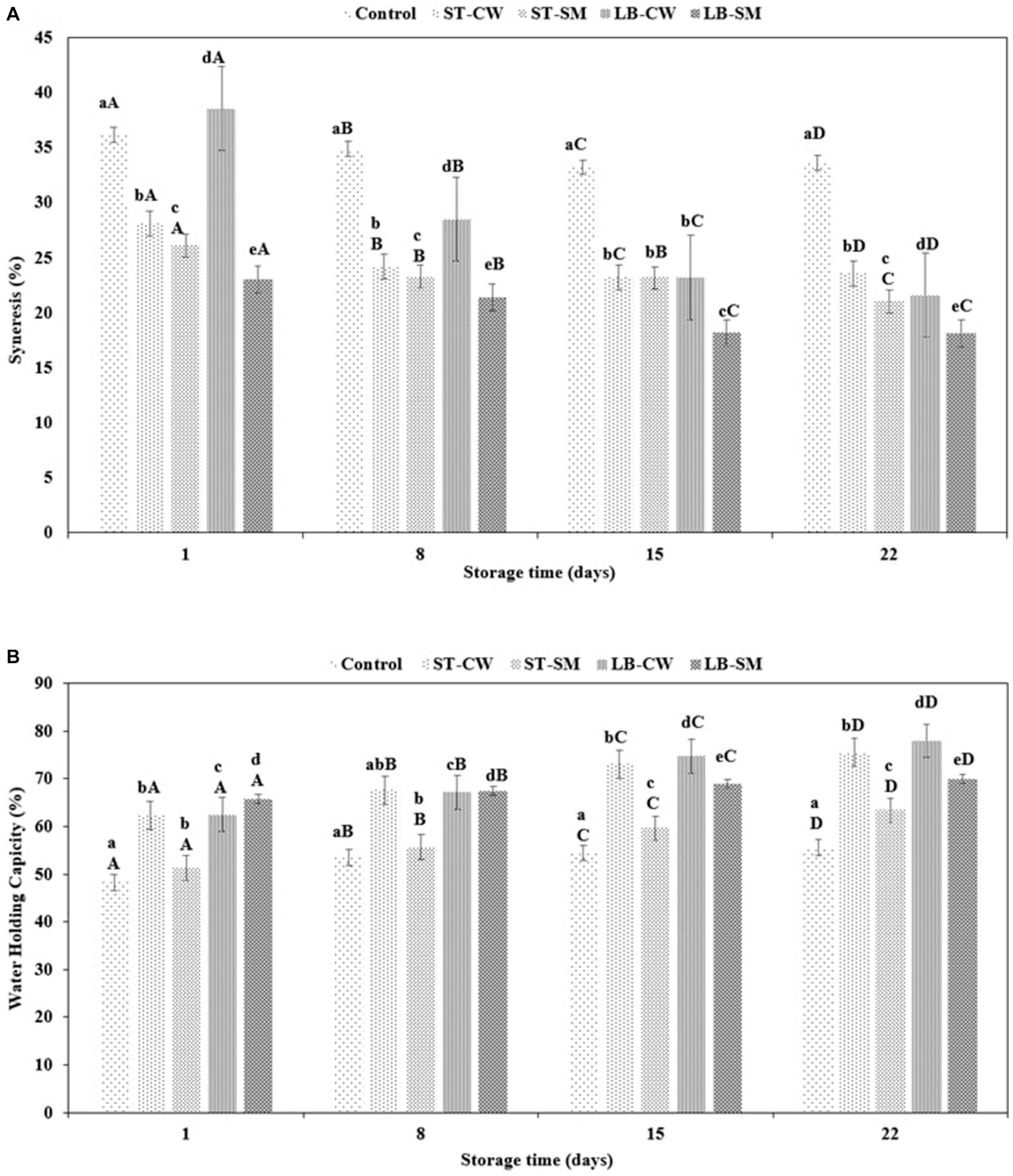

FIGURE 2
Syneresis (%) **(A)** and water holding capacity (%) **(B)** in different formulations of yoghurt during storage at 4°C. Control (yoghurt without postbiotic); ST-SM (*S. thermophilus* postbiotic-containing skim milk); ST-CW (*S. thermophilus* postbiotic-containing cheese whey); LB-CW (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey); and LB-SM (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk). Lowercase letters indicate significant differences ($p < 0.05$) between the storage days of each yoghurt sample. Uppercase letters indicate significant differences ($p < 0.05$) between different samples at the same storage time. Error bars represent the mean ($n = 3$) ± standard deviation (SD).

with prior research conducted by Akalin et al. (2012) on yoghurt fortification using skim milk powder, whey protein concentration (WPC), and sodium calcium caseinate. The study reported a WHC index of 68.78% for yoghurt fortified with WPC during a 28-day storage period, indicating the highest water holding capacity among all formulations. Also, in line with our finding, a study by Delikanli and Ozcan (2014) stated that yoghurt samples enriched with CW exhibited the highest WHC (83.32%) compared to other formulations during a 14-day storage period. Another recent study revealed the impact of adding CW to yoghurt samples, noting a significant increase in WHC values during storage (Brodziak et al., 2020). As discussed in the previous paragraph, ESPs can also affect WHC of yoghurt. Khider et al. (2022) demonstrated the impact of EPSs on the water holding capacity of low-fat yoghurt that was fortified with varying concentrations of EPS derived from Leuconostoc strains, in comparison to a control sample. The study revealed a noticeable trend:

as the concentration of EPS was elevated, there was a corresponding increase in the water holding capacity of the yoghurt.

3.3. Antioxidant activity

Postbiotics have been shown to possess a variety of functional/bioactive properties, including antioxidant activity, either directly (by interacting with the intestinal microbiota or immune cells) or indirectly (by interacting with other organs outside the gastrointestinal tract) (Sharma and Shukla, 2016; Aguilar-Toalá et al., 2018). EPSs and peptides are well-known postbiotic compounds with antioxidant properties. EPSs have been shown to reduce oxidative stress, lipid peroxidation, and inflammation. Peptides have been found to have anti-aging, anti-inflammatory, and anti-microbial effects. Peptides and EPSs both have potential applications in health-promoting foods and beverages (Sabeena Farvin et al., 2010; Amiri et al., 2019; Chang et al., 2021; Krunić and Rakin, 2022). In all yoghurt samples enriched with postbiotic supplement, the high rate of DPPH scavenging activity was significantly affected by yoghurt formulation and storage time ($p < 0.05$). As seen in Figure 3A, the LB-CW yoghurt sample showed the highest radical scavenging activity with 18.71% inhibition on day 15 of storage, which was significantly greater than all other yoghurt samples ($p < 0.05$) except ST-CW. The scavenging activities of DPPH radicals significantly increased with the addition of postbiotic powder compared to Control yoghurt. These findings are in agreement with Demirci et al. (2017), who reported that addition of rice bran, which has antioxidative properties, to yoghurt increased scavenging activities of DPPH radical (12.75%). Interestingly, DPPH activity of LB-CW was higher than the other samples on the last day of storage. In support of our findings, several previous studies have reported CW-enriched yoghurts can increase antioxidant activity (Bierzuńska and Cais-Sokolińska, 2018; Zoidou et al., 2019; Krunić and Rakin, 2022). However, Roumanas et al. (2016) stated that addition of cheese whey did not increase DPPH levels during storage.

In addition to the DPPH method, the ABTS method was also used to quantify the radical scavenging value to support quantified antioxidant activity. The initial ABTS activity ranged from 7.7 to 9.21% on the first day of storage, and it exhibited an exponential increase throughout the storage period, eventually reaching a relatively stable state after day 15 (Figure 3B). Yoghurts fortified with LB-CW and ST-SM showed higher ABTS activity on the final day of storage, with 51.78 and 51.19%, respectively ($p < 0.05$). The LB-CW sample exhibited the highest antioxidant activity in both DPPH and ABTS assays. This may be attributed to the ABTS radical inhibition capacities of EPSs produced by *L. delbrueckii* ssp. *bulgaricus* in postbiotic solutions. Abedfar et al. (2018) and El-Newary et al. (2017) reported that the percentage of ABTS radical scavenging activity of EPS increased with a rise in the concentration of EPS.

3.4. Viability of yoghurt cultures during yoghurt storage

As demonstrated in Figure 4, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* cell proportions were similar (approximately 10^8 cfu/mL each) and maintained the same cell counts during the cold storage period. It is generally accepted that the standard count for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* should fluctuate around 10^7 in yoghurt products (Fadela et al., 2009). In the current study, the viability of both yoghurt cultures was studied during a storage time of 22 days at 4°C. This cultures' growth and survival were influenced by CW and SM addition during cold storage. After the first storage day, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* counts of ST-SM samples were 8.46 and 8.46 log cfu/g, respectively, which were higher than in other samples ($p > 0.05$) (Figures 4A,B). These results agree with the yoghurt culture counts reported in the literature: *S. thermophilus* counts in skim milk-fortified yoghurt increased to 9.78 log cfu/g on day 1 of storage (Marafon et al., 2011). During the first week of storage, the *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* counts decreased slightly and continued to gradually decrease until the end of storage. Similarly, it was also found by Marafon et al., 2011 and Batawy and Khalil (2018) that the growth of yoghurt cultures decreased during cold storage. The viability of both starter cultures remained higher in the yoghurt fortified with LB-SM powder compared to the other samples during the storage period ($p > 0.05$) (Figures 4A,B). It is possible that LB-SM powder had more nutritional compounds that support yoghurt cultures. In the ST-CW and LB-CW yoghurt samples, the viable counts of starter cultures were 7.72 and 7.53 log cfu/g for *S. thermophilus* and 7.69 and 8.07 log cfu/g for *L. delbrueckii* ssp. *bulgaricus,* respectively, on day 22 of storage. These findings showed that the viable counts of starter cultures in the yoghurt samples were at favorable concentrations. It has been shown by Ranok et al. (2021) that adding cheese whey to yoghurt and increasing its concertation improves the bacteria viability in yoghurt products during storage and transit in the gastrointestinal tract. Furthermore, in a similar study by Glušac et al. (2015), the effects of adding honey and cheese whey to yoghurt were investigated, which revealed that adding cheese whey improved the viability of the yoghurt starters, but the addition of honey did not show a significant improvement.

3.5. Sensory analysis of yoghurts

The scores collected for sensory analyses (appearance, flavor, mouthfeel, body and texture, and overall acceptability) are displayed in Table 2. In the sensory analyses, the ST-SM samples received the lowest ratings in all indices except flavor by evaluators, while the highest ratings were given for the LB-CW and LB-SM yoghurt samples. This can be attributed to the development of texture and a more pleasant taste as a result of the postbiotic characteristics. The desirable body and texture in yoghurt samples (Table 2) could be associated with higher amounts of exopolysaccharide in the postbiotics powders (Aziznia et al., 2008; Amiri et al., 2019; Yousefvand et al., 2022). Our findings were in line with the studies reported by Salih and Hamid (2013) and Antunes et al. (2005) who showed addition of skim milk in the products has a positive impact on the flavor and viscosity of the samples. In terms of flavor, texture, mouthfeel, and overall acceptability, LB-CW-fortified yoghurt showed the highest scores ($p > 0.05$). Nevertheless, Akalin et al. (2012) reported no significant differences between experimental yoghurts containing and excluding CW in terms of sensory attributes. In order to determine overall acceptability, different sensory attributes must be considered, including flavor, texture, and appearance perceptions. In a related context, Ozma et al. (2022) unveiled that the

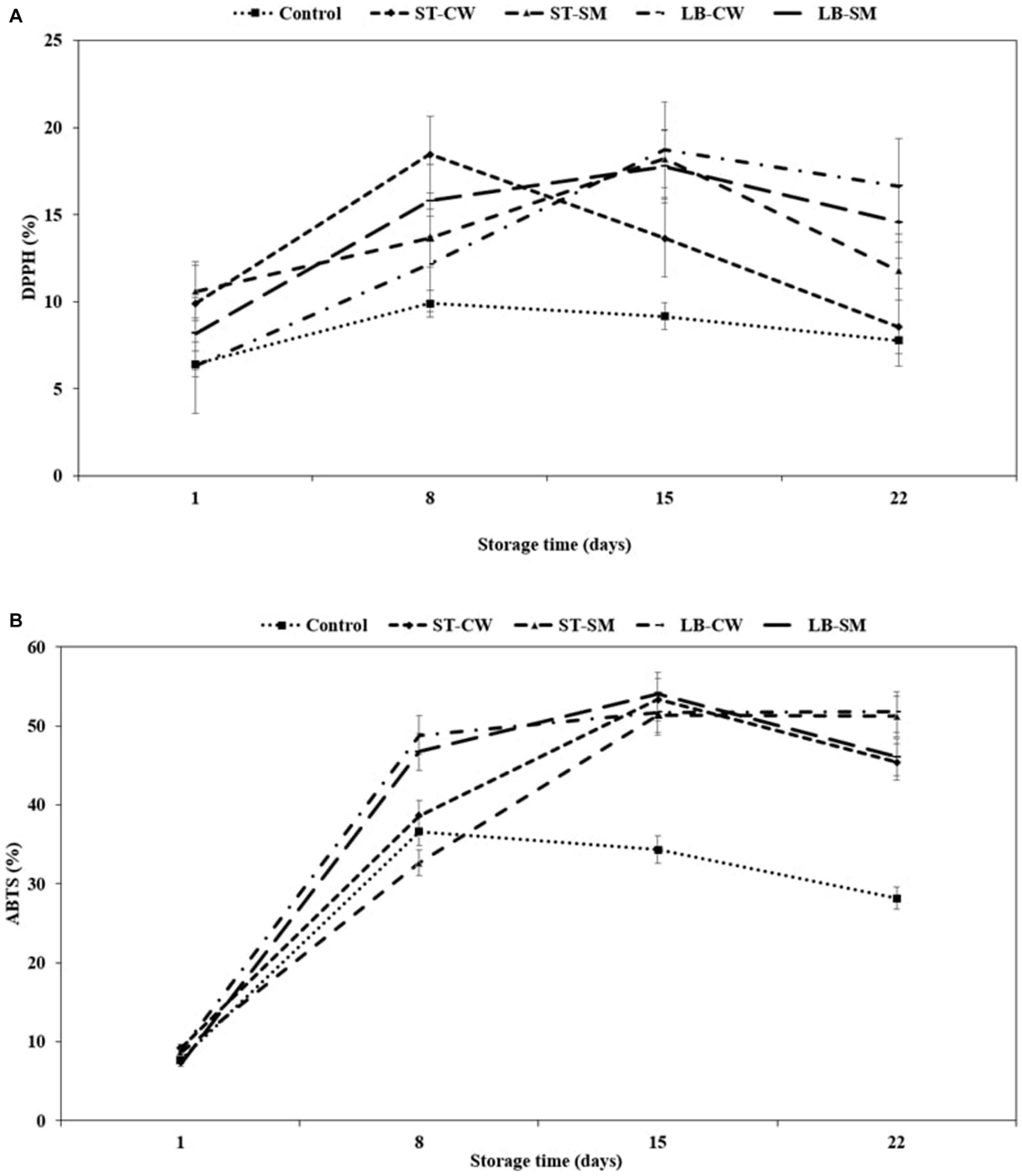

FIGURE 3
DPPH (%) **(A)** and ABTS (%) **(B)** in different formulations of yoghurt during storage at 4°C. Control (yoghurt without postbiotic); ST-SM (*S. thermophilus* postbiotic-containing skim milk); ST-CW (*S. thermophilus* postbiotic-containing cheese whey); LB-CW (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey); and LB-SM (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk) ($n = 3$).

application of an 8% solution of postbiotic derived from *Lactobacillus paracasei* ATCC 55544 as a coating for lamb meat slices resulted in consistent color, appearance, and overall consumer satisfaction ratings over the duration of storage. There were no notable alterations observed in these attributes for the lamb meat slices coated with the postbiotic. ST-SM and Control formulations were disliked slightly by panelists, while LB-CW and LB-SM formulations were preferred significantly by them. Antunes et al. (2005) found that the addition of WP and SM supplements had a positive impact on overall impressions.

4. Conclusion

Emphasizing the significance of utilizing affordable and easily accessible sources for postbiotic production, whey – a byproduct regularly generated in cheese plants – is often overlooked and discarded as waste within the food industry. However, recognizing its potential, whey can serve as a valuable resource for postbiotic preparation. This study explored the use of cheese whey and skim milk as alternative sources for postbiotic preparation. Specifically, postbiotics were derived from *S. thermophilus* and *L. delbrueckii*

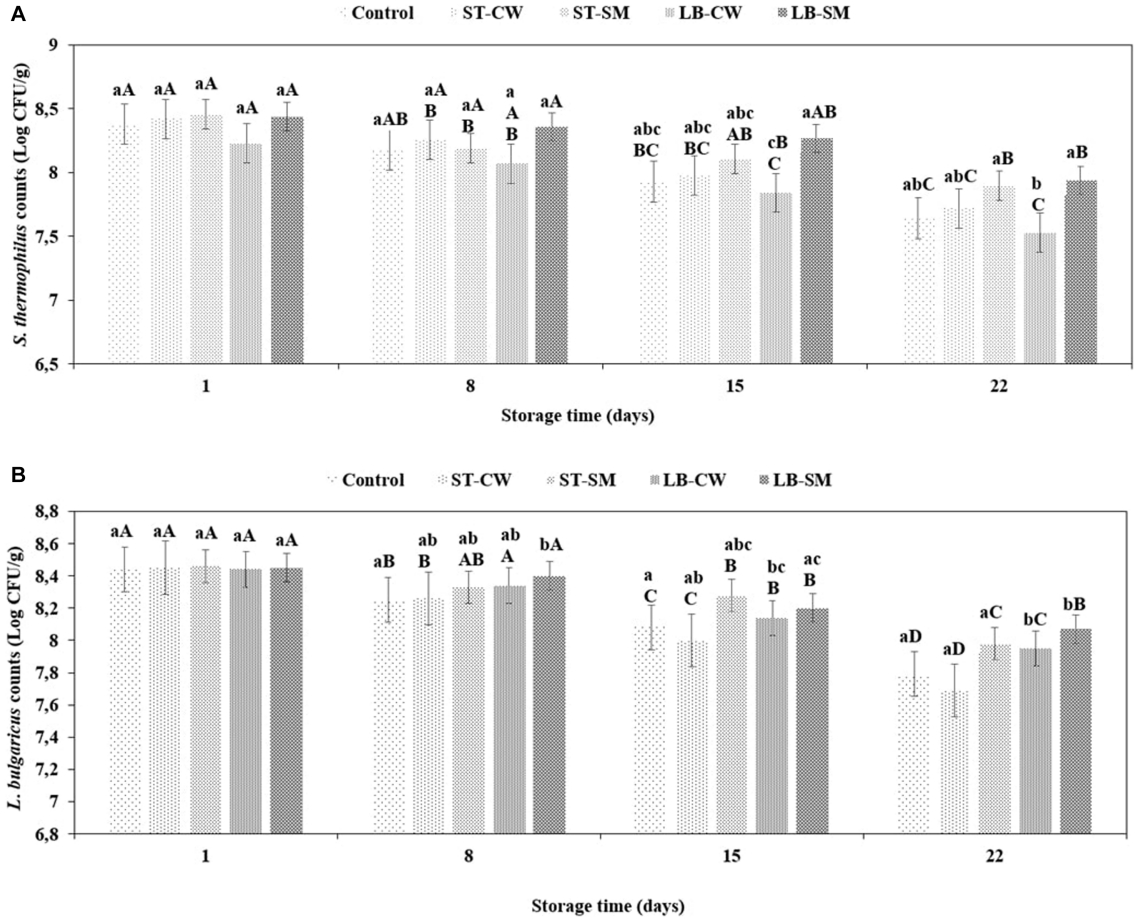

FIGURE 4
Viability of *S. thermophilus* **(A)** and *L. delbrueckii* ssp. *bulgaricus* **(B)** in different formulations of yoghurt during storage at 4°C. Control (yoghurt without postbiotic); ST-SM (*S. thermophilus* postbiotic-containing skim milk); ST-CW (*S. thermophilus* postbiotic-containing cheese whey); LB-CW (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey); and LB-SM (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk). Lowercase letters indicate significant differences ($p < 0.05$) between the storage days of each yoghurt sample. Uppercase letters indicate significant differences ($p < 0.05$) between different samples at the same storage time. Error bars represent the mean ($n = 3$) ± standard deviation (SD).

TABLE 2 Sensory scores of low-fat yoghurts on day 11 of storage at 4°C.

Sensory attributes					
Yoghurt formulation[1]	Appearance	Flavor	Mouthfeel	Body and texture	Overall acceptability
Control	7.20 ± 2.01^{ab}	6.33 ± 1.89^{a}	7.53 ± 2.08^{a}	7.73 ± 1.70^{a}	7.40 ± 1.66^{ab}
ST-CW	7.53 ± 2.18^{ab}	7.33 ± 1.88^{a}	7.46 ± 1.92^{a}	7.13 ± 2.36^{ab}	7.53 ± 2.06^{ab}
ST-SM	6.26 ± 1.48^{b}	6.93 ± 1.69^{a}	7.00 ± 1.26^{a}	6.06 ± 1.56^{b}	6.66 ± 1.49^{b}
LB-CW	8.00 ± 1.48^{ab}	8.46 ± 1.69^{a}	8.40 ± 1.26^{a}	8.60 ± 1.56^{a}	8.73 ± 1.49^{a}
LB-SM	8.46 ± 1.54^{a}	7.80 ± 1.46^{a}	8.26 ± 1.48^{a}	8.06 ± 1.56^{a}	8.33 ± 1.34^{ab}

$^{a\text{-}b}$Values (average ± SD) in the same column with the same superscript letter are not significantly different ($p > 0.05$).
[1]Abbreviations of different yoghurt formulations: control (yoghurt without postbiotic); ST-SM (*S. thermophilus* postbiotic-containing skim milk); ST-CW (*S. thermophilus* postbiotic-containing cheese whey); LB-CW (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing cheese whey); and LB-SM (*L. delbrueckii* ssp. *bulgaricus* postbiotic-containing skim milk).

ssp. *bulgaricus* in cheese whey and skim milk. Subsequently, the impact of these postbiotic-enriched cheese whey and skim milk supplements on the quality of yoghurt was thoroughly investigated. Postbiotic-enriched yoghurt showed high levels of antioxidant activity during 21 days of storage at 4°C. In addition to this beneficial property, sensory analysis conducted after 11 days of storage revealed that postbiotic-enriched yoghurt from *L. delbrueckii* ssp. *bulgaricus* in cheese whey and in skim milk were rated as highly acceptable – scores nearly reached the maximum rating. Moreover, the remaining yoghurt products also achieved

satisfactory sensorial acceptance. Drawing upon observations related to syneresis, water holding capacity, and sensory evaluations throughout a refrigerated storage period, our results suggests that the postbiotic-enriched formula has the potential for practical use as a product. The incorporation of postbiotic-enriched powder into yoghurt did not exert a significant impact on the overall properties of the yoghurt, supporting its feasibility for application in the final product. Postbiotic solutions obtained from probiotics in cheese whey and skim milk show promising potential as nutritious liquids. Nevertheless, exploring postbiotic preparation using alternative animal and plant-based sources, particularly waste or byproducts, warrants further investigation. It is crucial to emphasize that regulations and proper labeling guidelines for food products containing postbiotics are essential prerequisites to enable their commercial utilization in the food industry.

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

SS: Investigation, Validation, Visualization, Conceptualization, Methodology, Writing – review & editing. PS: Validation, Visualization, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Resources. SA: Conceptualization, Methodology, Project administration, Validation, Visualization, Writing – review & editing. AY: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing, Data curation, Formal analysis, Investigation, Software, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Open access is funded by the Helsinki University Library.

Acknowledgments

The Finnish Food Research Foundation, Faculty of Agriculture and Forestry Proof-of-concept funding (University of Helsinki), The Finnish Society of Sciences and Letters, and Oskar Öflund Foundation are acknowledged for the stipend to support this project.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1276268/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 29 September 2023
DOI 10.3389/fmicb.2023.1239837

OPEN ACCESS

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RECEIVED 14 June 2023
ACCEPTED 14 September 2023
PUBLISHED 29 September 2023

CITATION
Wu D, Fu L, Cao Y, Dong N and Li D (2023) Genomic insights into antimicrobial potential and optimization of fermentation conditions of pig-derived *Bacillus subtilis* BS21. *Front. Microbiol.* 14:1239837. doi: 10.3389/fmicb.2023.1239837

Genomic insights into antimicrobial potential and optimization of fermentation conditions of pig-derived *Bacillus subtilis* BS21

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Bacillus spp. have been widely used as probiotic supplements in animal feed as alternatives to antibiotics. In the present study, we screened a *Bacillus subtilis* strain named BS21 from pig feces. Antimicrobial activities, whole genome mining and UHPLC-MS/MS analysis were used to explore its antimicrobial mechanism. Strain BS21 showed Significant growth inhibition against a variety of animal pathogens, including *Escherichia coli*, *Salmonella enterica Pullorum*, *Salmonella enterica Typhimurium*, *Citrobacter rodentium*, *Shigella flexneri* and *Staphylococcus aureus*. Seven gene clusters involved in antimicrobial biosynthesis of secondary metabolites were encoded by strain BS21 genome, including four non-ribosomal peptides (bacillibactin, fengycin, surfactin and zwittermicin A), one ribosomal peptide (subtilosin A), one dipeptide (bacilysin) and one polyketide (bacillaene). Among them, production of surfactin, fengycin, bacillibactin, bacilysin and bacillaene was detected in the supernatant of *B. subtilis* strain BS21. To develop the potential application of BS21 in animal production, medium components and fermentation parameters optimization was carried out using response surface methodology (RSM). Production of antimicrobial secondary metabolites of strain BS21 was increased by 43.4%, and the best medium formula after optimization was corn flour 2%, soybean meal 1.7% and NaCl 0.5% with optimum culture parameters of initial pH 7.0, temperature 30°C, rotating speed at 220 rpm for 26 h. Our results suggested that strain BS21 has the potential for large-scale production and application as a potential source of probiotics and alternative to antibiotics for animal production.

KEYWORDS

***Bacillus subtilis*, antimicrobial activity, secondary metabolites, fermentation conditions, response surface methodology, antibiotic alternative**

1. Introduction

Antibiotics have been added to animal feed to improve livestock production performance and reduce disease since the 1950s (Low et al., 2021). Extensive use of antibiotics leads to the development of resistant bacteria and residues in animal-derived food (Van et al., 2020). Safe and effective antibiotic alternatives are therefore urgently needed. Probiotics, one of the most promising antibiotic alternatives, are reported to act against pathogenic bacteria, improve

immune system functions and restore the intestinal microbial balance of farm animals (Plaza-Diaz et al., 2019).

Bacillus spp. are important sources of probiotics in animal feed. The ideal *Bacillus* species applied in animals feed are usually isolated from gastrointestinal tracts and feces of different animals, such as pigs, chickens, ruminants and aquatic animals (Bahaddad et al., 2023). *B. subtilis*, *B. licheniformis*, *B. velezensis*, *B. coagulans*, and *B. amyloliquefaciens* have been used to feed animals and shown to exert beneficial effects on the host (Du et al., 2019; Li et al., 2019; Shang et al., 2022). Antimicrobial properties of probiotic strains are important to prevent infection caused by pathogenic bacteria, which is a functional property used to select potential probiotics (Fijan, 2023). In recent years, *Bacillus* species have been demonstrated to exhibit broad-spectrum activity against microbes, because they produce multiple antimicrobial metabolites, mainly antimicrobial peptides (AMPs) and polyketides (Chen et al., 2019).

AMPs from *Bacillus* can be divided into non-ribosomally synthesized peptides (NRPSs) or ribosomally synthesized peptides based on their synthesis pathway (Puan et al., 2023). Fengycins, iturins, surfactin, bacillibactin and bacitracin derived from *Bacillus* are all members of a class of molecules known as NRPSs. These NRPSs are commonly made by large, multisubunit enzymes, often known as non-ribosomal peptide synthetases (Shahid et al., 2021). These peptides are often cyclized, chained, or branched and can be further modified by N-methylation, glycosylation, acylation or heterocyclic ring formation (Riccardo et al., 2022). AMPs synthesized by ribosomes, including subtilin, bacthuricin and cerecin, commonly known as bacteriocins, exist as either post-translationally modified or unmodified peptides. These bacteriocins exhibit a range of activities, including cell lysis, quorum sensing mediation and induction of genetic competence (Rebuffat, 2020). In general, these AMPs are cationic and hydrophobic or amphiphilic, and the cellular membrane of bacteria, in most cases, is the main target for AMPs to exert antimicrobial activity (Zhang et al., 2021). In addition to anti-bacterial and anti-fungal properties, *Bacillus*-derived AMPs have also shown antiviral, antitumor and immunoregulatory activities, making them another attractive alternative to antibiotics in recent years (Caulier et al., 2019; Basi-Chipalu et al., 2022). Polyketides are another important antimicrobial compound produced by the *B. subtilis* group. *Bacillus* species secrete three antimicrobial polyketides and their variants (bacillaene, difficidin and macrolactin) exhibit antibacterial activities by selectively inhibiting protein synthesis. Moreover, these antimicrobial metabolites could have synergistic effects against pathogens (Chen et al., 2019).

The production of secondary metabolic products in microorganisms is influenced by the strain's genetic traits as well as the nutritional and growth conditions (Puan et al., 2023). Response surface methodology (RSM) is a computational method used for the identification of interactions between response values and defined factors as well as the combinations of factors responsible for an optimal response (Zhou et al., 2023). RSM is commonly employed in food, chemistry and environmental engineering for improved efficiency of production and lower production costs (Yolmeh and Jafari, 2017).

In this research, we isolated a *B. subtilis* strain from pig feces named BS21 with broad-spectrum antibacterial activity. The genome of BS21 was sequenced and analyzed and a total of seven gene clusters of antimicrobial secondary metabolites were predicted, including six antimicrobial peptides (bacillibactin, fengycin, surfactin, zwittermicin A, subtilosin A and bacilysin) and one polyketide (bacillaene). In addition, we successfully detected surfactin, fengycin, bacillibactin, bacilysin and bacillaene from fermentation supernatant. Optimization studies using the RSM showed that optimal factors affected the maximal production of antimicrobial secondary metabolites from strain BS21, providing a theoretical basis for large-scale fermentation. This study will lay the foundation for researching molecular mechanisms of antimicrobial activity, and provide theoretical support that *B. subtilis* BS21 has the potential to be developed as an antibiotic alternative in animal production.

2. Materials and methods

2.1. Isolation and screening of strain BS21

The feces sample was collected from free-range pigs for isolation and screening of bacteria. Briefly, 1 g feces sample was homogenized in 10 mL normal saline (0.9% NaCl) by vortex mixture, 100 μL aliquot from selected tenfold serial dilutions were spreadly plated on Luria-Bertani (LB, Qingdao Hope Bio-technology Co., Ltd., Qingdao, China) solid medium (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar). These plates were then incubated at 37°C for 18–24 h. Colonies with distinct morphology were selected and cultured in LB at 37°C for 24 h. Oxford cup method was used to test antimicrobial activity against *E. coli* of these bacteria. A potent isolate (BS21) was selected for further studies.

2.2. Microbial strains and growth conditions

Strain BS21 was stored at the China General Microbiological Culture Collection Center (No. CGMCC 20391). Pathogenic bacteria *Escherichia coli* (*E. coli*) K88, *E. coli* O127:H6, *Salmonella pullorum* (*S. pullorum*) CVCC1791, *Salmonella typhimurium* (*S. typhimurium*) SL1344, *Citrobacter rodentium* (*C. rodentium*) DBS100, *Shigella flexneri* (*S. flexneri*) 2457T, *Staphylococcus aureus* (*S. aureus*) CVCC1882 and CVCC43300 were acquired from the China Veterinary Culture Collection Center. All microbial strains were grown aerobically in LB medium with constant shaking (220 rpm) at 37°C.

2.3. Antimicrobial activity assay

Bacillus subtilis BS21 was cultured in LB medium with constant shaking (220 rpm) at 37°C for 24 h. Suspensions were centrifuged at 12,000 *g* for 15 min to remove bacteria, and the supernatant was filter sterilized using a 0.22 mm filter (Merck Millipore, Darmstadt, Germany) to obtain cell-free supernatant. Oxford cup method was used to test the antimicrobial activity of BS21 supernatant. Briefly, 100 mL of LB media agar was mixed with 10^8 pathogenic bacteria to attain 10^6 CFU/mL, and then the mixture was placed into petri dishes with Oxford cups (Mostafa et al., 2018; Xiang et al., 2021). Oxford cups were removed when LB media agar solidified. A volume of 150 μL of cell-free supernatant was dispensed into each well, followed

by co-culturing alongside various pathogens at 37°C for 12 h, and then the diameters of the inhibition zone were measured.

2.4. Identification of strain BS21

The total DNA from strain BS21 was extracted through the use of a bacterial genomic DNA kit (CWBIO, Beijing, China) following the directions provided by the manufacturer. PCR amplification of the extracted DNA was conducted using the forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-GGTTACCTTG TTACGACTT-3′). Sequencing of the resultant PCR products was performed by Beijing Ruibio Biotech Co., Ltd. The obtained sequences were subjected to a BLAST search, and the selected homologous *Bacillus* sequences were aligned using MEGA 7.0 software. Subsequently, a phylogenetic tree was constructed based on neighbor-joining method.

2.5. Genomic DNA extraction

BS21 was grown in LB medium at 30°C for around 10 h, with a shaking speed of 220 rpm. Following centrifugation at 12,000 *g* for 15 min, the cells were collected. The total DNA was isolated from strain BS21 using a bacterial genomic DNA kit (CWBIO, Beijing, China). The isolated genomic DNA was quantified using a TBS-380 fluorometer (Turner BioSystems Inc., CA, USA).

2.6. Genome sequencing and assembly

Genomic DNA of strain BS21 was sequenced (Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China) using a combination of PacBio Sequel II and Illumina sequencing platforms. In preparation for PacBio sequencing, the DNA fragments were subjected to purification, end-repair and ligation with SMRT bell sequencing adapters. The sequencing libraries underwent triplicate purification. An approximately 10 kb insert library was constructed and sequenced using one SMRT Cell and standard methods. Additionally, DNA samples were fragmented into 400–500 bp fragments for Illumina sequencing. Libraries constructed via these sheared fragments were used for Illumina sequencing. The prepared libraries were then used for paired-end Illumina sequencing on an Illumina Novaseq6000 machine. The Illumina data were used to evaluate the complexity of the genome. The data generated from PacBio and Illumina platforms were used for bioinformatics analysis.

2.7. Genome annotation

The coding sequences (CDSs) of the bacterial genome were predicted using Prodigal v2.6.3[1] and GeneMarkS.[2] tRNA and rRNA genes were predicted by tRNA-scan-SE v2.0[3] and Barrnap v0.9.[4] In addition, genomic islands, prophages and CRISPR-Cas were predicted using Island-Viewer,[5] PHAST[6] and CRISPRFinder,[7] respectively. The functions of genes were annotated using the COG (Clusters of Orthologous Genes) database through sequence alignment tools such as Diamond,[8] BLAST+[9] and HMMER.[10] AntiSMASH v4.0.2[11] was used to predict the secondary metabolite gene clusters in the genome of strain BS21.

2.8. UHPLC–MS/MS analysis of antimicrobial secondary metabolites

The crude extraction method of antimicrobial secondary metabolites refers to Al-Dhafri and Ching (2022) and Chen et al. (2018). Ammonium sulfate (Macklin Biochemical Technology Co., Ltd., Shanghai, China) was carefully added into the cell-free supernatant of *B. subtilis* BS21 to a final concentration of 70%. This was then allowed to stir overnight at 4°C. The precipitate was obtained by centrifugation at 12,000 *g* for 15 min followed by an ethanol extraction. Ethanol extract was subjected to rotating-drying at 45°C. Crude extract was dissolved in deionized water for further analysis.

An ultrahigh-performance liquid chromatography machine coupled to 6460 triple quadrupole mass spectrometry (UHPLC–MS/MS) (Agilent Technologies Inc., CA, USA) were used to detect secondary metabolites produced by strain BS21. UHPLC was conducted with a C18 column (100 × 2.1 mm, 1.7 μm) (Waters Corp., MA, USA). Eluent A and B were composed of H_2O/0.1% formic acid (Macklin Biochemical Technology Co., Ltd., Shanghai, China) and CH_3CN (Thermo Fisher Scientific Inc., Waltham, USA)/0.1% formic acid, separately. The flow rate was set at 300 μL/min, and the elution gradient ranged from 95% A/5% B at 0 min to 5% A/95% B at 60 min. For the 6460 triple quadrupole MS, the following conditions were applied: QQQ gas temperature of 350°C, gas flow rate of 10 L/min, sheath gas temperature of 350°C, sheath gas flow rate of 11 L/min and capillary voltage of 3500 V. Ions were accumulated using the MS2 scan mode with a scan time of 500 ms and a mass range between 200 and 2000 m/z.

2.9. Single-factor experiment

Bacillus subtilis BS21 was initially grown in 50 mL LB medium with constant shaking (220 rpm) at 30°C for 24 h. Single-factor experiment was used to select medium components (including different carbon sources, nitrogen sources, inorganic ions and their

1 https://github.com/hyattpd/Prodigal
2 http://topaz.gatech.edu/GeneMark
3 http://trna.ucsc.edu/software/
4 https://github.com/tseemann/barrnap/
5 http://www.pathogenomics.sfu.ca/islandviewer/
6 http://phast.wishartlab.com/
7 http://crispr.i2bc.paris-saclay.fr/
8 https://github.com/bbuchfink/diamond
9 https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/
10 http://www.hmmer.org/
11 https://dl.secondarymetabolites.org/releases/4.0.2/

proportions) and fermentation parameters (temperature, initial pH, rotational speed and fermentation time). After each single factor was selected, the fermentation medium or parameter was formulated according to experimental needs. The diameter of inhibition zone was tested using Oxford cup method and the indicator pathogen was *E. coli* K88. The inhibition zone diameter was used to characterize the production of antimicrobial secondary metabolites.

2.10. Response surface methodology

2.10.1. Plackett-Burman design (PBD)

Based on the data from single-factor experiment, the PBD was applied to analyze the main effect factors of medium composition: carbon source (corn flour), nitrogen source (soybean meal), mineral salts (NaCl) and fermentation parameters (fermentation temperature, initial pH, rotating speed and fermentation time). To perform the PBD, Design-Expert 13.0 software was used.

2.10.2. Box–Behnken design (BBD)

To further improve the production of antimicrobial secondary metabolites, BBD of response surface methodology was used to evaluate the most optimum level, impact and interactions of the screened principal component factors (soybean meal, temperature and time). BBD was conducted using Design-Expert 13.0 software.

2.11. Growth curve of BS21 under optimum conditions

Strain BS21 was cultured in optimum medium (2% corn flour, 1.7% soybean meal and 0.5% NaCl) under optimum fermentation parameter (30°C, pH 7.0, 220 rpm). Samples were carried out every 4 h for 36 h and were diluted appropriately to count bacteria cell numbers in LB solid medium plates.

2.12. Statistical analyses

The data were analyzed by one-way analysis of variance using SPSS 23.0 statistical software (IBM Corp., Armonk, NY, USA). A Duncan's test was employed in our testing for significant differences between treatments. Statistical differences of the mean value were defined at $p < 0.05$.

3. Results

3.1. Antimicrobial activity of strain BS21

The antimicrobial activities of *Bacillus subtilis* BS21 against pathogens are shown in Figure 1. BS21 exhibited strong antimicrobial activities against Gram-negative pathogens, including *E. coli* K88, *E. coli* O127:H6, *S. pullorum* CVCC1791, *S. typhimurium* SL1344, *C. rodentium* DBS100 and *S. flexneri* 2457T. In addition, strain BS21 had an obvious inhibiting effect on Gram-positive pathogens (*S. aureus* CVCC1882 and *S. aureus* CVCC43300).

3.2. Strain identification

The 16S rRNA sequence of strain BS21 showed 100% homology to *B. subtilis* strain NGS-STR-5 (GenBank number MF083067) and *B. subtilis* strain NG4-17 (GenBank number KR999961). The BS21 strain formed a cluster which was closely related to *B. subtilis* strains based on the neighbor-joining tree (Figure 2). Therefore, strain BS21 was identified as *B. subtilis* and named *B. subtilis* BS21. It was deposited in the China General Microbiological Culture Collection Center (strain No. 25977).

3.3. Genomic features of strain BS21

Genomic features of BS21 are represented in Figure 3. The complete genome of strain BS21 contained a circular chromosome with no plasmid. The genome size of BS21 was 4,780,609 bp and the GC content (guanine-cytosine percentage in the genome) was 43.85%. A total of 3,788 genes were identified that occupied 81.11% of the genome. Moreover, 4,670 protein CDSs, 30 rRNAs, 87 tRNAs, 1 prophage, 5 genomic islands and 4 CRISPR-Cas were predicted in the genome of strain BS21.

3.4. COG classification

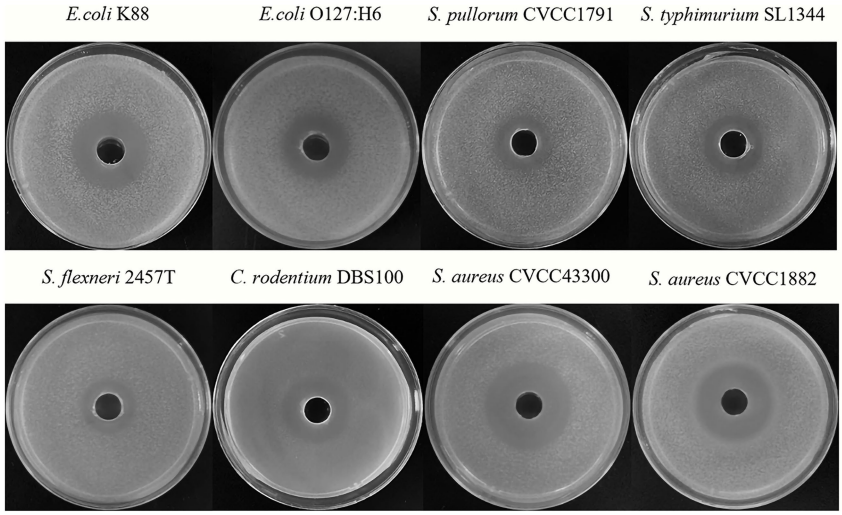
A high percentage of genes (81.11%) were annotated by COG. 23 COG functional categories contained a total of 3,788 genes (Figure 4). Many genes were classified into functional categories, including amino acid transport and metabolism (378 genes), carbohydrate transport and metabolism (351 genes), transcription (344 genes), general function prediction (330 genes) as well as translation, ribosomal structure and biogenesis (295 genes).

3.5. Genetic basis and UHPLC–MS/MS analysis for pathogen inhibition

A total of 13 gene clusters of secondary metabolites were predicted by antiSMASH in the genome of strain BS21, and seven were found to be associate with synthesis of antimicrobial secondary metabolites (Table 1). Among them, four gene clusters were predicted to synthesize the non-ribosomal peptides surfactin, zwittermicin A, bacillibactin and fengycin with 82, 22, 100 and 100% similarity, respectively. Two gene clusters had 100% similarity with one bacteriocin (subtilosin A) and one dipeptide (bacilysin). One gene cluster showed 100% similarity with a polyketide (bacillaene). These highly similar gene clusters (with more than 80% similarity) and their core genes are presented in Figure 5. Surfactin, bacillaene, fengycin, bacillibactin and bacilysin were further detected using UHPLC–MS/MS, verifying their successful production by BS21 (Figures 6A–E).

3.6. Optimization of fermentation conditions by single-factor experiment

In this study, carbon sources, including glucose, fructose, sucrose, lactose, soluble starch and corn flour were supplemented in



Indicator bacteria	Inhibition zone diameter (mm)
E. coli K88	17.5 ± 0.21
E. coli O127:H6	16.3 ± 0.51
S. pullorum CVCC1791	14.4 ± 0.29
S. typhimurium SL1344	14.9 ± 0.24
S. flexneri 2457T	12.5 ± 0.12
C. rodentium DBS100	15.1 ± 0.30
S. aureus CVCC43300	18.6 ± 0.54
S. aureus CVCC1882	18.2 ± 0.37

FIGURE 1
The antimicrobial activities of strain BS21 culture supernatant against different pathogens.

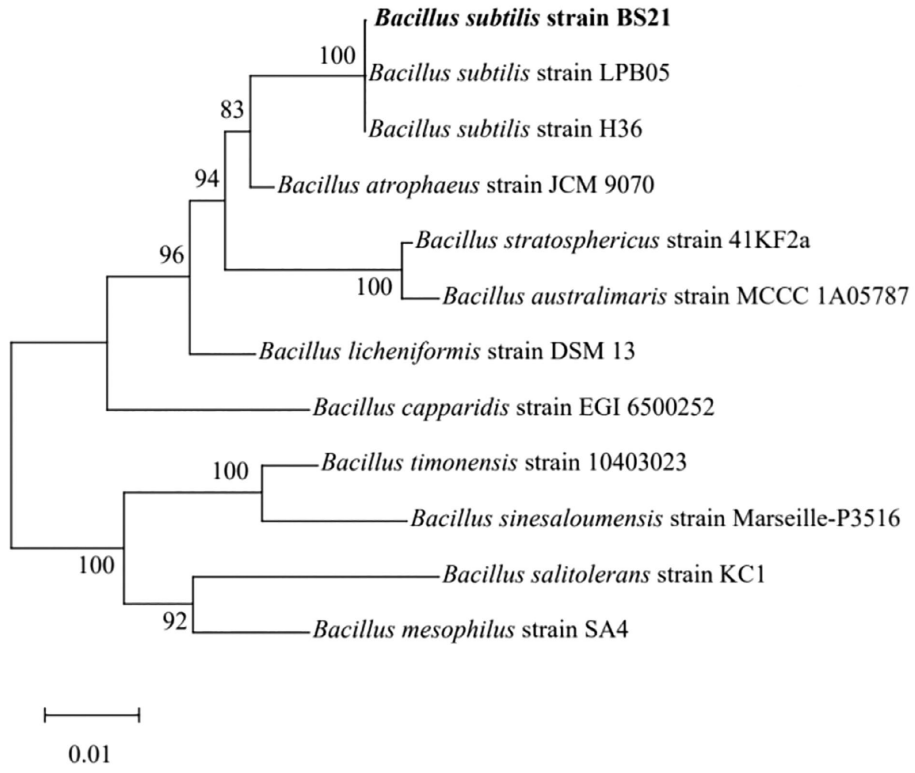


FIGURE 2
The neighbor-joining tree of strain BS21 based on 16S rRNA sequences.

the culture medium. Supplementation of corn flour showed the maximum antimicrobial activity at an optimal concentration of 2% (Figures 7A,B). Yeast extract, peptone, soybean meal, fermented soybean meal, rapeseed meal and ammonium sulfate as sole nitrogen sources were added to culture strain BS21. The maximum inhibition zone diameter was detected when soybean meal was added as the carbon source, followed by fermented soybean meal with the optimal concentration of 1.5% (Figures 7C,D). Addition of $MnSO_4$ led to the loss of antibacterial activity of strain BS21. The inhibition zone diameter in the NaCl group with the optimal concentration of 0.5% was greater than that in $CaCl_2$, KH_2PO_4, $MgSO_4$ and $ZnSO_4$ groups (Figures 7E,F). Cell-free supernatant of strain BS21 showed no antimicrobial activity when cultured at 20°C, while the inhibition zone diameter was maximum at 30°C and

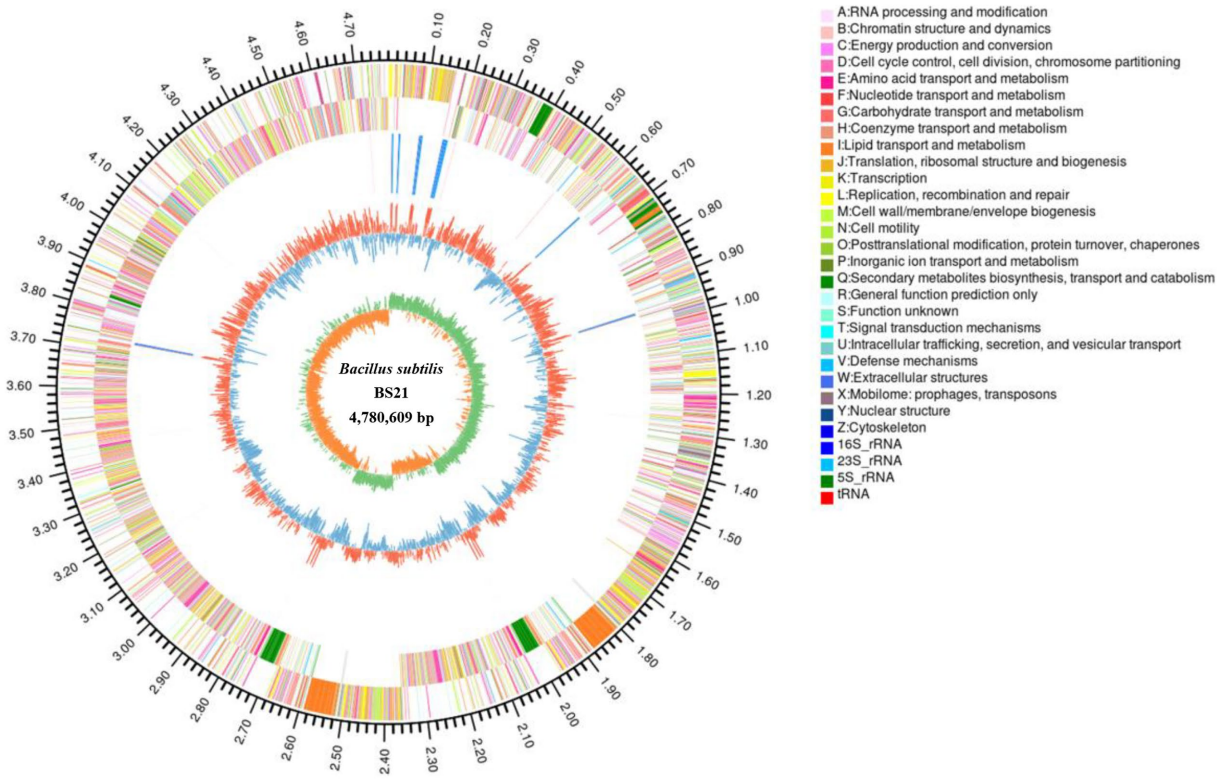


FIGURE 3
The circular genome map of strain BS21. From outside to inside, circle 1, the size of complete genome; circles 2 and 3, the predicted protein-coding genes on the positive and negative strands (different colors indicate different COG functions); circle 4, tRNA and rRNA; circle 5, GC content (red or blue represent the GC content higher or lower than average of GC content); the inner circle, GC-skew (green >0, yellow <0).

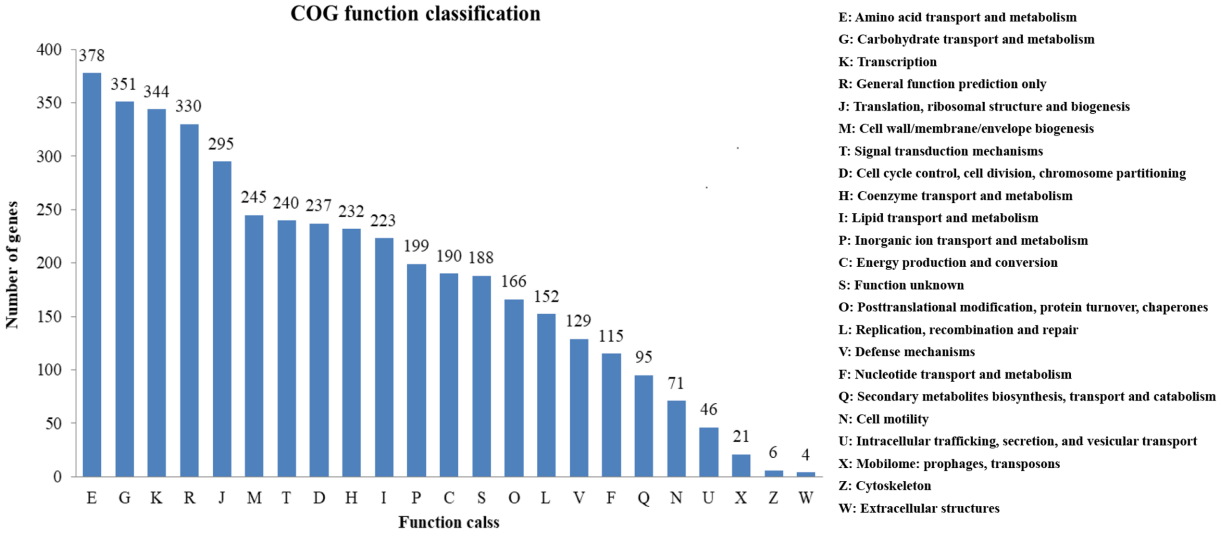


FIGURE 4
COG functional classification for strain BS21.

decreased as temperature dropped (Figure 8A). Antimicrobial activity was high at pH 7.0 with a rotating speed of 220 rpm (Figures 8B,C). Inhibition zone diameter peaked at 24 h, indicating that antimicrobial secondary metabolites accumulated most at 24 h (Figure 8D).

3.7. Optimization of fermentation conditions by RSM

PBD is generally used to screen significant factors and evaluate their main effects. In this study, seven factors (corn flour, soya bean

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TABLE 1 Antimicrobial secondary metabolite clusters predicted in strain BS21.

No	Similar cluster	Cluster type	Similarity (%)	Size (kb)	Features	MIBiG accession
1	Surfactin	NRPS	82	63.27	Antibacterial, antifungal	BGC0000433
2	Zwittermicin A	NRPS	22	80.62	Antibacterial	BGC0001059
3	Unkown	Terpene	–	20.36	–	–
4	Bacillaene	TransAT-PKS	100	114.25	Antibacterial	BGC0001089
5	Fengycin	NRPS	100	82.17	Antifungal	BGC0001095
6	Unkown	Terpene	–	20.82	–	–
7	Unkown	T3PKS	–	40.65	–	–
8	Unkown	Terpene	–	21.90	–	–
9	Unkown	T3PKS	–	41.10	–	–
10	Bacillibactin	NRPS	100	49.74	Antibacterial, siderophore	BGC0000309
11	Unkown	Thiopeptide	–	30.11	–	–
12	Subtilosin A	Sactipeptide	100	21.61	Antibacterial	BGC0000602
13	Bacilysin	Other	100	41.42	Antibacterial, antifungal	BGC0001184

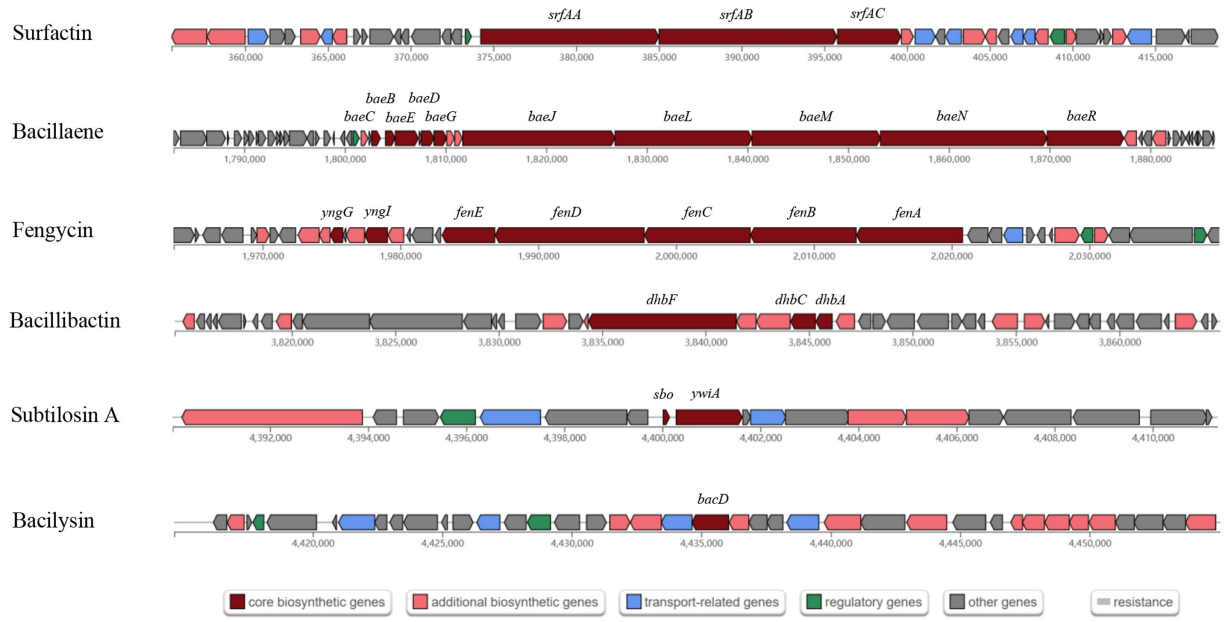

FIGURE 5
The antimicrobial secondary metabolite biosynthetic gene clusters in strain BS21. Red, pink, blue, green and gray indicate core biosynthetic genes, additional biosynthetic genes, transport related genes, regulatory genes and other genes, respectively. The core biosynthetic genes are marked in red.

meal, NaCl, temperature, initial pH, rotational speed and fermentation time) were evaluated for their effect on antimicrobial activity of strain BS21. PBD and its experimental responses are shown in Table 2. The effect and significance of culture condition factors for PBD are shown in Table 3. The *p*-value in this model was below 0.05, suggesting that the regression equation carried statistical significance. The regression coefficient (R^2) was 0.9731, demonstrating that 97.31% of the total variation could be explained by the developed model. The significance order of the seven tested factors on inhibition zone diameter was as follows: temperature > time > soybean meal > pH value > rotating speed > NaCl > corn flour, of which temperature, time and soybean meal were significant factors. Therefore, temperature, time and soybean meal were chosen for RSM to determine an optimum value and analyze their interactive effects.

RSM had been proven to be a practical tool in the optimization of significant fermentation condition factors. In order to find out the optimum fermentation condition for production of antimicrobial secondary metabolites, BBD with three factors and three levels was adopted to optimize conditions further. BBD and its experimental responses are shown in Table 4. Analysis of

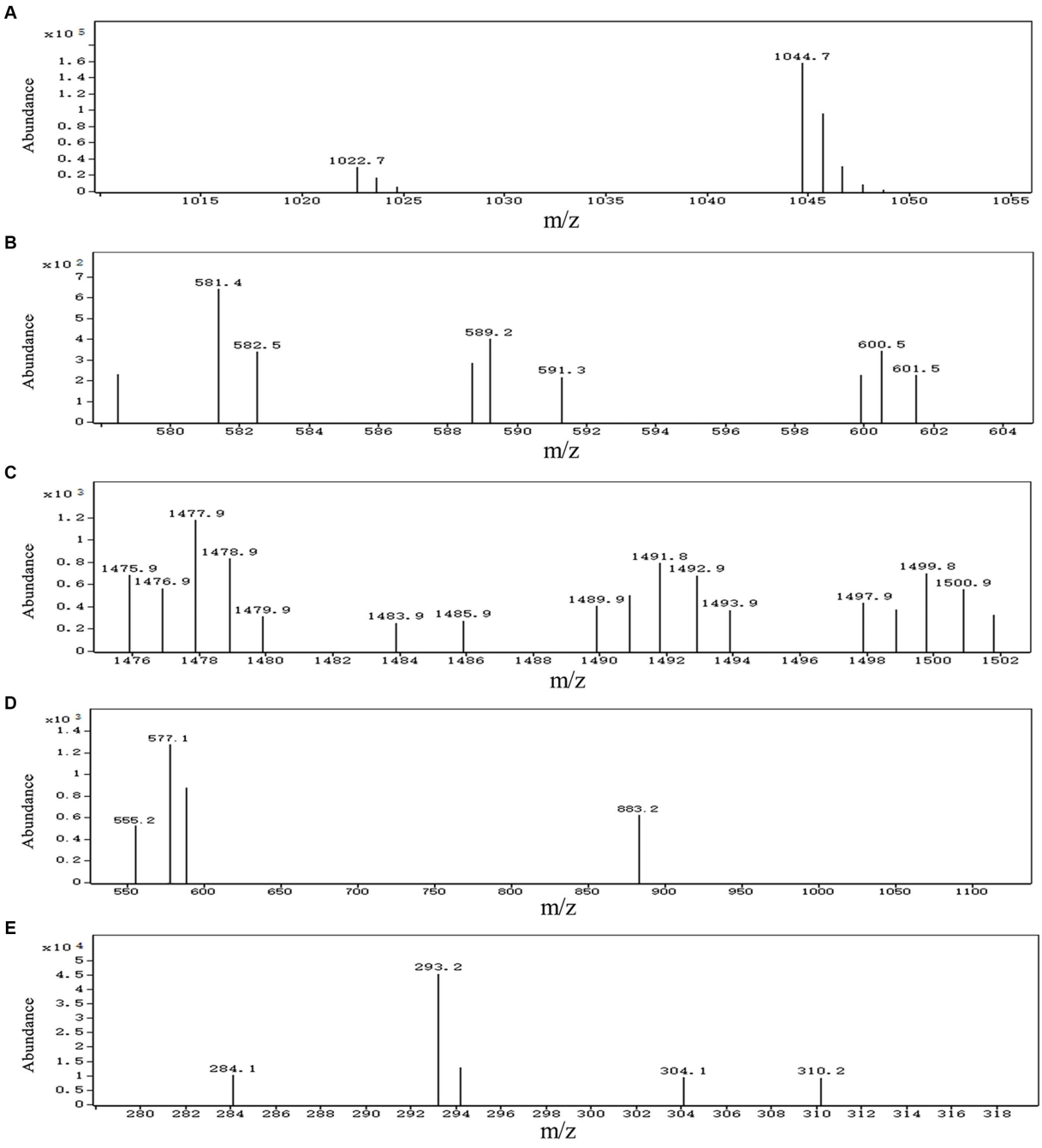

FIGURE 6
UHPLC–MS/MS analysis of antimicrobial secondary metabolites synthesized by strain BS21. **(A)** Ions of m/z values 1,022.7 correspond to C_{14} surfactin A $[M + H]^+$, and ions of m/z values 1,044.7 correspond to C_{14} surfactin A $[M + Na]^+$, respectively. **(B)** Ions of m/z 581.4 correspond to bacillaene A $[M + H]^+$. **(C)** Ions of m/z values 1,477.9 correspond to C_{15} fengycin B $[M + H]^+$, and ions of m/z values 1,499.8 correspond to C_{15} fengycin B $[M + Na]^+$, respectively. **(D)** Ions of m/z 883.2 correspond to bacillibactin $[M + H]^+$. **(E)** Ions of m/z value 293.2 correspond to bacilysin $[M + Na]^+$.

variance for the response surface model is shown in Table 5. The *p*-value in this model was below 0.05, indicating that the regression equation was significant. The R^2 was 0.9807, demonstrating that 98.07% of the total variation could be explained by the developed model. The response equation obtained was as follows:

$$Y = 24.36 + 1.25^{*}A + 0.79^{*}B + 1.01^{*}C - 0.05^{*}AB$$
$$-0.5^{*}AC - 0.83^{*}BC - 2.47^{*}A^2 - 3.64^{*}B^2 - 1.54^{*}C^2$$

The response surface plots showed that there is an interaction among three selected factors to affect antimicrobial activity of strain BS21 (Figures 9A–C). Moreover, the response surface was convex, meaning the existence of an optimal value for each variable. Based on our response surface analysis, the ideal values for the three tested factors were determined as: soybean meal at 1.7%, temperature at 30°C and time at 26 h. Under the optimal conditions, the inhibition zone diameter was predicted a theoretical maximum of 24.7 mm. The predicted values by RSM were confirmed through experimentation. At

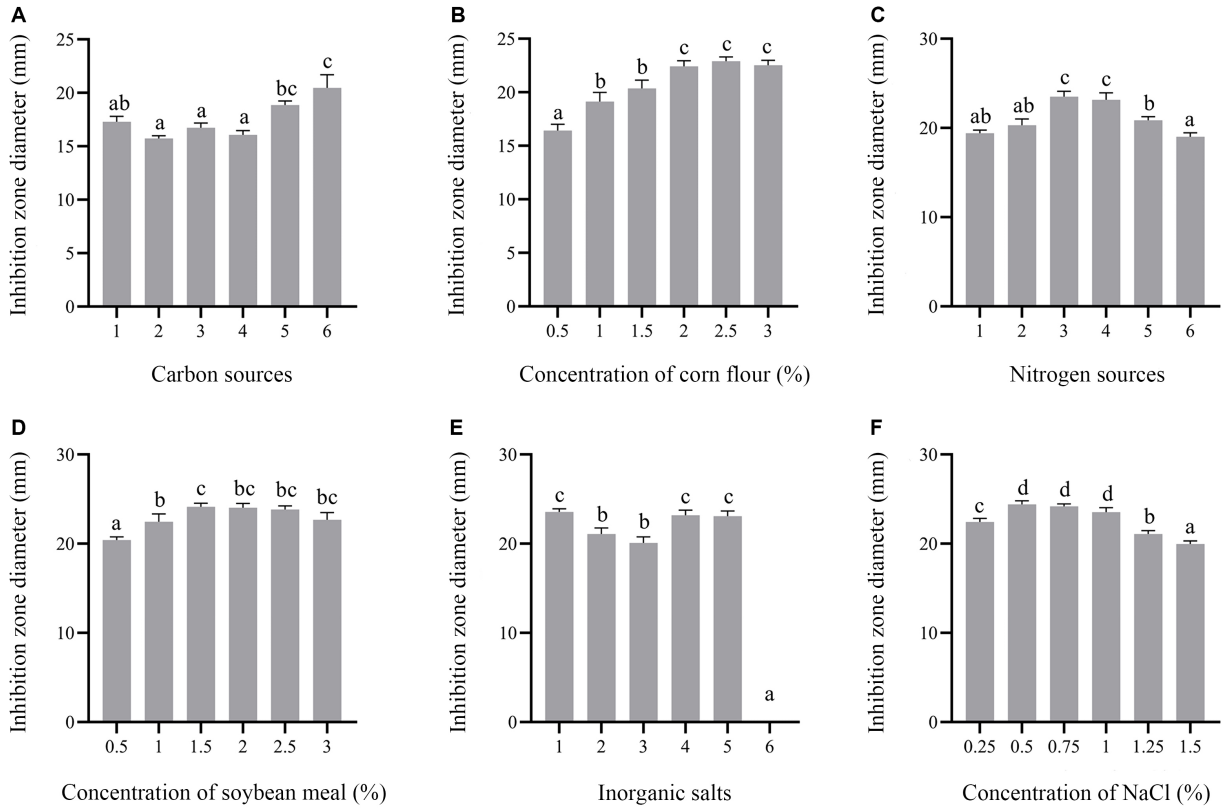

FIGURE 7
Effect of medium composition on antimicrobial secondary metabolite production. **(A)** Effect of different carbon sources (1%) (1: Glucose; 2: Fructose; 3: Sucrose; 4: Lactose; 5: Soluble starch; 6: Corn flour). **(B)** Effect of corn flour concentration. **(C)** Effect of different nitrogen sources (1%) (1: Yeast extract; 2: Tryptone; 3: Soybean meal; 4: Fermented soybean meal; 5: Rapeseed Meal; 6. Ammonium sulfate). **(D)** Effect of soybean meal concentration. **(E)** Effect of different mineral salts (1%) (1: NaCl; 2: $CaCl_2$; 3: KH_2PO_4; 4. $MgSO_4$; 5: $ZnSO_4$; 6: $MnSO_4$). **(F)** Effect of NaCl concentration on inhibition zone diameter.

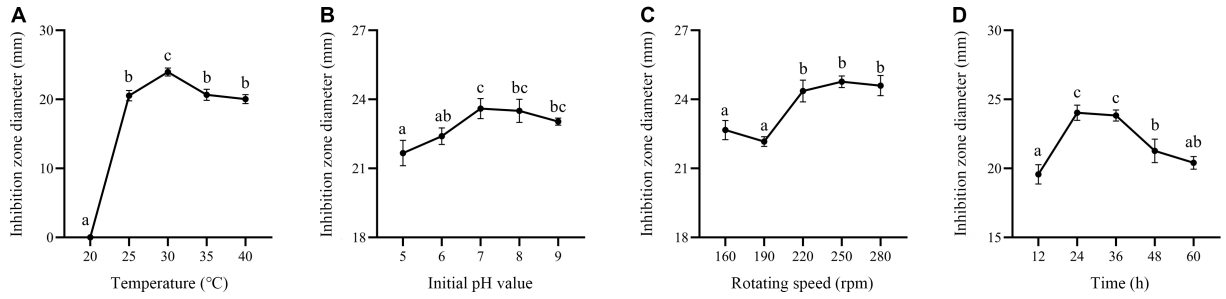

FIGURE 8
Effect of fermentation conditions on antimicrobial secondary metabolite production. **(A)** Effect of fermentation temperature. **(B)** Effect of initial pH value. **(C)** Effect of rotating speed. **(D)** Effect of fermentation time.

the optimum level, the inhibition zone diameter reached 25.1 mm, which was similar to the predicted maximum value, which showed a 43.4% increase compared to the original culture conditions (17.5 mm).

3.8. Growth curve of BS21 under optimum conditions

The growth curve of BS21 under optimum condition is presented as number of bacteria cells and showing a sigmoid growth pattern (Figure 9D). The rate of BS21 growth is slow at lag phase (0–4 h). The growth of BS21 is exponential from 4 to 28 h. After 28 h, BS21 transit to stationary phase, and the number of bacteria cell stabilized around 2.5×10^{11}.

4. Discussion

Banning antibiotics in animal feed has resulted in reduced growth performance and higher morbidity and mortality. Probiotics are reported

TABLE 2 Design and results of Plackett-Burman experiment.

Run	A	B	C	D	E	F	G	Inhibition zone diameter (mm)
1	2	1.5	0.5	25	5	160	24	17.5
2	2	0.5	0.25	25	7	160	24	15.9
3	2	1.5	0.25	30	7	220	12	20.9
4	0.5	1.5	0.5	30	5	160	12	18.3
5	2	0.5	0.5	30	5	220	24	19.2
6	2	0.5	0.5	30	7	160	12	16.3
7	0.5	0.5	0.25	30	5	220	24	20.6
8	0.5	1.5	0.5	25	7	220	24	21.2
9	0.5	0.5	0.5	25	7	220	12	11.6
10	2	1.5	0.25	25	5	220	12	14.7
11	0.5	0.5	0.25	25	5	160	12	12.0
12	0.5	1.5	0.25	30	7	160	24	23.5

A = Corn flour (%), B = Soybean meal (%), C = NaCl (%), D = Temperature (°C), E = pH value, F = Rotating speed (rmp), G = Time (h). For each factor, the small values represent its low level (−1) and the large values represent its high level (+1).

TABLE 3 Analysis of variance for Plackett-Burman experiment.

Source	Sum of square	df	Mean square	*F*-value	*P*-value
Model	146.99	7	21	20.71	0.0054
A-Corn flour (%)	0.6075	1	0.6075	0.599	0.4822
B-Soybean meal (%)	35.02	1	35.02	34.53	0.0042
C-NaCl (%)	1.02	1	1.02	1.01	0.3725
D-Temperature (°C)	55.9	1	55.9	55.12	0.0018
E-pH value	4.2	1	4.2	4.14	0.1115
F-Rotating speed (rmp)	1.84	1	1.84	1.82	0.2492
G-Time (h)	48.4	1	48.4	47.72	0.0023
Residual	4.06	4	1.01		
Cor Total	151.05	11			

The *p*-values less than 0.05 are significant.
$R^2 = 0.9731$, Adj $R^2 = 0.9261$.

to improve animal health through stimulation of intestinal microbiota, host immunity and other factors (Ding et al., 2021). Their metabolites, also known as postbiotics, have strong activity against multiple pathogenic bacteria (Yadav et al., 2022). Therefore, probiotics and natural antimicrobial products from microorganisms seem to be a favorable alternative to antibiotics. Du et al. (2019) demonstrated that *B. subtilis* WS-1 inhibits the growth of *E. coli in vitro*, dietary supplementation with strain WS-1 significantly reduced diarrhea rates and death in piglets from *E. coli* infection and ameliorated small intestinal lesions. The yak feces-derived *B. velezensis* JT3-1 was strongly antagonistic against *E. coli*, *S. typhimurium*, *S. aureus* and *Mannheimia haemolytica*. The average weight, cure rate of diarrhea and levels of immunoglobulins (IgA, IgG, IgM) in Angus calves supplemented with *B. velezensis* JT3-1 were significantly improved (Li et al., 2019).

In this research, *Bacillus subtilis* BS21 isolated from pig feces exerted antibacterial activity on Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella* and *Citrobacter*, which are major pathogens causing diarrhea in young animals (Li et al., 2021). In addition, strain BS21 also inhibited the growth of Gram-positive bacteria *S. aureus*, which can lead to mastitis in dairy cows (G Abril et al., 2020). These results indicated that strain BS21 has the potential to act as probiotic dietary supplements when added to animal feed and will show positive effects on animal health.

The strong antimicrobial activity of *Bacillus* species may be attributed to their property to produce multiple antimicrobial compounds, which favors their distribution in gut microbiota of animals to exert prebiotic functions (Caulier et al., 2019). A complete understanding in the genome of *B. subtilis* will contribute to the utilization of antimicrobial activity in strains of this species. The 43.85% GC content of BS21 was similar to previously reported *Bacillus subtilis* GM5 and 9407, which were 43.3 and 43.7%, respectively, (Hadieva et al., 2018; Gu et al., 2021). COG functional annotation of BS21 indicated that many genes are predicted to participate in amino acid transport and metabolism, carbohydrate transport, transcription as well as translation, ribosomal structure and biogenesis, which are important to the biological functionality of BS21. In addition, through antiSMASH tool and UHPLC–MS/MS analysis, secondary metabolites, including four antimicrobial peptides (surfactin,

TABLE 4 Design and results of Box–Behnken experiment.

Run	A-Soybean meal (%)	B-Temperature (°C)	C-Time (h)	Inhibition zone diameter (mm)
1	1.5	30	24	23.7
2	0.5	30	30	20.1
3	1.5	30	24	24.0
4	1.5	35	30	20.5
5	2.5	30	30	22.2
6	1.5	30	24	24.2
7	2.5	30	18	21.6
8	1.5	30	24	24.8
9	1.5	25	30	20.3
10	0.5	35	24	18.0
11	2.5	25	24	18.6
12	1.5	30	24	25.1
13	2.5	35	24	19.8
14	0.5	30	18	17.5
15	1.5	35	18	19.7
16	0.5	25	24	16.6
17	1.5	25	18	16.2

bacillibactin, fengycin, bacilysin) and one polyketide (bacillaene) were identified from culture supernatants, thus demonstrating that the corresponding biosynthetic gene clusters are functional in strain BS21.

Bacillibactin, a siderophore-type antimicrobial, show considerable antibacterial activity against *E. coli*, *S. aureus*, vancomycin-resistant *E. faecalis*, *P. aeruginosa*, and *K. pneumoniae* depending on its capability of complexing iron, which means that less iron was available to pathogens (Chen et al., 2019; Chakraborty et al., 2022). Fengycin, a lipopeptide, is known for its antifungal properties (Fu et al., 2022). In addition to directly restrict the growth of mycotoxigenic fungi, fengycin also inhibits mycotoxin production (Bertuzzi et al., 2022). Moreover, fengycin was reported by Piewngam et al. (2018) to eliminate *S. aureus* by blocking its quorum sensing. Surfactin is a common antimicrobial lipopeptide secreted by *Bacillus* species with antifungal, antiviral, anti-mycoplasma and antiprotozoal activities (Chen et al., 2022). The antibacterial mechanism of surfactin mainly relies on damage to bacterial cell membranes, inhibition to bacterial protein synthesis and bacterial enzyme activity (Theatre et al., 2021). It was reported that dietary surfactin supplementation improved growth performance and gut health of broilers challenged with *Clostridium perfringens* and tilapia (*Oreochromis niloticus*) fingerlings, suggesting surfactin as a potential substitute for antibiotics in poultry (Zhai et al., 2016; Cheng et al., 2018). In addition, surfactin also extends the shelf life of vegetables, fruits, meat, cereals and milk, functioning as a preservative with minimal degradability and toxicity (Huang et al., 2008; Kourmentza et al., 2021). Bacilysin is a simple antimicrobial peptide containing an L-alanine residue at the N-terminus and a non-proteinogenic amino acid L-anticapsin at the C-terminus (Islam et al., 2022). Although having a simple structure, it exhibits broad activity against various bacteria and fungi, attributing to its ability to induce cell lysis (Wang et al., 2018). Bacillaene, a polyene antibiotic, selectively inhibits prokaryotic protein synthesis (Olishevska et al., 2019). More valuably, these antimicrobial secondary metabolites may have synergistic effects against pathogens, therefore, have great potential to maintain microbiota homeostasis of intestine and regulate immune response of animals (Chen et al., 2019; Liu et al., 2022).

Production of antimicrobial secondary metabolites by microbial fermentation is strongly associated with culture conditions. Slight modifications in the composition of the medium or fermentation parameters can have a big impact on both the quality and quantity of secondary metabolites, also the overall metabolic characteristics of microorganisms (Sharma et al., 2020). Temperature can alter cell growth and product formation efficiencies (Vehapi et al., 2023). The temperature at which bacteria produce maximum antimicrobial secondary metabolites may not be the optimal temperature for their growth (Tan et al., 2022). It was reported that antibacterial compounds production of *Bacillus* spp. maximized at a temperature of 25–30°C (Puan et al., 2023). The antibacterial secondary metabolites produced by bacteria are reported to be synthesized during the exponential or early stationary phase (Boottanun et al., 2017; Regmi et al., 2017; Johny and Suresh, 2022). Therefore, optimizing fermentation conditions is a necessary strategy to develop biological additives. As a powerful mathematical tool for multiple regression analysis and statistical experimental designs, RSM has proven to be highly effective in optimizing formulation conditions (Mohammed and Luti, 2020). In this study, temperature, time and soybean meal were significant factors to effect antimicrobial activity of BS21 by screening of PBD. The optimal levels of them was predicted at 30°C, 26 h and soybean meal at 1.7% by BBD, respectively. The maximum antimicrobial activity of BS21 was predicted at the end of the exponential phase of growth (at 26 h) by BBD experiment. The significant interactions among temperature, time and soybean meal were obtained from the response surface plots.

In our investigation, the diameter of inhibition zone reached 25.1 mm after optimization using single-factor experiments and RSM with BBD, representing a 43.4% increase compared to the

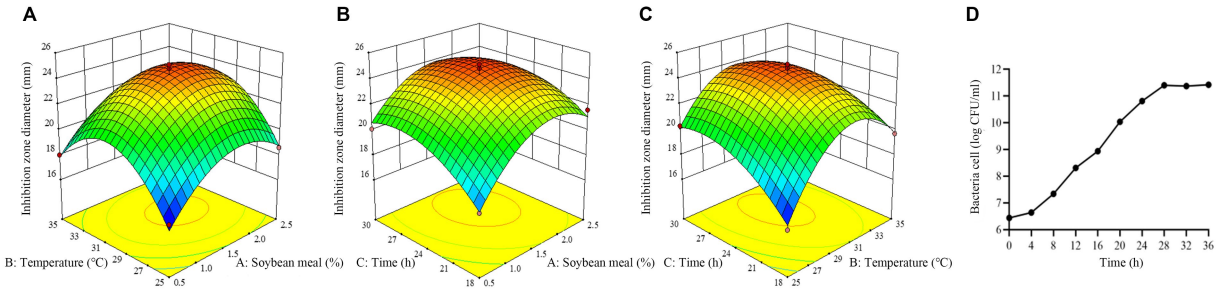

FIGURE 9
Response surface plot of the effect of three variables on antimicrobial activity of strain BS21. **(A)** Effect of soybean meal and temperature. **(B)** Effect of soybean meal and time. **(C)** Effect of temperature and time. **(D)** Time-dependent growth curve of strain BS21 under optimum condition.

TABLE 5 Analysis of variance for the response surface model.

Source	Sum of square	df	Mean square	*F*-value	*p*-value
Model	130.12	9	14.46	39.46	<0.0001
A-Soybean meal (%)	12.5	1	12.5	34.12	0.0006
B-Temperature (°C)	4.96	1	4.96	13.54	0.0079
C-Time (h)	8.2	1	8.2	22.39	0.0021
AB	0.01	1	0.01	0.027	0.8734
AC	1	1	1	2.73	0.1425
BC	2.72	1	2.72	7.43	0.0295
A^2	25.64	1	25.64	69.98	<0.0001
B^2	55.86	1	55.86	152.49	<0.0001
C^2	10.02	1	10.02	27.35	0.0012
Residual	2.56	7	0.37		
Lack of Fit	1.23	3	0.41	1.23	0.4072
Pure Error	1.33	4	0.33		
Cor Total	132.68	16			

The *p*-values less than 0.05 are significant.
$R^2 = 0.9807$, Adj $R^2 = 0.9558$.

original formulation conditions. Similarly, RSM had let to improved production of antibacterial compounds in different bacterial strains. In a recent study by Sa et al. (2022), the formulation conditions of *Paenibacillus polymyxa* DS-R5 strain were optimized by using RSM experimental design. This optimized conditions (medium volume 51.0 mL; initial pH 6.7; fermentation temperature 33.1°C) led to a remarkable 77.6% increase in the titer of antifungal substances, with the production reaching 8036 mg/L compared to the initial formulation conditions of 4357 mg/L. Ju et al. (2018) demonstrated that the inhibition zone diameter of cell-free culture supernatant of the *Streptomyces rimosus* AG-P1441 strain was enhanced from an initial 15 to a 29 mm under the optimized culture conditions (3% glucose, 3.5% corn starch, 2.5% soybean meal, 1.2 mM $MgCl_2$ and 5.9 mM glutamate) by using RSM. As a result, RSM showed a high accuracy of the developed model and model validation under the present study, it could be a reliable way to optimize the formulation conditions for antimicrobial secondary metabolites of the BS21 strain.

In conclusion, strain BS21 and its antimicrobial secondary metabolites have the potential to be developed as antibiotic alternatives to control pathogenic bacteria and improve growth performance of animals. Optimization studies using the single-factor design and RSM successfully increased the production of antimicrobial secondary metabolites of strain BS21. This study not only lays an experimental basis for subsequent development and application of strain BS21 as an antibiotic alternative in animal production, but also provides a reference for improving the production of antimicrobial secondary metabolites in other bacterial strains.

Data availability statement

The complete genome sequence of Bacillus subtilis BS21 has been deposited in GenBank (Accession number PRJNA962810).

Author contributions

DW, ND, and YC conceived and designed the research. DW and LF performed the experiments. ND and DL contributed analytical

tools and reagents. DW wrote the manuscript. All authors have read and approved the manuscript.

Funding

This research was funded by NSFC grand (32172754) and the National Key Research and Development Program of China (2022YFD1300404).

Acknowledgments

We thank Fan Ruiqi and Dr. Chen Yiqiang for the UHPLC-MS/MS analysis.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 23 October 2023
DOI 10.3389/fmicb.2023.1289819

OPEN ACCESS

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RECEIVED 06 September 2023
ACCEPTED 09 October 2023
PUBLISHED 23 October 2023

CITATION
Bai X, Ya R, Tang X and Cai M (2023) Role and interaction of bacterial sphingolipids in human health.
Front. Microbiol. 14:1289819.
doi: 10.3389/fmicb.2023.1289819

Role and interaction of bacterial sphingolipids in human health

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Sphingolipids, present in both higher animals and prokaryotes, involving in cell differentiation, pathogenesis and apoptosis in human physiological health. With increasing attention on the gut microbiome and its impact on wellbeing, there is a renewed focus on exploring bacterial sphingolipids. This review aims to consolidate the current understanding of bacterial sphingolipids and their impact on host health. Compared to mammalian sphingolipids, bacterial sphingolipids are characterized by odd chain lengths due to the presence of branched alkyl chains. Additionally, intestinal microbial sphingolipids can migrate from the gut to various host organs, affecting the immune system and metabolism. Furthermore, the intricate interplay between dietary sphingolipids and the gut microbiota is explored, shedding light on their complex relationship. Despite limited knowledge in this area, this review aims to raise awareness about the importance of bacterial sphingolipids and further our understanding of more uncharacterized bacterial sphingolipids and their significant role in maintaining host health.

KEYWORDS

sphingolipids, bacteria, gut microbiome, human health, interaction

1. Introduction

Sphingolipids are a group of lipids characterized by long-chain bases serving as the backbone, along with an amine group and two or three hydroxy groups at the structural end (Merrill, 2011). Sphingolipids, in the beginning, were thought to only exist in higher animals. However, subsequent research discovered sphingolipids in flagellates, rumen bacteria, protozoa, and specific Bacteroidaceae species such as *Bacteroides (B.) melaninogenicus* and *B. thetaiotaomicron*. These species are characterized by branched-chain sphingolipids (Stoffel et al., 1975; Olsen and Jantzen, 2001; Johnson et al., 2020; Le et al., 2022). As the gut microbiome and human health garner more attention, bacterial sphingolipids, particularly intestinal microbial sphingolipids, have gained interest due to their potential connections to gut microbiome and host health. Studies have shown that bacterial sphingolipids can be absorbed and detected in various organs throughout the body (Fukami et al., 2010; Johnson et al., 2020; Le et al., 2022). Additionally, sphingolipids produced by prominent *Bacteroidetes* in the gut have been found to impact host lipid metabolism and liver function (Johnson et al., 2020). Moreover, intestinal microbial sphingolipids play a significant role in maintaining host immune homeostasis (An et al., 2014; Brown et al., 2019). Despite the work of laboratories like Elizabeth Johnson's Lab at Cornell University, there have been relatively fewer reports on bacterial sphingolipids compared to mammal sphingolipids.

There is evidence suggesting that many microorganisms, microbial toxins, and viruses bind to cells through sphingolipids (Vesper et al., 1999) and bacteria can translocate to host

organs (Brown et al., 2019; Johnson et al., 2020). Several intriguing clinical phenomena have emerged, indicating the potential prospects of studying intestinal microbial sphingolipids in clinical research. For instance, it has been reported that dietary gangliosides may play a crucial role in modifying the intestinal microflora and promoting the development of intestinal immunity in neonates, thereby preventing infections during early infancy (Rueda, 2007). Another study examined the gut microbiome and neurodevelopment in infants from a general population birth cohort at two critical periods during infancy. The findings revealed that infants with a dominance of *Bacteroidetes* displayed enrichment in multiple metabolic functions, including sphingolipid metabolism and glycosphingolipid biosynthesis. This group of infants also achieved higher scores in cognitive, language, and motor development at the age of 2 years old (Tamana et al., 2021). These fragmented pieces of information emphasize the importance of investigating bacterial sphingolipids, including their unidentified compounds and structures, their impact on host physiology, and their role in connecting the gut microbiome with host health. Understanding how the daily diet influences the gut microbiome and its sphingolipids, as well as comprehending the mechanisms by which intestinal microbial activity affects host health, are important areas that require extensive research. It is clear that addressing these complex questions will require considerable effort and time.

This review provides a comprehensive overview of current knowledge regarding bacterial sphingolipids and their impact on host health. We will begin by introducing sphingolipids in general and highlight the structural differences between bacterial sphingolipids and those found in mammals. Additionally, we examine the influence of bacterial sphingolipids on the host's immune system and metabolites. Furthermore, we will explore the intricate relationship between dietary sphingolipids and the gut microbiota. Despite limited knowledge in this area, we aim to contribute to the understanding of bacterial sphingolipids. Our ultimate objective is to raise awareness and further investigate the importance of bacterial sphingolipids, particularly intestinal microbial sphingolipids, and provide potentials to develop targeted therapeutics for uncharacterized sphingolipids. Additionally, we aim to elucidate the underlying mechanisms by which these sphingolipids interact with the gut and influence host health.

2. An overview of sphingolipid structure and categories

2.1. Definition and categories of sphingolipids

Sphingolipids are a group of lipids characterized by a common structural feature: they all consist of "long-chain" bases, also known as "sphingoid" bases, as the backbone, along with an amine group and two or three hydroxy groups at the structural end. The representative sphingoid base is sphingosine, specifically (2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol, which is also referred as (E)-sphing-4-enine (Wieland Brown et al., 2013; Taniguchi and Okazaki, 2021). The sphingoid bases can vary in terms of the number and arrangement of hydroxyl groups, the length of alkyl chains, and the presence of saturated or unsaturated bonds (Merrill, 2011; **Figure 1**). Free sphingoid bases are present in minimal amounts, making them complex compounds. In case of complex sphingolipids, the amino group of the sphingoid bases undergoes acylation with fatty acids and/or a substituent at position 1 hydroxyl. The fatty acid chains can vary in length and number of double bonds, typically ranging from 14 to 36 carbon atoms and can be saturated, have a single double bond, or possess an α-hydroxyl group (Pruett et al., 2008). Common substituents include -H, fatty acids, phosphates, phosphocholine, phosphoethanolamine, galactose, glucose, sialic acid, sulfate, glucuronic acid, or a combination of these groups. Consequently, the potential diversity of lipid species is vast (Merrill et al., 2007). However, the actual number of species produced in biological systems is significantly lower, primarily due to the limited number of synthases involved in complex sphingolipid synthesis and their substrate specificities. In order to illustrate the sphingolipid structures clearly, the common structures were summarized in **Figure 1**.

Common sphingolipids include ceramide, sphingomyelin, glucosylceramide (GlcCer), lactosylceramide (LacCer), and galactosylceramides (GalCer). Additionally, there are more complex glycosphingolipids that contain a varying number of sugar residues. In addition to these, small amounts of sphingolipid analogs are present, along with "lyso-" sphingolipids. "Lyso-" sphingolipids refer to sphingoid bases combined with a headgroup but lacking the N-acyl substituent. Examples of "lyso-" sphingolipids include sphingosine 1-phosphate, sphingosine 1-phosphocholine, and lyso-glycosphingolipids (**Figure 1**). Moreover, there are *N*-methyl derivatives of sphingolipids and covalent adducts with proteins (Vesper et al., 1999; Merrill, 2011).

2.2. Bacterial sphingolipids

Initially, sphingolipids were believed to be present only in higher animals. However, further investigations have unveiled the presence of branched-chain sphingolipids in bacteria, including *Bacteroides*, *Prevotella*, *Sphingomonas*, *Sphingobacterium*, *Porphyromonas*, *Fusobacterium*, *Bdellovibrio*, *Cystobacter*, *Mycoplasma*, and *Flectobacillus*, which is noteworthy that a majority of these bacteria are anaerobes (Stoffel et al., 1975; Olsen and Jantzen, 2001).

In Stoffel et al. (1975) conducted an initial investigation on the lipid composition of anaerobic *B. thetaiotaomicron* metabolites. The analysis revealed that approximately 50% of the total lipid extract was sphingolipids, including sphingomyelin, ceramide phosphinicoethanolamine, free even-numbered and branched chain sphingosine bases and ceramide (Stoffel et al., 1975). More recently, in a germ-free mice mono-colonized with wild type *B. thetaiotaomicron* (BTWT) or *SPT* knocked *B. thetaiotaomicron* ($BT_{\Delta}SPT$), 144 unique bacterial lipids were identified using Tandem Mass Spectrometry, dependent on Spt, with 35 unique *Bacteroides* sphingolipids present in BTWT but absent in the $BT_{\Delta}SPT$. Among the differential sphingolipids, the most abundant ones in the BTWT-colonized mouse cecum were ceramide phosphoethanolamine (Cer-PE) and dihydroceramide (DHCer). Additionally, an abundant ceramide phosphoinositol (Cer-PI) was observed, a sphingolipid newly reported to be produced by gut *Bacteroides* strains and not detected in mammalian cells.

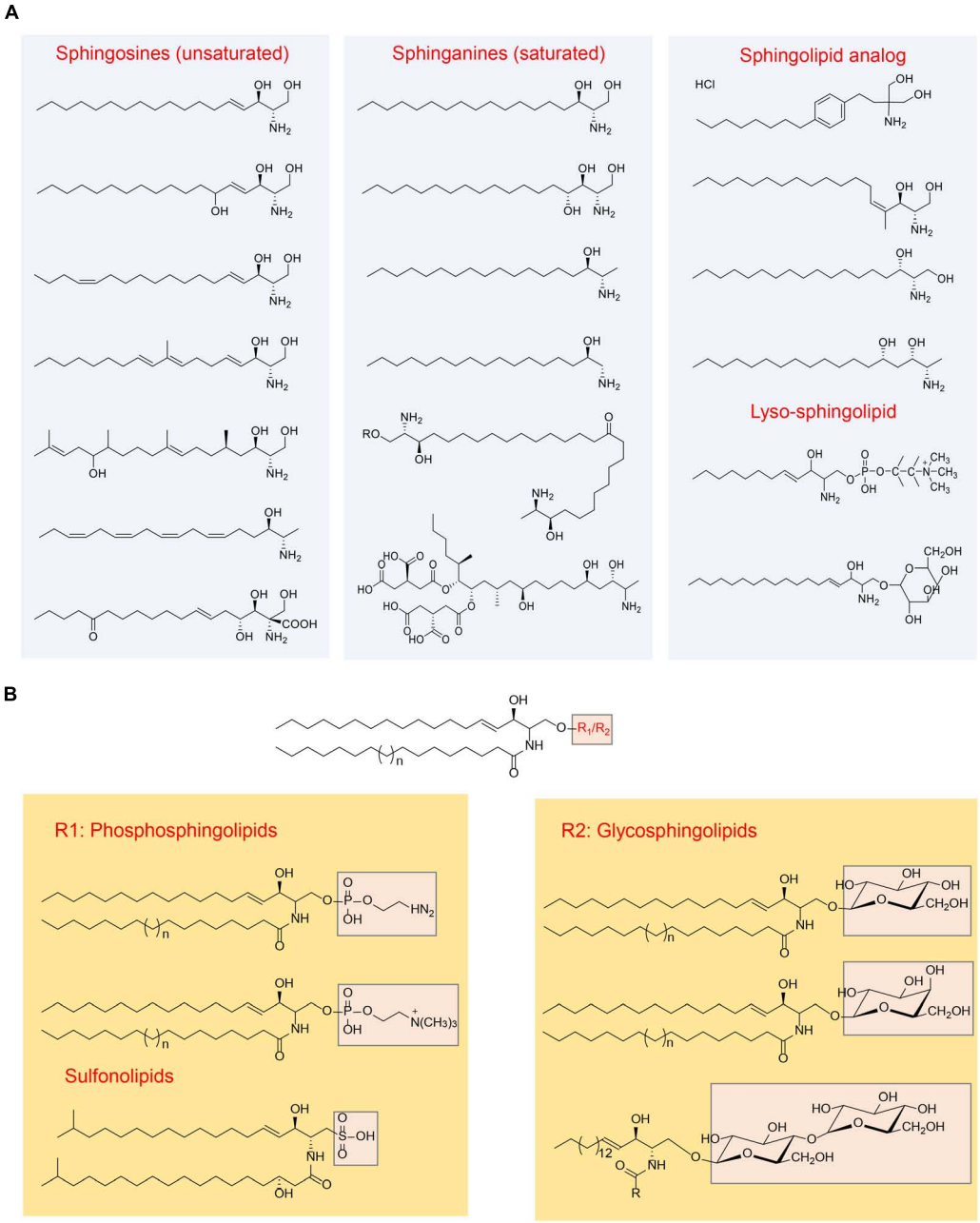

FIGURE 1
Overview of sphingolipids structure (Merrill, 2011). **(A)** Categorization of sphingolipid structures based on the "long chain" base; **(B)** categorization of sphingolipid structures based on the head group.

Furthermore, a subset of phosphatidylethanolamine (PE) that were significantly more abundant in $BT_{\Delta}SPT$-colonized mouse cecum was discovered, with PE 32:0 and PE 35:07 being the most differential *in vivo* (Brown et al., 2019). Another study found that *B. thetaiotaomicron* can synthesize a set of uncharacterized homoserine-containing sphingolipids that are transferred to the host liver (Le et al., 2022).

Lipidomics analysis of the outer membrane vesicles (OMVs) from *B. thetaiotaomicron* VPI-5482 revealed the presence of diverse sphingolipids, glycerophospholipids, and glycine-serine dipeptide lipids (GS). The most abundant sphingolipids identified were ethanolamine phosphoceramide (EPC) and inositol phosphoceramide (IPC) (Sartorio et al., 2022). Another recent lipidomic analysis of four Bacteroides species reported that Dihydroceramide Phosphoethanolamine (DHCer-PE) is the most abundant sphingolipid across all four Bacteroides strains. Sphingolipids accounted for 19–29% of the total lipids detected in Bacteroides species, with DHCer making up approximately 20% of the sphingolipid fraction in *B. ovatus*, *B. vulgatus*, and *B. thetaiotaomicron*, but only 2% in *B. fragilis*. Instead, *B. fragilis* appeared to accumulate higher levels of keto-sphinganine (keto-sph). Additionally, detectable levels of sphinganine (sph) and deoxy-sph were found in both *B. thetaiotaomicron* and *B. ovatus*. α-Galactosylceramide (α-GalCer) was detected solely

in *B. fragilis* and *B. vulgatus* (Ryan et al., 2023). Hence, there exists a diverse array of sphingolipid compounds, suggesting the potential existence of multiple pathways involved in sphingolipid biosynthesis across various *Bacteroides* species.

In addition to the previous mentioned *Bacteroides*, a few members of the Chlorobi phylum, especially within genus *Chlorobium*, which are also capable of sphingolipid producing sphingolipids (Gupta and Lorenzini, 2007). Moreover, certain Alpha-Proteobacteria (such as *Acetobacter, Sphingomonas*, and *Novosphingobium*) and Delta-Proteobacteria (including *Myxococcus* and *Bdellovibrio*) are known to produce sphingolipids as well. While there are thousands of bacteria, only a small percentage of them are capable of producing sphingolipids (**Table 1**). Bacterial sphingolipids can be found in various environments, indicating the successful adaptation of sphingolipid producers in the biosphere (Fredrickson et al., 1995; Schubotz et al., 2009; Sollich et al., 2017; Heaver et al., 2018). For example, the human gastrointestinal tract is often heavily colonized by Bacteroidetes, including species like *Bacteroides* and *Prevotella* species, resulting to the existence of approximately 1 gram of sphingolipids produced by intestinal bacteria at any given time (Schnorr et al., 2014; Smits et al., 2017). Within the human gut, members of the Bacteroidetes phylum (e.g., *Bacteroides*, *Prevotella*, *Porphyromonas*) are known to synthesize sphingophospholipids that resemble sphingomyelin, a sphingolipid abundant in mammalian membranes. They also produce glycosphingolipids and DHCers (Stoffel et al., 1975; Olsen and Jantzen, 2001; Wieland Brown et al., 2013; Moye et al., 2016). Additionally, within the Bacteroidetes phylum, species such as *Chryseobacterium gleum*, *Alistipes*, and *Odoribacter* spp. were reported to synthesize sulfonolipids, as well (Walker et al., 2017; Heaver et al., 2018; Hou et al., 2022). Although the adaptation driving factors are unclear, it was reported that some pathogens, like *Bacillus cereus* (Flores-Díaz et al., 2016), *Clostridium perfringens* (Urbina et al., 2011), *Helicobacter pylori* (Chan et al., 2000), *Mycobacterium tuberculosis* (Speer et al., 2015), can survive in extreme environment because they produce sphingomyelinase, which degrade sphingomyelin (a kind of sphingolipids in cell membranes) and help pathogens invade into the host or escape from macrophage (Simonis and Schubert-Unkmeir, 2018). Moreover, certain opportunistic human pathogens, such as Sphingomonas species, are known to produce sphingolipids that likely originate from the plant rhizosphere (Berg et al., 2005). In the past years, increased human infections caused by opportunistic pathogens originating from the rhizosphere, which refers to the zone surrounding plant roots, have been noticed. It is worth noting that certain strains of Proteobacteria that are associated with plant roots can engage in interactions with both plants and humans. These particular strains have been found to possess beneficial effects on plant growth, displaying plant growth-promoting properties. Moreover, they have also shown excellent antagonistic properties against plant pathogens, offering potential benefits for both agricultural practices and the development of novel disease-control strategies (Berg et al., 2005; Bodenhausen et al., 2013; Erkosar and Leulier, 2014). This highlights the emerging significance of sphingolipid-producing *Proteobacteria* as important colonizers of both plants and animals. Examples include *Sphingomonas* spp. found on plant and root surfaces (Bodenhausen et al., 2013), as well as *Acetobacter* spp. associated with *Drosophila melanogaster* and *Caenorhabditis elegans* (Ogawa et al., 2010; Erkosar and Leulier, 2014; Zhang et al., 2017). The bacteria mentioned above are just a subset of the numerous species known to produce sphingolipids and more information are illustrated in **Table 1** and **Figure 2**.

2.3. The distinction between bacterial sphingolipids and eukaryotic sphingolipids

Recent research has uncovered that ability of both bacteria and eukaryotic organisms to produce sphingolipids (Olsen and Jantzen, 2001; Brown et al., 2019, 2023). However, there are notable distinctions between bacterial and eukaryotic sphingolipids. In mammals, the primary type of synthesized sphingolipids consists of even-chained linear structure. Conversely, bacterial sphingolipids exhibit a unique characteristic in which their sphingoid base has an odd chain length due to the presence of branched alkyl chains (Le et al., 2022; Ryan et al., 2023), like Threonine C_{19}, Homoserine C_{19}, and Serine/Alanine C_{35} DHCer, as well as Serine C_{37}/C_{35} DHCer-PE in wild *B. thetaiotaomicron* (Le et al., 2022).

The initial step of sphingolipid synthesis in both bacteria and eukaryotes involves the condensation of an amino acid (typically serine in mammals) and a fatty acid (typically palmitate in mammals) through the action of the serine palmitoyltransferase (SPT) enzyme. This enzyme is highly conserved in both eukaryotes and bacteria (Yard et al., 2007). However, after the initial step, the pathways and products of sphingolipid biosynthesis diverge between eukaryotes and bacteria, including 3-Ketodihydrosphingosine reductase (KDSR), Sphingosine kinases (SKs), Sphingosine 1-phosphate lyase (S1PL) (Harrison et al., 2018; Heaver et al., 2018). Nevertheless, our understanding of bacterial sphingolipids is still less comprehensive compared to their eukaryotic counterparts, and therefore, the exact differences remain unclear. The specific variations in bacterial sphingolipid synthesis compared to eukaryotic sphingolipid synthesis have yet to be fully elucidated due to the relatively limited knowledge about bacterial sphingolipids. Further research is required to gain a more comprehensive understanding of bacterial sphingolipids and their distinctions from eukaryotic sphingolipids.

3. Interaction of bacterial sphingolipids with the host

Efforts were undertaken to investigate the potential of bacterial sphingolipids to undergo transformation within the host organs. Fukami et al. (2010) conducted a study where ^{13}C-labeled ceramide extracted from *Acetobacter malorum* were orally administered to mice. And these sphingolipids were readily absorbed and metabolized in the liver, ultimately forming complex sphingolipids (Fukami et al., 2010). Recent experiments utilizing fluorescently labeled bacteria demonstrated the presence of sphingolipids originating from bacteria in various tissues such as the liver, colon, ileum, brain, and skin, through the intestinal epithelial cells (Brown et al., 2019; Hussain et al., 2019; Johnson et al., 2020;

TABLE 1 Known bacterium producing sphingolipids.

Bacterium	Sphingolipids	References
Acetobacter malorum	Ceramide	Ogawa et al., 2010
Alistipes finegoldii	Sulfonolipids	Radka et al., 2022
Algoriphagus machipongonensis	Sulfonolipids	Alegado et al., 2012
Alistipes and Odoribacter	Sulfonolipids	Walker et al., 2017
Bacteroides thetaiotaomicron	Cer-PE, Cer-PG, Cer PI, Cer-PC, DHCer, DHcer-PE, DHcer-PI, sphingoid base,	Olsen and Jantzen, 2001; Le et al., 2022; Ryan et al., 2023
Bacteroides fragilis	Cer-PE, Cer-PG, DHCer, α-GalCer	Olsen and Jantzen, 2001; Ryan et al., 2023
Bacteroides ovatus	Cer-PE, Cer-PG, Cer-PI DHCer Sphingoid base,	Olsen and Jantzen, 2001; Ryan et al., 2023
Bacteroides uniformis	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Bacteroides caccae	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Bacteroides eggerthii	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Bacteroides stercoris	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Bacteroides. vulgatus	DHCer, Cer PE, Cer PI, α-GalCer	Ryan et al., 2023
Bdellovibrio bacteriovorus	Sphingophospholipids	Olsen and Jantzen, 2001
Capnocytophaga ochracea	Sulfonolipid Capnine	Liu et al., 2022
Caulobacter crescentus	Cer-PG	Dhakephalkar et al., 2023
Chlamydia psittaci	Sphingomyelin	Koch-Edelmann et al., 2017
Chlamydia trachomatis	Sphingomyelin	Koch-Edelmann et al., 2017
Chryseobacterium gleum	Sulfonolipids	Chaudhari et al., 2009; Hou et al., 2022
Cystobacter fuscus	Sphingolipids	Olsen and Jantzen, 2001
Flectobacillus major	Amino sphingophospholipids (namely Cer-PC)	Olsen and Jantzen, 2001
Mycoplasma spp.	Sphingophospholipid	Olsen and Jantzen, 2001
Sphingobacterium spp.	Sphingolipids	Olsen and Jantzen, 2001
Porphyromonas gingivalis	DHCer	Moye et al., 2016
Prevotella melaninogenica	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Prevotella intermedia	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Prevotella bivia	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Porphyromonas gingivalis	Cer-PE, Cer-PG	Olsen and Jantzen, 2001
Sphingomonas spp	Sphingolipids	Olsen and Jantzen, 2001
Sphingomonas paucimobilis	GlcCer, α-GalCe	Yard et al., 2007
Sphingobacterium spiritivorum	Ceramide	Minamino et al., 2003
Salinibacter ruber	Sulfonolipid	Corcelli et al., 2004

Cer-PE, ceramide phosphoethanolamine; Cer-PG, ceramide phosphoglycerol; Cer-PC, ceramide phosphocholine; Cer PI, ceramide phosphoinositol; DHCer, dihydroceramide; α-GalCer, α-Galactosylceramide; DHCer-PE, dihydroceramide phosphoethanolamine; DHCer-PI, dihydroceramide phosphoinositol, GlcCer, glucosylceramide.

Le et al., 2022). These findings suggest that bacterial sphingolipids play an active role in maintaining are actively utilized in maintaining sphingolipid homeostasis and promoting symbiosis within the host. Furthermore, these results provide evidence for a direct connection between host and microbial sphingolipids, as depicted in **Figure 3**.

3.1. Implications of bacterial sphingolipids on the immune system

The interaction between bacterial sphingolipids and the host began with the identification of a lipid produced by members of the Bacteroidetes that is analogous to alpha-GC, a known CD1-binding lipid derived from *Sphingomonas* species (Brown et al., 2023). Early studies revealed that alpha-GC derived from *Bacteroides fragilis* has the ability to bind to both mouse CD1d and the T-cell receptor (TCR) on invariant natural killer T (iNKT) cells, thereby inhibiting iNKT cell proliferation during neonatal development (Wieland Brown et al., 2013; An et al., 2014). Subsequent investigations have further elucidated the structural requirements for efficient binding between alpha-GC and CD1d, as well as the functional consequences of alpha-GC recognition by the TCR. These studies have highlighted the role of alpha-GC in regulating colonic NKT cells, suggesting its structure-specific immunomodulatory activity (Oh et al., 2021). Moreover, administration of these sphingolipids

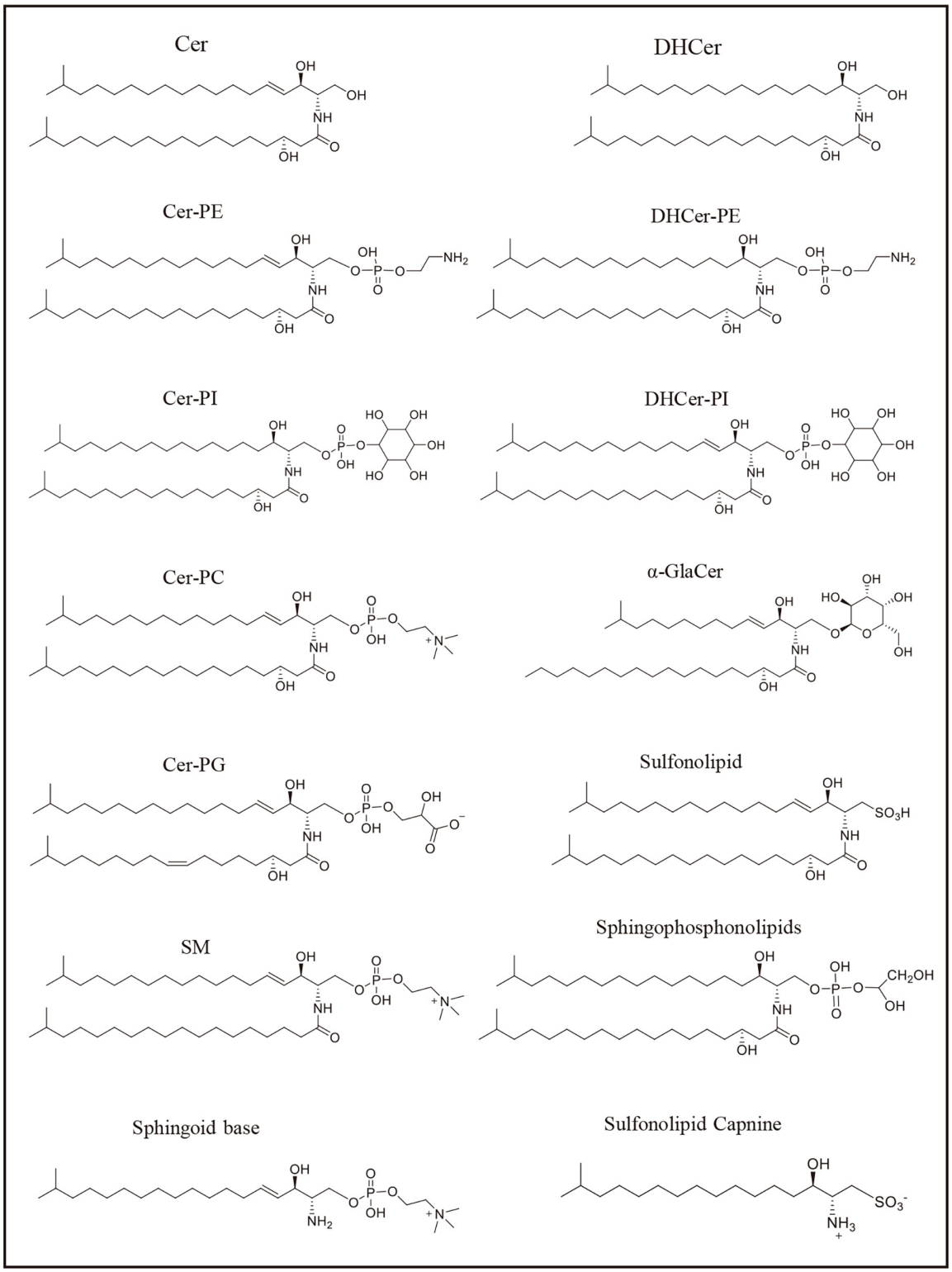

FIGURE 2
Sphingolipids represented structure mentioned in the **Table 1**. The formulas in the figure represent the representative structure, not the exact formulas.

to mice has been found to induce an anti-inflammatory effect and reduce the number of colonic NKT cells (Oh et al., 2021).

Further support for the impact of microbiome sphingolipids on host inflammatory and metabolic pathways was obtained through experiments conducted on germ-free mice colonized with sphingolipid-deficient bacteria. These mice exhibited gut inflammation and alterations in host ceramide levels (Brown et al., 2019). The interaction of *Bacteroides* sphingolipids with

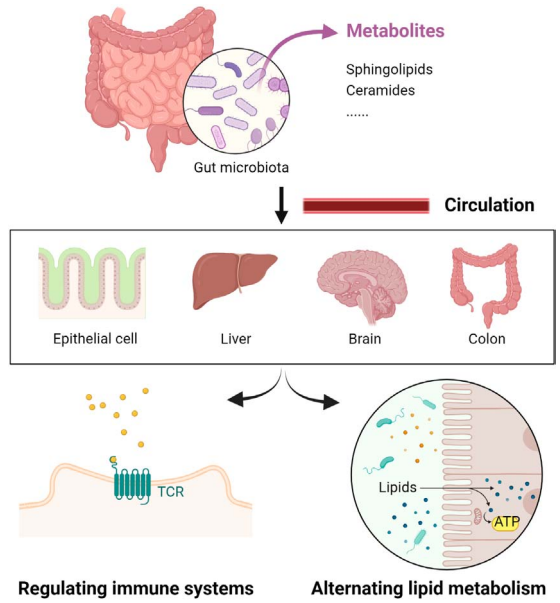

FIGURE 3
The potential link between host and microbial sphingolipids.

the innate immune system was also observed, as sphingolipids present in the outer membrane of *Bacteroides* facilitated a tolerant immune response. Studies on Bacteroidetes demonstrated that sphingolipids in outer membrane vesicles (OMVs) acted as agonists for TLR2 signaling in macrophages, thus playing a critical role in limiting inflammatory signaling (Rocha et al., 2021). Analysis of an inflammatory bowel disease (IBD) metabolomic dataset indicated reduced abundance of *Bacteroides* sphingolipids in IBD cases, along with negative correlations with inflammation and host sphingolipid production (Brown et al., 2019). These findings, coupled with the observation that bacterial sphingolipids are capable of translocating into the host (Yatsunenko et al., 2012), highlight the significance of bacterial sphingolipids in maintaining immune homeostasis.

3.2. Influence of bacterial sphingolipids on host metabolites

A recent study demonstrated that exposure of mice to *B. thetaiotaomicron*, a producer of sphingolipids, resulted in a reduction in the *de novo* production of sphingolipids by the host, while liver ceramide levels increased. Furthermore, experiments conducted on human cell cultures and mouse models confirmed that *Bacteroides* sphinganine can be taken up by host epithelial cells and incorporated into sphingolipid metabolic pathways. These findings suggest that gut-derived sphingolipids have an impact on host lipid metabolism and liver function (Johnson et al., 2020). Further investigation into the relationship between the gut and liver regarding sphingolipid signaling unveiled that the mere presence of microbiome sphingolipids was sufficient to reverse fatty liver disease in mice (Le et al., 2022). This indicates that microbiome sphingolipids not only exert local effects within the gut but also possess systemic effects by trafficking to organs beyond the intestine, thereby modulating host sphingolipid signaling.

4. Microorganisms and dietary sphingolipids

Sphingolipids are essentially widespread components found in various foods, especially in eggs and milk, that we consume on a daily basis (Andrieu-Abadie and Levade, 2002; Hussain et al., 2019), While it is believed that most sphingolipids, including sphingomyelin (SM), are not absorbed intact in the upper intestine, they do enter the distal small intestine, where they undergo degradation and give rise to bioactive lipids such as ceramide and sphingosine (Duan et al., 2007; Fischbeck et al., 2009; Norris et al., 2019). Research studies have indicated the presence of approximately 10% undegraded sphingomyelin and 30–90% ceramide in mouse feces (Norris et al., 2019). Furthermore, analysis of fecal metabolome of urban-dwelling Italians and the Hadza people of Tanzania revealed an overall abundance of sphingolipids (Turroni et al., 2016).

A study discovered that the addition of gangliosides, a type of sphingolipid, to infant formula had a significant impact on the intestinal ecosystem of preterm newborns. This supplementation led to an increase in the presence of *Bifidobacteria* and a decrease in the abundance of *Escherichia coli* (Rueda, 2007). *Bacteroidetes*, including *Bifidobacterium* strains, possess enzymes that can break down gangliosides, such as sialidases that release free sialic acid. This biochemical process contributes to the immunological functions and prevention of infections (Rueda, 2007; Lewis and Lewis, 2012). Interestingly, *Bifidobacterium* strains, despite lacking the ability to synthesize sphingolipids, can still import and utilize sphingolipids to generate DHCer (Lee et al., 2021). On the other hand, the pathogenic bacterium *Clostridium perfringens*, which can be found in stool samples, produces an enzyme called sphingomyelinase. This enzyme hydrolyzes sphingomyelin into ceramide and phosphocholine (Urbina et al., 2011; Wang et al., 2021).

In more recent findings, conclusive evidence of the microbial uptake of dietary sphinganine in the mouse gut has been established using a click-chemistry based method to trace the incorporation of bio-orthogonal dietary omega-alkynyl sphinganine into the gut microbial community. The study identified several bacterial genera, including *Bacteroides*, *Prevotella*, *Bifidobacterium*, *Lactobacillus*, and *Turicibacter*, as participants in the assimilation process of sphinganine. Of particular interest, over 99% of the bacteria involved in the assimilation were identified as *Bacteroides*, with *Prevotella* being the second most prevalent, albeit in much lesser abundance compared to *Bacteroides* (Lee et al., 2021). Based on these findings, it can be inferred that known sphingolipid-producing bacteria, primarily *Bacteroides*, play a dominant role in the metabolism of dietary sphinganine.

5. Conclusion

Among sphingolipids characterized by long-chain bases with an amine group and hydroxy groups at the structural end, bacterial sphingolipids consist of odd chain lengths due to the presence of branched alkyl chains, unlike mammalian sphingolipids. The interaction between bacterial sphingolipids and the host is a complex and dynamic process, although our understanding of

it is limited. Current knowledge suggests that bacterial sphingolipids have the ability to translocate from epithelial cells to body organs, thereby impacting the immune system and metabolism of the host. Furthermore, undegraded dietary sphingolipids in the distal small intestine are exposed to the gut microbiome, which can break them down into bioactive lipids. These findings emphasize the intricate interplay of sphingolipids within the host, underscoring the importance of bacterial sphingolipids in maintaining inner homeostasis and overall health.

However, our understanding of microbial sphingolipids in the gut is still limited, and further research is needed to fully explore their potential. Several key areas warrant investigation. Firstly, it is important to identify the specific bacteria responsible for sphingolipid production and characterize the types of sphingolipids they produce. Understanding the translocation mechanisms of these sphingolipids and their transformation processes is also essential. Additionally, investigating the functions of bacterial sphingolipids within the host, including their impact on the immune system and metabolic homeostasis, is crucial. Since the gut microbiome functions as a vital organ in the host, conducting more comprehensive studies in this field will provide valuable insights that can potentially lead to the development of therapeutics targeting sphingolipid metabolism and improving the various diseases. Advancements in these areas could have profound implications for human health and overall wellbeing.

Author contributions

XB: Writing – original draft. RY: Writing – review and editing. XT: Writing – review and editing. MC: Writing – review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by the National Key R&D Program of China (2021YFC2102500) and Science and Technology program of Inner Mongolia (2021GG0340).

Acknowledgments

We are grateful to Dr. Hafiz Arbab Sakandar for revising the article and providing invaluable advice.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 07 November 2023
DOI 10.3389/fmicb.2023.1290015

OPEN ACCESS

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RECEIVED 06 September 2023
ACCEPTED 23 October 2023
PUBLISHED 07 November 2023

CITATION
Ying Z-H, Mao C-L, Xie W and Yu C-H (2023) Postbiotics in rheumatoid arthritis: emerging mechanisms and intervention perspectives. *Front. Microbiol.* 14:1290015. doi: 10.3389/fmicb.2023.1290015

Postbiotics in rheumatoid arthritis: emerging mechanisms and intervention perspectives

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Rheumatoid arthritis (RA) is a prevalent chronic autoimmune disease that affects individuals of all age groups. Recently, the association between RA and the gut microbiome has led to the investigation of postbiotics as potential therapeutic strategies. Postbiotics refer to inactivated microbial cells, cellular components, or their metabolites that are specifically intended for the microbiota. Postbiotics not only profoundly influence the occurrence and development of RA, but they also mediate various inflammatory pathways, immune processes, and bone metabolism. Although they offer a variety of mechanisms and may even be superior to more conventional "biotics" such as probiotics and prebiotics, research on their efficacy and clinical significance in RA with disruptions to the intestinal microbiota remains limited. In this review, we provide an overview of the concept of postbiotics and summarize the current knowledge regarding postbiotics and their potential use in RA therapy. Postbiotics show potential as a viable adjunctive therapy option for RA.

KEYWORDS

intestinal microbiota, metabolites, short chain fatty acids, Treg cells, Th17 cells

1. Introduction

Rheumatoid arthritis (RA) is a long-term autoimmune disorder that causes persistent inflammation in the joints' synovial membranes. It is often accompanied by subsequent destruction of the joint cartilage and erosion of bone (Alivernini et al., 2022). RA can develop at any age, with 80% of patients developing the disease between the ages of 35 and 50. Additionally, the number of female patients is two to three times higher than that of male patients (Sparks et al., 2023). Apart from impairing motor function, RA also has systemic effects on various organs including the respiratory, renal, and cardiac systems. Additionally, it can give rise to complications such as dry eye syndrome, pericarditis, anemia, and necrotizing vasculitis, significantly impacting patients' everyday activities and professional life. Up to now, the pathogenesis of rheumatoid arthritis remains unclear. But most scholars believe that genetics and environmental factors may be the main triggers for RA (Desai et al., 2022; Guo et al., 2023; Lin et al., 2023).

Intestinal dysbiosis is one of the important causes of RA. Once the balance between beneficial bacteria, such as Bifidobacterium, and harmful bacteria, such as *Prevotella copri*, is disturbed, it can lead to an imbalanced ratio of intestinal microbiota and the proliferation of

microorganisms related to RA. This imbalance can trigger inflammation and ultimately accelerate the progression of RA (Opoku et al., 2022; Romero-Figueroa et al., 2023). Probiotic intervention and fecal microbial transplantation (FMT) can restore the balance of the intestinal microbiota, improve intestinal dysbiosis, and prevent the occurrence and development of RA, and even achieve a cure. The connection with the gut microbiome has led to the use of oral probiotics or FMT as therapeutic strategies for RA (Wang et al., 2023; Zhao et al., 2023). However, as viable microorganisms, those strategies have some potential biosafety risks. Postbiotics are being used as novel food supplements and provide safer and higher quality products for controlling the microbial population compared to probiotics. Oral administration of postbiotics can enhance immunity, regulate intestinal microbiota, improve growth performance, and reduce the occurrence of diarrhea (Yeom et al., 2021; Jung et al., 2023). Especially, the metabolites derived from intestinal microbiota improved the intestinal barrier integrity and mediated the balance of Treg/Th17 cell ratio, resulting in the decreased release of serum IL-17 and expedite bone repair (Chen et al., 2021; Hanlon et al., 2022). Therefore, non-viable postbiotics have recently been considered as a better alternative. This paper summarizes the different types of postbiotics and their potential benefits in improving health and preventing diseases. It specifically focuses on the biological functions of postbiotics in preventing RA and highlights recent advancements in their clinical applications.

2. Roles of the gut dysbiosis on RA occurrence and development

The roles of intestinal microbiota in the occurrence of RA are mainly manifested in mucosal immunity and are associated with T cell differentiation, including regulatory T cells (Treg) and helper T (Th) cells. During intestinal dysbiosis, intestinal T cells undergo auto-activation, which can increase susceptibility to arthritis. Additionally, the metabolites produced by the intestinal microbiota can indirectly promote the development of RA (Figure 1).

2.1. Gut microbiota affects the Treg/Th17 balance

Gut microbiota is critical to affect the balance between Th17 cells and Treg cells. IL-17, which is secreted by Th17 cells, promotes the development of RA, while IL-10 and TGF-β1, which are secreted by Treg cells, control the progression of RA. Increasingly, studies have shown that the gut microbiota can influence the immune response to RA by regulating the homeostasis of Th17/Treg cells (Kotschenreuther et al., 2022; Dagar et al., 2023). In particular, the differentiation and expansion of Treg/Th17 cells are independently controlled by specific members of anaerobic bacteria. *P. copri* influences the ratio of Th1 to Th17 cells by mediating T helper cell differentiation, while *Bacteroides fragilis* enhances the anti-inflammatory effects of Treg cells through the expression of polysaccharide A, which interacts with TLR2 (Guerreiro et al., 2018; Alpizar-Rodriguez et al., 2019; Kitamura et al., 2021).

Compared to the germ-free mice, collagen-induced arthritis (CIA) mice showed a significant increase in serum IL-17 levels, as well as elevated levels of splenic $CD8^+$ T cells and Th17 cells. Conversely, the levels of dendritic cells, B cells, and Treg cells were significantly reduced in CIA mice (Liu et al., 2016). Under germ-free conditions, the symptoms of the K/BxN mouse with autoimmune arthritis exhibited remarkable improvement, which was accompanied by notable reductions in serum autoantibody levels, splenic autoantibody-secreting cell, germinal centers, and splenic Th17 cell populations. Once a specific type of intestinal microbiota, such as segmented filamentous bacteria, is introduced, it becomes possible to reintroduce Th17 cells into the lamina propria of the small intestine. This, in turn, leads to the rapid production of antibodies and the development of arthritis (Wu et al., 2010). In addition, SKG mice harboring microbiota from patients with RA had increased numbers of Th17 cells in their intestines and developed severe arthritis when treated with yeast zymosan. T cells derived from naive SKG mice were co-cultured with *P. copri*-stimulated dendritic cells, thereby promoting the production of IL-17 in response to the arthritis-associated autoantigen RPL23A, leading to the rapid induction of arthritis (Maeda et al., 2016).

2.2. Gut microbiota affects the permeability of intestinal mucosa

The presence of intestinal dysbiosis can lead to an increase in the permeability of the intestinal mucosa, allowing conditionally pathogenic bacteria to translocate. This can result in heightened autoimmune inflammation and an increased risk of rheumatoid arthritis (Matei et al., 2021). The analysis of 16S ribosomal DNA sequencing data revealed a robust association between the prevalence of *Collinsella* and elevated concentrations of alpha-aminoadipic acid, asparagine, and IL-17A. More importantly, *Collinsella aerofaciens* enhances intestinal mucosal permeability and increases arthritis severity in HLA-DQ8 mice susceptible to collagen-induced arthritis. This effect is accomplished through the downregulation of tight junction proteins ZO-1 and occludin, as well as the upregulation of IL-17-mediated network cytokines, such as IL-1LA, CXCL1, CXCL5, and NF-κB1, in CACO-2 cells (Chen et al., 2016).

2.3. Gut microbiota affects the production of sex hormones

In the clinic, there are more women than men with RA, which may be attributed to the influence of intestinal microbiota on the regulation of sex hormones. Estradiol is dose-dependent and generally stimulates the production of pro-inflammatory factors, such as TNF-α and IL-1β, at low doses. When estrogen levels are elevated, such as during pregnancy, it can produce anti-inflammatory effects by inhibiting the signaling of pro-inflammatory factors, inducing the expression of anti-inflammatory factors (resulting in a Th2 phenotypic shift), and activating Tregs cells, respectively. Progesterone may reduce the severity of RA during pregnancy by promoting the production of Treg cells and inhibiting the differentiation of Th17 cells (Rizzetto et al., 2018; Brettle et al., 2022). Previous studies have demonstrated that *Clostridium difficile* encodes hydroxysteroid dehydrogenase and certain enzymes involved in the conversion of glucocorticoids to androgens. This can have an impact on the metabolism and activity of sex hormones, resulting in an immunomodulatory effect (Schmidt et al., 2020).

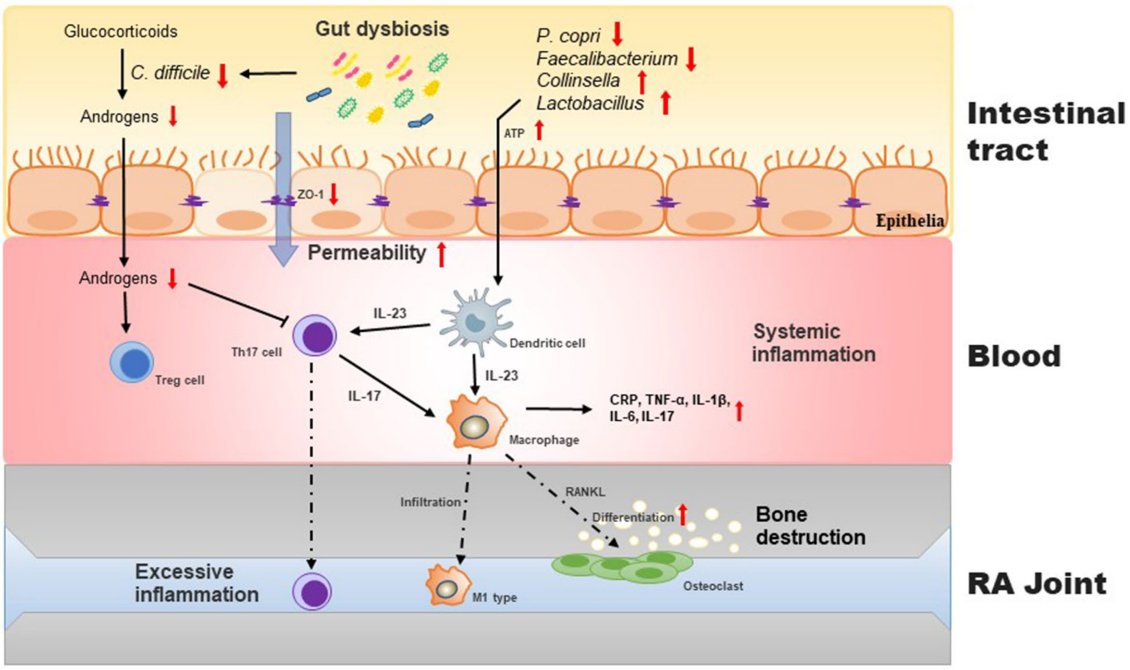

FIGURE 1
Intestinal barrier disrupted by gut dysbiosis facilitates induces imbalance of Th17/Treg cells and exacerbates inflammatory process in the pathogenesis of RA. During gut dysbiosis, the increased APT levels in gut stimulates the production of IL-23 in dendritic cells, which in turn promotes the differentiation of macrophage to osteoclast, resulting the bone destruction. Moreover, gut dysbiosis increases the intestinal permeability and subsequently aggravates host inflammatory responses. *C. difficile* regualtes the conversion of glucocorticoids to androgens that promote the proliferation of Treg cells.

3. Application of postbiotics in RA treatment

Probiotics refer to non-pathogenic microorganisms that are widely recognized for their ability to provide health benefits when consumed in adequate amounts by the host. As the scope and function of probiotics have expanded and deepened, researchers have discovered that not only can live bacteria perform probiotic functions, but some "non-live" bacterial components also exhibit significant health-promoting effects. These components include inactivated bacterial cells, components released by bacterial lysis after death, and bacterial metabolites. The bacterial components can include lipophosphatidic acid, cell surface proteins, and peptidoglycan, while metabolites include enzymes, peptides, short-chain fatty acids (SCFAs), and polysaccharides (e.g., extracellular polysaccharides). Depending on the source of the components, both the cell-free supernatant and the metabolites obtained from bacterial fermentation are usually referred to parabiotics. In contrast, the inactivated bacterial cells (lysed or unlysed), including cell wall fragments, bacteriophage cells, and cellular components, are referred to paraprobiotics. These inactivated bacteria and metabolites belong to the category of "postbiotics." Although the mechanisms by which postbiotics are beneficial to human health have not yet been fully elucidated, it has been proven that postbiotics have various beneficial functions. These functions include protecting the epithelial barrier and possessing antitumor, antioxidant, and immunomodulatory properties. When consumed in sufficient quantities by the host, postbiotics can have a positive impact on microbiota homeostasis and/or host metabolic and signaling pathways, thereby influencing specific physiological, immunological, and metabolic functions (Figure 2).

Recently, postbiotic preparations can be obtained through various methods, including heat treatment, enzyme treatment, solvent extraction, exposure to γ or UV light, ultrasound, etc. In most cases, heat treatment is the common method used to inactivate probiotic organisms. Heat-inactivated probiotic cells, cell-free supernatants, and other active ingredients can produce beneficial effects, such as immunomodulation, balancing the intestinal microbiota, and regulating physiological functions. At the clinical level, products containing inactivated bacteria have been utilized to treat various gastrointestinal disorders, including bloating, diarrhea, and infantile colic. Inactivated bacteria have also proven to be useful in managing skin or respiratory allergies. Therefore, postbiotics have become a new means of improving intestinal microecology in the past 3 years.

3.1. Cell-free supernatant (CFS)

After centrifugation and filtration, CFS containing active metabolites can be obtained from microbial cultures. The supernatant of lactic acid bacteria usually includes low molecular weight compounds (e.g., hydrogen peroxide, organic acids, carbon dioxide, etc.) and high molecular weight compounds (such as bacteriocins, fine-like bacteriocins, and bacteriocin-like substances). *Lactobacillus acidophilus* and *Lactobacillus casei* supernatants have the ability to penetrate the intestinal mucosal barrier, decrease the secretion of TNF-α, and increase the production of anti-inflammatory IL-10,

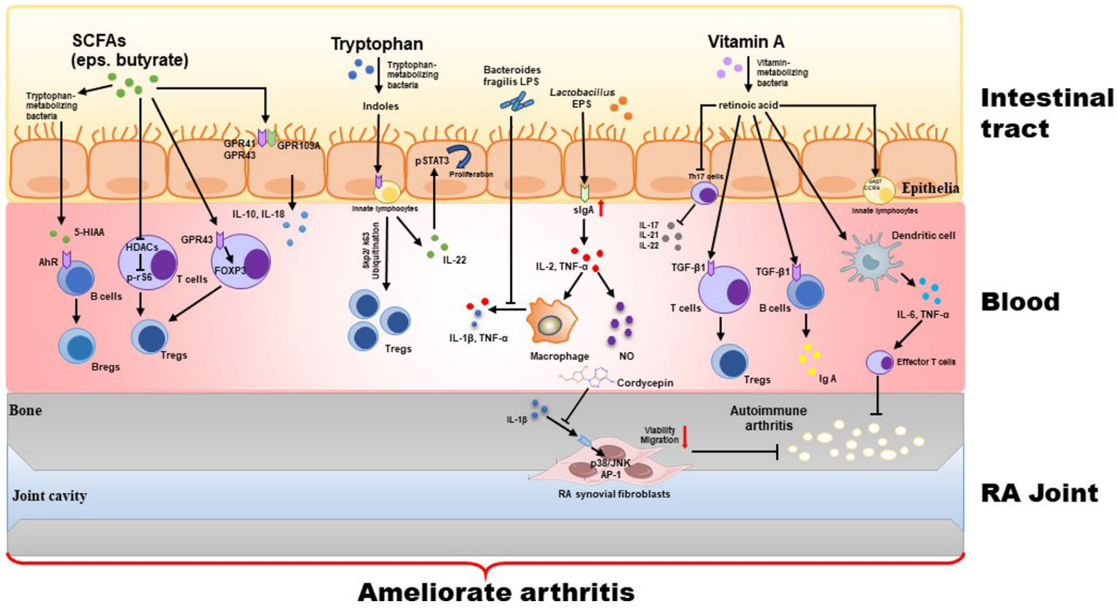

FIGURE 2
Treatment with postbiotics (including inactivated bacterial cells, cellular components, and their metabolites) strengthens intestinal tight junction via regulation of GPRs and HDACs signaling, and inhibits inflammatory response via mediating the Th17/Treg balance.

indicating its anti-inflammatory and antioxidant properties. *Bifidobacterium shorteri* supernatants can reduce the release of various inflammatory mediators in dendritic cells by activating the Toll-like receptor, thus safeguarding the immune system against pathogenic bacteria. Secreted protein HM0539 from *Lactobacillus rhamnosus* GG supernatant enhances intestinal mucin expression and prevents TNF-α-induced intestinal mucosal barrier damage.

3.2. SCFAs

SCFAs, the products of dietary fiber fermentation by intestinal microbiota, include acetic, propionic, and butyric acids, which are the most well-known components of postbiotics. Acetate and propionate are primarily used as substrates for mitochondrial oxidation in the liver and muscle, while butyrate is mainly utilized in the colon to provide energy to intestinal epithelial cells (Palmnäs-Bédard et al., 2022; Tan et al., 2023).

It is well known that autoimmune diseases, including RA, are often accompanied by intestinal barrier dysfunction. Zonulin is a peptide that regulates intestinal tight junctions, and is known to be closely linked with an impaired intestinal barrier, dysbiosis (an imbalance in the gut microbiota), and inflammation in both autoimmune mice and humans. Excitedly, the oral administration with zonulin antagonist larazotide acetate or butyrate, a specific intestinal microbiota metabolite, has been found to enhance intestinal barrier integrity. This restoration of the intestinal barrier has shown potential in reducing the onset of arthritis, indicating a protective effect of intestinal microbiota metabolites in the early stages of arthritis development (Tajik et al., 2020; Hecquet et al., 2023).

Compared to healthy controls, supplementation with microbial-derived butyrate reduces the severity of arthritis in mice. The levels of stool propionate and butyrate were significantly lower in RA patients and arthritic mice. Moreover, supplementation with butyrate has been observed to reduce the severity of RA through a Breg cell-dependent mechanism. This effect is achieved by increasing the levels of a serotonin-derived metabolite called 5-Hydroxyindole-3-acetic acid (5-HIAA), which in turn activates the aryl-hydrocarbon receptor (AhR) and then skews the B cell population. However, it was observed that mice deficient in B cells did not experience any benefits, highlighting the distinct role of B cells in the suppression of arthritis mediated by butyrate. Thus, butyrate could be used as a viable candidate for the treatment of systemic autoimmune diseases (Rosser et al., 2020). Furthermore, the regulation of butyrate mediated the differentiation of $CD4^+$ T cells into Treg cells. These Treg cells can generate the anti-inflammatory cytokine IL-10, which in turn affects the function of Th17 cells and inhibits the expression of other inflammatory cytokines, and ultimately alleviated RA-related symptoms of the arthritic mice. However, it was also found that butyrate could not inhibit the expression of IFN-γ, although it could selectively inhibit IL-17A. Propionate ameliorated CIA by promoting the proliferation of Treg cells and elevating IL-10 levels both (Hui et al., 2019). Additionally, the consumption of a high-fiber diet enriched with resistant starch led to an increase in serum levels of intestinal acetate, propionate, and isobutyrate (Jiang et al., 2022).

In addition, alterations in bone metabolism are another significantly characteristic pathology in the progression of RA. Butyrate produced by *Firmicutes* bacteria, has been found to modulate the differentiation of Treg cells and promote bone formation in experimental animals. Clinical trial also demonstrated that purine metabolism is significantly reduced in RA patients when the intestinal microbiota is dominated by *P. copri*, indicating that the high abundance of *P. copri* in gut may affect the therapeutic efficacy of methotrexate to RA patients (Scher et al., 2013). Similarly, the abundant of *Prevotella copri* was closely related to the severity of the new-onset RA patients. The mice colonized with *P. copri* can increase

the sensitivity to chemically induced colitis. However, there were not any significant changes observed in osteoblasts, suggesting the inhibition of SCFAs on bone loss of arthritic mice. Moreover, reduced bone erosion markers and improved Th1/Th17 ratios were also observed in individuals who followed a high-fiber diet for 4 weeks, leading to improved outcomes associated with RA. This intervention induces up-regulation of insulin-like growth factor 1 (IGF-1), which stimulates osteoblast proliferation and bone remodeling (Häger et al., 2019). Additionally, SCFAs produced by microbial catabolism have been shown to have the same effects in mice (Dürholz et al., 2020; Rosser et al., 2020).

Mechanically, SCFAs can activate immunity-related pathways by binding to G protein-coupled receptors (GPCRs) including GPR41, GPR43 and GPR109A. Acetic, propionic, and butyric acids primarily activate GPR41 and GPR43, while butyric acid also activates GPR109A (D'Souza et al., 2017). Butyrate can bind to GPR109A on the intestinal epithelial cells (IEC), macrophages, and dendritic cells, causing the secretion of IL-10. This, in turn, induces the differentiation of Treg cells. SCFAs can stimulate inflammation by binding to GPR43 on the vesicles of IEC, thereby activating downstream of IL-18, which is involved in repairing and maintaining the integrity of the intestinal barrier (Singh et al., 2014; Macia et al., 2015). Furthoremore, SCFAs can enhance the ability of intestinal T cells to express the transcription factor Foxp3 by activating GPR43 on T cells, thereby regulating the development and differentiation of Treg cells (Balakrishnan et al., 2021; Duan et al., 2023). Notably, SCFAs also can act as the inhibitors of HDACs and subsequently accelerate the differentiation and function of T cells to regulate the immune tolerance of the body. Studies have shown that butyric acid, as a violent inhibitor of HDACs, can increase the acetylation of p70S6K and phosphorylation of rS6, thereby expediting T-cell differentiation into effector and regulatory cells via activation of mTOR-S6K pathway (Park et al., 2015). It has been shown than SCFAs can promote IL-22 production by splenic $CD4^+$ T cells and innate lymphocytes in wild-type C57BL/6 J mice through the inhibition of HDACs and binding to GPR41 (Li et al., 2018; Yang et al., 2020). Notably, those inhibitory effects of SCFAs on HDAC activity is not limited to immune cells in the intestine, but also extends to other tissues and organs through the circulation. The inhibition of HDACs leads to the inactivation of NF-κB, which subsequently reduces the release of inflammatory cytokines in RA patients and arthritic mice (Kim et al., 2018; Mao et al., 2023; Vijaykrishnaraj et al., 2023).

3.3. Exopolysaccharides (EPS)

Microorganisms can secrete extracellular polysaccharides (also called as exopolysaccharides) to the outside of their cells during growth. Some EPS can act as virulence factors for pathogens or help pathogens to adhere and colonize the gut; on the other hand, EPS can also produce beneficial effects on the host (Liu et al., 2019; Dahiya and Nigam, 2022). In addition, EPS have antioxidant, anti-infective and anticancer effects (Kawanabe-Matsuda et al., 2022; Sharma et al., 2023; Srinivash et al., 2023; Zeinivand et al., 2023).

As previously reported, EPS isolated from *Lactobacillus* in yogurt can stimulate NK cell activation and regulate immune response. It also increased phagocytosis of macrophages, stimulated NO secretion, and resisted immunosuppression in cyclophosphamide-exposed mice by promoting the production of sIgA, IL-2 and TNF-α in the intestinal mucosae (Makino et al., 2016). Ebosin, a novel EPS isolated from *Lactobacillus rhamnosus* remarkably inhibited IL-1β-mediated MAPK and NF-κB pathways in rat fibroblast-like synoviocytes and then reduced arthritogenic autoantibodies in collagen+lipopolysaccharide (LPS)-induced arthritic mice (Nowak et al., 2012; Zhang et al., 2016, 2022).

3.4. Tryptophan metabolites

Tryptophan is an essential amino acid that cannot be synthesized by the animal body itself. Food is the primary source of intake. Most intestinal microbiota in animals, except for viruses and archaea, can metabolize tryptophan through various pathways to produce a range of biologically active molecules, including indole, tryptamine, indole ethanol, indole propionic acid, indole lactic acid, indole acetic acid, and indole acrylic acid which are primarily produced by specific strains as summarized in Supplementary Table S1.

AhR is a sensor of microbial metabolites that plays a role in the development of innate lymphocytes and intraepithelial lymphocytes, thereby exerting antimicrobial and anti-inflammatory effects (Stone and Williams, 2023). Many microbial tryptophan metabolites could activate the AhR. Indole, 2-indolone, indoleacetic acid, and kynurenine can activate the AhR, thereby mediating a variety of immune responses (Li, 2023). Indole-3-aldehyde, a metabolite of *Lactobacillus Royce* D8, activates the AhR, promotes the secretion of IL-22 by innate lymphocytes, and upregulates the expression of p-STAT3 in IEs that in turn promotes the proliferation of intestinal epithelial cells and facilitates the repair of damaged intestinal mucosa. Activation of AhR by tryptophan metabolites in the intestine enhances the function of group 3 innate lymphoid cells (Hou et al., 2018), and promotes Treg cell generation via the Skp2/K63-ubiquitination pathway to prevent and treat RA, indicating the potential of AhR agonists and tryptophan metabolites (Zhang et al., 2023).

3.5. Vitamins and their metabolites

In addition to the vitamins obtained from foods, animals can also metabolize and synthesize some vitamins (mainly vitamin K2 and B family) by intestinal microbiota as summarized in Table 1 (Yoshii et al., 2019). It is known that vitamin B1 can be synthesized by *Bifidobacterium fragilis*, *Prevotella*, and *Bifidobacterium*, vitamin B2 by *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus fragilis*, vitamin B9 by *Bifidobacterium bifidum* and *Bifidobacterium longum*, while vitamin B12 by *Propionibacterium feldsponenum*, *Salmonella*, and *Lactobacillus roehlis* (Barone et al., 2022). Vitamin K2 is endogenously synthesized by intestinal bacteria, such as *Viridans Streptococci*, *B. subtilis natto*, *L. lactis*, *L. reuteri*, *Pichia pastoris*, and *Flavobacterium* sp.

After being absorbed and metabolized by the body, retinoic acid, the main metabolites of vitamin A, promotes the production of Treg cells by $CD4^+$ T cells and immunoglobulin A by B cells via activation of TGF-β1 pathway (Qiu et al., 2023; Shri Preethi et al., 2023). A deficiency in retinoic acid inhibits the differentiation of Th17 cells in the lamina propria of the small intestine in mice and reduces the secretion of IL-17, IL-21, and IL-22. In addition, retinoic acid induces

TABLE 1 The origin and function of vitamin B family and vitamin K.

Vitamins	Metabolizing bacteria	Function	References
Vitamin B1	*Bifidobacterium fragilis*	Promoting immune defense of the intestinal mucosa; Promoting macrophage proliferation and B cell differentiation	Kunisawa et al. (2015), Sabui et al. (2022), and Stambuk et al. (2009)
	Prevotella copri		
	Clostridium difficile		
	Lactobacillus casei		
	L. curvatus, *L. plantarum*		
	Ruminococcus lactaris		
	B. bifidum, *B. infantis*		
	Fusobacterium varium		
Vitamin B2 and its intermediate 6-hydroxy methyl-8-d-ribityllumazine	*L. plantarum*, *L. fermentum*	Promoting differentiation of naïve B cells; Inducing IL-17 and IFN-γ production by MAIT cells	Vogl et al. (2007) and Birkenmeier et al., 2015
	B. fragilis		
	P. copri		
	C. difficile		
	R. lactaris		
Vitamin B6	*B. fragilis*, *B. longum*	Maintaining Th1/Th2 balance	Lee et al. (2023) and Wan et al. (2022)
	P. copri		
	Collinsella aerofaciens		
	Helicobacter pylori		
Vitamin B9 and its metabolite 6-FP	*B. fragilis*	Promoting Treg cell differentiation via activating FR4; Inhibition of MAIT cells	Kunisawa et al. (2012) and Wan et al. (2022)
	P. copri		
	C. difficile		
	L. plantarum, *L. delbrueckii*, *L. reuteri*		
	Streptococcus thermophilus		
	Fusobacterium varium		
	S. enterica		
Vitamin B12	*Bacteroides fragilis*	Activation of $CD8^+$ cells and NK-T cells	Barone et al. (2022), Degnan et al. (2014) and Tanaka et al. (2017)
	P. copri		
	C. difficile		
	Faecalibacterium prausnitzii		
	R. lactaris		
	L. plantarum, *L. cortniformis*, *L. reuteri*		
	B. animalis, *B. infantis*, *B. longum*		
	F. varium		
Vitamin K2	*Viridans Streptococci*	Inhibit osteoclastogenesis via Osteocalcin, Gla, nuclear steroid and xenobiotic receptor	Yuan et al. (2020) and Mladěnka et al. (2022)
	B. subtilis natto		
	L. lactis, *L. reuteri*		
	Pichia pastoris		
	Flavobacterium sp.		

innate lymphocytes to express α4β7 and CCR9 molecules, which helps these cells home to the intestine and promotes a balance of Th17/Treg cells in the intestinal-associated lymphoid tissue (Manhas et al., 2022). When the body is in an inflammatory state, retinoic acid can induce dendritic cells to produce the pro-inflammatory cytokines IL-6 and TNF-α. This, in turn, promotes the differentiation of effector T cells and helps protect the intestinal mucosal barrier function against collagen-induced autoimmune arthritis (McBride et al., 2023).

3.6. Others

It has been reported that RA patients had remarkably lower levels of gut *Bacteroides* species. Treatment with *Bacteroides fragilis* LPS significantly suppressed TNF-α and IL-1β production in *Escherichia coli* LPS-exposed macrophages and improved the development of CIA in mice (Kitamura et al., 2021).

FIGURE 3
Advantages of postbiotic.

Cordycepin (3′-deoxyadenosine), one of the major bioactive compounds in *Cordyceps militaris* and fermented *Hirsutella sinensis* mycelium extract, has been demonstrated to exert anticancer and anti-inflammatory activities as well as immunomodulation (Alhakamy and Fahmy, 2022; Liu et al., 2022; Cheng et al., 2023; Priya et al., 2023). Cordycepin significantly inhibited the viability and migration of RA synovial fibroblasts via blocking IL-1β-induced p38/JNK and AP-1 activation, thereby preventing inflammation of RA (Noh et al., 2009).

4. Dosage and safety in use

Due to the complex composition and significant variations in composition produced by different strains, there are challenges in standardizing the dosage of postbiotics in current production practices, as well as in relevant formulation standards. Although national and international standards on the recommended intake of probiotics are relatively mature, no standards have been issued yet to regulate or recommend the intake of the postbiotics.

According to the clinical trials, studies focusing on parabiotic bacteria have shown that the number of dead bacteria corresponds to the number of live bacteria. However, due to the variability of metabolites, studies focusing on postbiotic metabolites have not yet determined specific dosages. Whether the dosage of postbiotics can be recommended based on the dosage of probiotics during practical application requires further observation.

From an economic standpoint, the advantages of parabiotics over probiotics include an extended shelf life, easier storage and transportation, and a decreased requirement for low temperatures. The possibility of utilizing a repetitive production process and implementing more precise dosing controls are additional advantages of postbiotics in comparison to probiotics. The undeniable advantage of postbiotics is that it circumvents the issue of the organism potentially acquiring antibiotic resistance genes and virulence factors, which can accumulate in the body when using probiotics. Postbiotics eliminate the need for exposure to live microorganisms, which is particularly crucial for children with underdeveloped immune systems and compromised gut barriers. Compared to paraprobiotics, the composition of parabiotic components is easier to determine, the dosage and efficacy are more stable, and there is relatively less variation among different probiotic sources (Figure 3).

5. Future challenges and prospects

Postbiotics, derived from intestinal microorganisms, can serve various functions in the intestinal tract and throughout the body to treat and prevent a variety of autoimmune diseases. Recently, there has been a growing focus on studying the effects of probiotics and their derivatives on bone metabolism and function. Many studies have shown the potential of postbiotics in the treatment of osteoarthritis and RA. Therefore, the current clinical reliance on antirheumatic drugs to manage the condition of RA could be changed by establishing an evidence-based link between RA and the intestinal microbiome. The clinical application of the new strategy "targeting intestinal microbiota" can not only alleviate the symptoms of RA, but also offer personalized treatment and reduce the economic burden on patients and society.

As a burgeoning field, there are still numerous challenges that need to be addressed. Firstly, the definition and scope of postbiotics still need to be refined and expanded. The specific bacterium-derived small RNA, polypeptides, and exosome-like nanoparticles also exert noticeable bioactivities. Secondly, most of the products are still in the experimental stage or are used as food supplements. The specific mechanism of postbiotics against arthritis induced by various genetic or environmental factors has not been fully elucidated. Only after clarifying the characteristic active ingredients that contribute to the effectiveness of postbiotics can qualitative and quantitative analytical methods be developed to control the quality of postbiotics. Finally, SCFAs can be obtained by fermenting plant polysaccharides with intestinal microorganisms. CFS are obtained through centrifugal filtration, and bacterial lysates are prepared through chemical or mechanical degradation. Therefore, the production of different postbiotics involves various methods, which makes it challenging to

establish standardized criteria for their industrial production. However, in any case, the potential of postbiotics in autoimmune diseases and other fields still deserves attention and anticipation.

Author contributions

Z-HY: Funding acquisition, Project administration, Writing – original draft. C-LM: Conceptualization, Writing – original draft. WX: Visualization, Writing – review & editing. C-HY: Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Zhejiang Provincial Science and Technology Project (No.2015C33164), Zhejiang Provincial Medicine and Technology Project (No. 2020KY527), and Zhejiang Key Laboratory of Traditional Chinese Medicine for Arthritis Diagnosis and Research (No.202206).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1290015/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 23 November 2023
DOI 10.3389/fmicb.2023.1273861

OPEN ACCESS

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RECEIVED 07 August 2023
ACCEPTED 08 November 2023
PUBLISHED 23 November 2023

CITATION
Motei DE, Beteri B, Hepsomali P, Tzortzis G, Vulevic J and Costabile A (2023) Supplementation with postbiotic from *Bifidobacterium Breve* BB091109 improves inflammatory status and endocrine function in healthy females: a randomized, double-blind, placebo-controlled, parallel-groups study. *Front. Microbiol.* 14:1273861. doi: 10.3389/fmicb.2023.1273861

Supplementation with postbiotic from *Bifidobacterium Breve* BB091109 improves inflammatory status and endocrine function in healthy females: a randomized, double-blind, placebo-controlled, parallel-groups study

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This study evaluated the effects of dietary supplementation with a postbiotic extract of *Bifidobacterium breve* BB091109 on pro-inflammatory cytokines levels and markers of endocrine function. A prospective, double-blind, placebo-controlled, randomized, single-centered, parallel study was conducted on a group of 40–55-year-old females. The study included 30 healthy females, divided into two groups: a supplement ($n = 20$) and a placebo ($n = 10$) groups. Blood and saliva samples were collected at baseline (wk0), after 4 weeks (wk 4) and 12 weeks (12wk) of daily supplementation (500 mg), and 4 weeks (wk 16) after termination of supplementation. The levels of fasting CRP, IL-6, IL-10, TNF-α, IFN-γ, DHEA, estradiol, estriol, progesterone, cortisol and human growth hormone were analysed. The results revealed a significant effect of the 90-day supplementation with *B. breve* postbiotic extract on changes in CRP, IL-6 levels, DHEA, estradiol and estriol. In conclusion, the supplementation with the *B. breve* postbiotic extract improved endocrine function in females over 40 years old and induced protective changes in inflammatory markers. These findings highlight the potential health benefits of this supplementation in promoting hormonal balance and reducing inflammation in this population.

KEYWORDS

chronic inflammation, sex hormones, postbiotics, bifidobacteria, intestinal epithelial barrier function, cell-wall polysaccharides, microbial associated molecular patterns

1 Introduction

Chronic inflammation has become a significant contributor to various physical and mental health problems, playing a substantial role in global illness and death rates (Netea et al., 2017; Bennett et al., 2018). Normally, inflammation is a temporary reaction to threats that resolves once the threat is eliminated. However, certain factors such as social, psychological, environmental, and biological influences can hinder the resolution of acute inflammation,

leading to persistent, non-infectious systemic chronic inflammation (Calder et al., 2013; Straub, 2017). This transition from acute to chronic inflammation can disrupt immune tolerance, impacting tissues, organs, and cellular processes, accelerating the aging process, and increasing the risk of various non-communicable diseases (Kotas and Medzhitov, 2015; Furman et al., 2017; Gisterå and Hansson, 2017). Systemic chronic inflammation can also impair immune function, making individuals more susceptible to infections, tumors, and reduced vaccine responses (Taniguchi and Karin, 2018). Depending on the duration and extent of inflammation, metabolic and neuroendocrine changes occur to conserve energy and support immune activity (Kotas and Medzhitov, 2015; Straub and Schradin, 2016). These changes, known as sickness behaviors, include symptoms like fatigue, disrupted sleep, decreased appetite, and social withdrawal (Straub et al., 2010). In addition to genetic factors and exposure to environmental triggers, two further components have been identified as contributors to chronic inflammatory disorders: an inappropriate increase in intestinal permeability, which may be influenced by the composition of the gut microbiota, and an overactive immune system that disrupts the balance between tolerance and immune responses (Bjarnason et al., 1995; Leclercq et al., 2014; Gonzalez-Gonzalez et al., 2018). The gut microbiota represents a central ecosystem that changes from one site to another (Geuking et al., 2011; Korpela and de Vos, 2018). Its composition is individualized and dynamic, and it depends on age, the influence of diet, environmental conditions of the intestine, lifestyle, and other host related factors (Derrien and van Hylckama Vlieg, 2015). The microbiota contains all three domains of life (i.e., fungi, yeast, protozoa), but consists predominately of anaerobic microorganisms, including thousands of bacterial species and millions of genes (Zoetendal et al., 2008) that play an important role in homeostatic mechanisms leading to the regulation of numerous physiological activities both in health and disease (de Vos et al., 2022). In addition to aiding in the digestion of foods to produce favorable by-products, the gut microbiota also has an important role in the development and function of innate and adaptive immunity (Chen et al., 2017) by essentially establishing a "tolerant" phenotype (Hooper et al., 2012). Reciprocally, the host immune system plays an important role in shaping the gut microbiota (Chen et al., 2017). Due to their proximity, it is essential that the gut microbiota and intestinal immune system tolerate one another in order to enable the continuation of a healthy host-microbe coexistence that maintains intestinal barrier function (Bischoff, 2011). Any disruption in the balance between the gut microbiome and the mucosal immune system will impair the intestinal barrier function and increase the risk of developing various local diseases, immune-mediated disorders, and extraintestinal diseases, characterized by chronic inflammation. Numerous bacterial products regulate intestinal barrier function by activating Toll-like receptors (TLR) and nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) pathways (Marques and Boneca, 2011; Mu et al., 2015). Microbial associated molecular patterns (MAMPs), like lipopolysaccharide (LPS), flagellin, peptidoglycans, exopolysaccharides, formyl peptides and unique nucleic acid structures, are detected by transmembrane and cytoplasmic pattern recognition receptors (PRRs), initiating conserved signaling cascades that drive stimulatory or regulatory effector responses crucial for host defense (Ali et al., 2020). The healthy intestinal barrier maintains a hypo-reactivity to those MAMPs, while in the underlying immune-stromal-rich layer of lamina propria, the innate immune cells exhibit greater reactivity to commensal and pathogenic microbial ligands (Iwasaki and Kelsall, 2000). Dysbiosis of the gastrointestinal microbiota, characterized by increased LPS concentration, has recently been highlighted as one of the major contributors to intestinal mucosal permeability, induction of innate defenses, and thus an environmental risk factor capable of triggering chronic inflammation (Dorrestein et al., 2014). LPS binding to TLR4 in intestinal cells initiates an inflammatory process that ultimately downregulates the levels of tight junction proteins and favors the translocation of LPS into the systemic circulation, inducing thus local and systemic inflammatory processes that can further affect distant organs, including the lung, liver, brain and skin (Ghosh et al., 2020). The crucial role for the LPS–TLR4 pathway in modulating intestinal barrier integrity has been demonstrated in several studies in experimental models using TLR4 inhibitors or Tlr4-knockout animals (Fort et al., 2005; Peterson et al., 2010; Wang et al., 2015). The administration of the main probiotic genera *Bifidobacterium* and *Lactobacillus* have been reported to lead to an improvement in several factors related to intestinal barrier integrity and inflammation (Zheng et al., 2023). The gut *Bifidobacterium* population is resident within the GI tract throughout our whole lifespan (Asnicar et al., 2017). As one of the first inhabitants of neonatal intestines, bifidobacteria play pivotal roles in the modulation of mucosal physiology and fine-tuning of the host innate and adaptive immune development (Lin et al., 2022) and are associated with immune well-being. *Bifidobacterium* spp. have been reported to suppress levels of proinflammatory cytokines, increase intestinal absorption of electrolytes, repair intestinal permeability, inactivate carcinogens, induce apoptosis, improve T-cell proliferation and cytotoxicity, and modulate natural killer (NK) cell and dendritic cell (DC) interactions, in a strain specific manner (Lee et al., 2017; Wilkins and Sequoia, 2017).

Research to date (Ruiz et al., 2017) has shown that bifidobacteria play a critical role in promoting host immune health by modulating intestinal epithelial and immune cells through the release of various MAMPs, including capsular polysaccharides (CPS) and exopolysaccharides (EPS), during their growth. Those MAMPs crosstalk with PRRs present on the membrane of epithelial/immune cells to configure the cellular structure of the intestinal epithelial barrier (Ruiz et al., 2017). Such polysaccharides have been reported to play an immunomodulatory role (Fanning et al., 2012). EPS from *Bifidobacterium longum* BCRC 14634 was able to stimulate macrophages J774A to produce increased level of anti- inflammatory cytokine IL-10 and to lower levels of pro-inflammatory TNF-α after LPS challenge (Wu et al., 2010), while the EPS-producing *B. breve* UCC2003 significantly decreased the production of pro-inflammatory cytokines both *in vitro* and *in vivo* (Hughes et al., 2017). Moreover, *B. breve* UCC2003 producing EPS was required to reduce pathological epithelial cell shedding in a mouse model, as EPS- knock out isogenic strain could not (Salazar et al., 2014). Using a mouse model to compare the effects of EPS-producing wild-type strain with its isogenic EPS negative mutant, it has been reported that the protection offered by *B. longum* 35,624 against the occurrence of colitis and respiratory allergy symptoms was dependent on the presence of EPS (Schiavi et al., 2016). More recently, a polysaccharide synthesized by *B. bifidum*, consisting of β-glucans/galactans was found to be the key in the induction of Treg cells. The polysaccharide efficiently repeated the activity of whole bacteria and was demonstrated to act via dendritic cells through TLR2 -mediated mechanism (Verma et al.,

2018). Overall, it has been postulated that bifidobacterial molecules, based on immune receptor ligand interactions and downstream signaling events, regulate T-cell responses and strengthen immune tolerance to the existing colonic environment (including the microbiota and its metabolites), preventing thus colonic tissue damage and inflammation (Knoop et al., 2017). Temporal variance as a feature of the microbiome has been discussed before (Bastiaanssen et al., 2020) and the term volatility has been scarcely used in the context of the microbiome. Recently, some studies have shown that volatility may be related to stress although the impact of stress on microbial volatility measures remains unknown (Clooney et al., 2020). Furthermore, given that re-enacting the gut microbiome's signaling function with postbiotics, such as CPS and EPS, rather than maintaining a specific bacterial species composition may be a more reliable and efficient approach to maintain intestinal epithelial barrier function reducing the risk of developing low-grade systemic inflammation and/or chronic inflammatory diseases. To this end, our aim was to conduct a double-blind, placebo-controlled intervention study in healthy females over 40 years old to evaluate the potential of a mixture of exopolysaccharides from *B. breve* BB091109 (that have been shown to act through MAMPs *in vitro* and barrier function based on data and role of TLR2/TLR4 presented in Supplementary Table S1) on markers of chronic systemic inflammation, during and following a 3-month supplementation period.

2 Materials and methods

2.1 Test product and placebo

Both the test product and the placebo were administered in identical HPMC capsules (size 0) and packaged in identical containers, providing a one-month supply of 30 capsules. The test product, VMK223 (veMico Ltd., UK) consisted of 500 mg of a β-glucans mixture extracted from *Bifidobacterium breve* BB091109 (NCIMB43992). In details, 51.7 g VMK223 was recovered per litre of *B. breve* fermentation, consisting of 74% (w/w) β-glucan mixture, 16% (w/w) other oligosaccharides and 1% (w/w) protein. VMK223 was obtained during the stationary stage of growth in a specially formulated fermentation medium containing 24 g/L yeast extract, 26 g/L soy peptone, 25 g/L lactose, 20 g/L fucose-containing oligosaccharides, 10 g/L glucose, 1 g/L Tween 80, 2 g/L K_2HPO4, 5 g/L sodium chloride, 3 g/L sodium acetate, 9 g/L bile salts and 20 mM taurocholic acid. Following anaerobic fermentation for 60 h at 37°C, *B. breve* cells were collected through centrifugation and dissolved in 1 N NaOH overnight at room temperature, while the supernatant was kept at 4°C for further treatment. The solution was further centrifuged, and the supernatant collected. The two collected supernatants were further treated with 1 vol 96% cold ethanol overnight and centrifuged to collect the precipitate, three times. All collected precipitates were dissolved in distilled water and heated to 100°C for 5 min. Following overnight dialysis (MWCO 10KDa), the retentate was freeze dried and used to prepare the 500 mg VMK223 containing capsules.

Cellulose microcrystalline (Alfa Aesar, UK), a common excipient in the pharmaceutical industry known for its lack of impact in the colonic environment, was chosen as the placebo. The test product and placebo were taken daily before or together with a meal. Subject' compliance was followed by daily questionnaires.

2.2 Study design and ethical aspects

This study utilized a single-center, double-blind, randomized, placebo-controlled and parallel study design. The protocol adhered to the Helsinki Declaration and received approval from the University of Roehampton Research Ethics Committee (Ethics reference number: LSC 18/274). The study was registered as a clinical trial (ID: NCT04267731).[1] Participants provided written consent, and the selection process involved a medical interview to determine health status and adherence to the inclusion/exclusion criteria. The primary and secondary outcomes of the study aimed to evaluate the efficacy of a 12-week consumption of 500 mg VMK 223 on plasma markers of chronic low-grade inflammation (Plasma C-reactive protein (CRP), IL-6), plasma inflammatory markers (TNF-α, IFNγ, IL-10), selected saliva hormone levels (oestradiol, DHEA, estriol, progesterone, cortisol) and plasma human growth hormone, respectively. Additionally, product tolerance was assessed for potential adverse events and gastrointestinal (GI) side effects following 12 weeks of intake.

2.3 Study participants

A total of 40 female subjects aged between 40 and 55 years participated in this study (Figure 1). The main exclusion criteria were as follows: significant health problems (e.g., hypercholesterolaemia, diabetes, GI disorders), the use of medications or supplements known to affect mineral or glucose metabolism within the month prior to the study and/or during the study, pregnancy or plans to become pregnant, breastfeeding, hormone replacement therapy, a history of anaphylaxis to food, known allergies or intolerance to foods and/or to the study materials (or closely related compounds) or any of their stated ingredients, ongoing dieting, having lost >5% body weight in the previous year, and abnormal eating behaviour.

Participants were instructed to maintain their usual living habits, and the intake of other products similar to the test product during the study was not allowed. The recruited subjects were then randomized into two groups to receive either VMK223 or an equivalent placebo comparator. The randomization process utilized an Excel-based covariate adaptive program (Kang et al., 2008) to ensure the volunteers were evenly distributed between the two intervention arms.

2.4 Study schedule

The total study duration was 16 weeks, comprising a 12-week treatment period and a 4-week follow up period. Additionally, a 2-week run-in period preceded the study, during which volunteers refrained from consuming any probiotic or prebiotic-containing food or drinks. During the run-in period, participants recorded a 4-day food diary, including one weekend day, to assess their habitual diet. The randomization process utilized and Excel-based covariate

1 http://clinicaltrials.gov

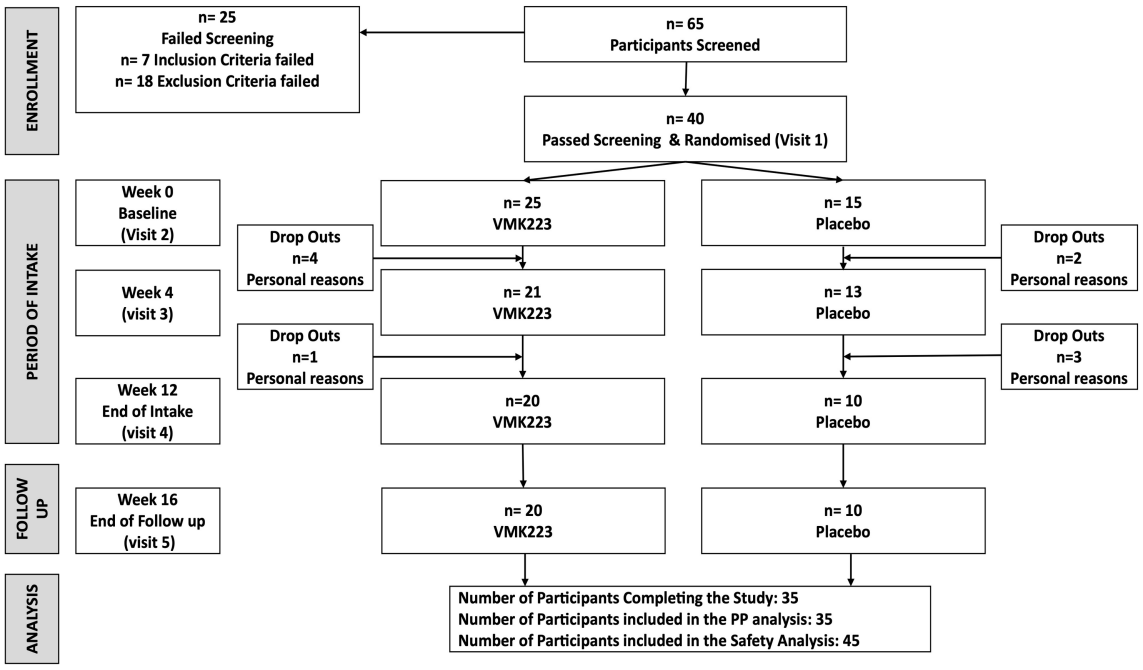

FIGURE 1
The study flow diagram. VMK223, *Bifidobacterium breve* BB091109 postbiotic; PP, per protocol.

adaptive program (Kang et al., 2008) to allocate volunteers into two intervention arms: VMK223 or placebo comparator stratified by age, gender, and Body Mass Index (BMI).

Data collection occurred before the first intake of the study product, after 4 weeks of intake, after the 12-week treatment period, and after the 4-week follow-up period. During these assessments, blood, saliva and tolerability data were collected. At the end of the follow-up period, the sustainability of the previously observed effects was evaluated in the test group of subjects who had taken the test product for at least 80% of the 12-week period.

2.5 Collection and analysis of blood and saliva samples

Fasted blood samples and saliva were collected from each participant at four time points: baseline (wk 0) before the intervention began, after 4 weeks of product intake, at the completion of the 12-week study treatment period (wk 12), and at the end of the 4-week follow-up period (wk 16).

For blood collection, participants underwent a 12-h fasting period. Trained phlebotomist obtained the blood samples from the participant's antecubital vein using a 23G butterfly needle (Greiner Bio-One GmbH, Kremsmünster, Austria). Four types of blood tubes were used: one 9 mL K2EDTA tube, one 9 mL Lithium Heparin tube, one 2 mL Sodium Fluoride/Potassium Oxalate tube, and one 2 mL K2EDTA tube (Vacuette®; Greiner Bio-One GmbH, Kremsmünster, Austria). After collection, all samples were immediately placed on ice until centrifugation.

Plasma samples were obtained by centrifugation at 2095 x g for 10 min, dispensed into 1.5 mL microcentrifuge tubes and promptly frozen at −80°C within 1 h from the collection.

2.6 Immune inflammatory markers

CRP, IL-6, IL-10, INFγ, TNF-α and human growth hormone (HGH), were measured using commercially available human selected biomarker kits (V-Plex Panel 2 Human Kit and CRP kit, Meso Scale Diagnostics LLC, USA) in accordance with the manufacturer's recommendations. This ultra-sensitive method has a detection limit of 1.33 pg/mL for CRP, 0.06 pg/mL for IL-6, 0.04 pg/mL for IL-10, 0.37 pg/mL for INF-γ, and 0.04 pg/mL for TNF-α.

Saliva samples were promptly stored in −80°C freezers following collection until further analysis. Participants were instructed to provide fasting saliva samples (approximately 5 mL) between 6 a.m. and 8 a.m. or within 30 min of waking up. Collection was done before eating, drinking liquids, or brushing teeth. The salivary hormones (estriol, oestradiol, DHEA, progesterone, and cortisol) were tested in duplicate using a fully automated enzyme-linked immunosorbent assay (ELISA) platform (Affinity Labs, London UK).

2.7 Anthropometric variables

The anthropometric variables monitored throughout the study included height, weight, body mass index and composition, waist and hip circumference and waist to hip ratio as reported by Keleszade et al. (2022).

2.8 Assessment of safety and tolerability

Safety and tolerability of the test material was assessed by two methods: (1) monitoring of adverse events during the study through

information collected in interviews and questionnaires; and (2) using a daily GI function questionnaire to record details of bowel habits including stool frequency and consistency (Bristol stool scale), stomach or intestinal bloating, abdominal pain, incidence, and frequency of flatulence.

2.9 Statistical analysis

The study was powered to provide 80% statistical power (MGH Biostatistics Hedwig Software) based on a treatment difference in C-Reactive Protein level (1.5 times the standard deviation), based on data from a previous VMK223 dose response study (Costabile et al., unpublished data). Given these calculations, 40 participants (to allow for 25% attrition due to the longer study period) were required to detect a treatment difference at a two-sided 0.05 significance level.

All baseline endpoints from the blood and saliva analysis were analysed using descriptive statistics and compared between groups by using independent samples *t*-tests. The location and scale statistics of all the baseline demographic characteristics and parameters were calculated, including the arithmetical mean, standard deviation and 95% confidence interval for mean (minimum, and maximum). From the calculated values of skewness and kurtosis it was concluded that all the parameters could be regarded as (approximately) normally distributed.

Data from all continuous endpoints were analyzed by using separate 2 × 4 repeated measures of analyses of variance (ANOVA) with Treatment (VMK223 vs. placebo) as the between-subject factor and Time (baseline, after 4 weeks of supplementation, after 12 weeks of supplementation, and after 4 weeks of terminating the supplementation) as the within-subject factor. Significant interactions were subsequently analyzed using Tukey's post-hoc test. The reliability of assumptions of this statistical test was checked using the Shapiro–Wilk test for normality, and Levene's test for the homogeneity of variance. Further, the effect size was estimated by eta-squared statistics ($\eta 2$). Values equal to or greater than 0.01, 0.06, and 0.14 indicated a small, moderate, and large effect, respectively. In order to adjust for multiple testing, the Bonferroni method was applied.

Additionally, intra-individual mean changes of (i) CRP and (ii) IL-6 (comparing T0 versus T4, T12, and T16), were evaluated using paired-samples *t* tests. The mean outcomes in the VMK223 and placebo groups were compared using the independent samples *t*-tests. The primary outcomes and test hypotheses of the trial were based on the comparison of the VMK223 and placebo groups with respect to the change (T12–T0) of: (i) CRP, and (ii) IL-6.

The significance threshold was set at $p < 0.05$. All statistical analyses were performed according to the principles of ICH (International Conference on Harmonization) guideline E9 "Statistical Principles for Clinical Trials" using JASP software (Version 0.17.1, JASP Team, Netherlands).

3 Results

3.1 Baseline characteristics of participants

Participants ($n = 40$) were randomly allocated to either the test group (VMK) ($n = 25$) or the placebo group ($n = 15$). At baseline, there were no significant differences between the two groups in terms of age, body weight, systolic and diastolic blood pressures, and all the selected fasting blood and saliva parameters (Table 1). Throughout the study, all recruited participants completed the trial, and no adverse events were reported. Both products were well tolerated by the participants.

Daily GI function questionnaires showed no significant effects of time or intervention on the bowel function, mood or dietary intakes

TABLE 1 Demographic and baseline fasting blood and saliva characteristics of all study participants at the beginning of the study.

	VMK223 group ($n = 20$)		Placebo group ($n = 20$)		*p*-value*
	Mean (SD)	Min, Max$	Mean (SD)	Min, Max$	
Age (years)	48.00 (4.5)	40, 59	48.35 (4.32)	43, 57	0.749
Weight (kg)	54.45 (5.25)	44.3, 70.5	56.73 (4.46)	48.1, 65.3	0.067
Systolic bp (mmHg)	117.61 (13.87)	98, 140	114.52 (11.41)	95, 140	0.336
Diastolic bp (mmHg)	71.79 (10.29)	48, 95	69.35 (9.32)	46, 91	0.326
CRP (mg/L)	5.09 (1.80)	4.31, 5.89	5.10 (1.82)	3.98, 6.23	0.997
TNF-α (pg/ml)	7.72 (1.37)	7.12, 8.32	7.80 (1.10)	7.12, 8.48	0.874
IL-6 (pg/ml)	3.92 (0.68)	3.62 4.21	4.09 (0.52)	3.78, 4.42	0.457
IL-10 (pg/ml)	2.39 (0.85)	2.02, 2.76	2.27 (0.94)	1.69, 2.85	0.724
IFN-γ (pg/ml)	138.85 (23.67)	128.48, 149.23	142.03 (38.17)	118.34, 165.67	0.782
Progesterone(pg/mL)	88.32 (14.08)	82.15, 94.49	99.30 (16.34)	79.17, 99.43	0.866
Oestradiol (pg/ml)	35.01 (12.57)	29.50, 40.52	31.00 (12.18)	23.45, 38.55	0.413
Estriol (pg/ml)	2.04 (0.17)	1.96, 2.11	2.16 (0.18)	2.05, 2.27	0.074
DHEA (ng/ml)	0.81 (0.26)	0.69, 0.92	0.70 (0.18)	0.59, 0.81	0.263
Cortisol (ng/ml)	53.95 (6.25)	51.21, 56.69	53.50 (8.33)	48.34, 58.66	0.868
HGH (ng/ml)	1.34 (0.57)	1.09, 1.59	1.54 (0.55)	1.19, 1.88	0.369

$, 95% Confidence Interval for Mean. *p-values for independent t-test comparisons between values in the VMK223 group and the placebo group. For a significant difference by t-test, $p < 0.05$. bp, blood pressure; CRP, Plasma C-reactive Protein; DHEA, dehydroepiandrosterone; HGH, human growth hormone.

10.3389/fmicb.2023.1273861

following daily supplementation with VMK223 and placebo (data not shown).

3.2 Effect of VMK223 on fasting plasma inflammatory markers and salivary hormones

The data of plasma and salivary biomarkers during the study period are shown in Table 2. The statistical analysis (ANOVA) revealed a significant effects of supplementation group, supplementation time, and their interaction for several biomarkers. For the plasma biomarkers, significant effects of the supplementation group were observed on IL-6, DHEA, oestradiol, estriol, progesterone and cortisol levels. Supplementation time had a significant effect on CRP, TNF-α, IL-6, progesterone, oestradiol and estriol levels. Additionally, the interaction between supplementation group and time showed significant effects on CRP, TNF-α, IL-6, IL-10, progesterone, oestradiol, estriol, cortisol and human growth hormone levels. Post-hoc analysis further examined specific differences between the groups and time points for the significant biomarkers. Notable, at the end of

TABLE 2 Two-factor ANOVA with repeated measures (2 groups × 4 time points) of the fasting plasma levels of inflammatory markers and fasting saliva levels of selected hormones before and after 12 weeks of supplementation with VMK223.

	Effect	*F*	df	*p*-value	Effect size (η^2)	Post-hoc outcome
CRP	GR	1.702	1, 28	0.203	0.048	
	RM	25.925	2, 57	<0.001**	0.064	B, I > E, F
	GR * RM	12.356	2, 57	<0.001**	0.031	VE < VB, PB, PE
TNF-α	GR	2.966	1, 28	.096	0.080	
	RM	5.474	3, 70	0.002**	0.021	B > I, E, F
	GR * RM	8.718	3, 70	<0.001**	0.034	VE < VB
IL-6	GR	6.221	1, 28	0.019*	0.125	V < P
	RM	14.312	2, 63	<0.001**	0.095	B, I > E; B > F
	GR * RM	4.511	2, 63	0.012*	0.030	VE < VB, PB, PE
IL-10	GR	0.336	1, 28	0.567	0.010	
	RM	1.070	3, 84	0.367	0.005	
	GR * RM	3.438	3, 84	0.020*	0.017	VE < VB
IFN-γ	GR	0.305	1, 28	0.585	0.008	
	RM	1.284	2, 65	0.286	0.011	
	GR * RM	2.399	2, 65	0.091	0.021	
Progesterone	GR	7.284	1, 28	0.012*	0.111	V > P
	RM	13.239	3, 84	<0.001**	0.129	B, I < E; B < F
	GR * RM	5.930	3, 84	0.001**	0.058	VE > VB, PB, PE
Oestradiol	GR	8.841	1, 28	0.006**	0.165	V > P
	RM	12.996	3, 84	<0.001**	0.083	B < I, E, F; I < F
	GR * RM	7.816	3, 84	<0.001**	0.050	VE > VB, PB, PE
Estriol	GR	6.173	1, 28	0.019*	0.059	V > P
	RM	7.779	2, 64	<0.001**	0.129	B, I, F < E
	GR * RM	4.834	2, 64	0.008**	0.080	VE > VB, PB, PE
DHEA	GR	GR	1, 28	0.001**	0.129	V > P
	RM	RM	3, 84	0.232	0.028	
	GR * RM	1.773	3, 84	0.159	0.034	
Cortisol	GR	10.978	1, 28	0.003**	0.139	V < P
	RM	1.317	2, 59	0.276	0.019	
	GR * RM	5.411	2, 59	0.006**	0.079	VE < VB, PB, PE
HGH	GR	5.289	1, 28	0.029	0.059	
	RM	2.451	2, 63	0.088	0.045	
	GR * RM	3.494	2, 63	0.032*	0.065	VE > VB

GR, treatment; RM, repeat measure; V, VMK223 group; P, placebo group; B, baseline; I, week 4; E, end of treatment; F, end of follow up. Significant difference at * $p < 0.05$ and ** $p < 0.01$.

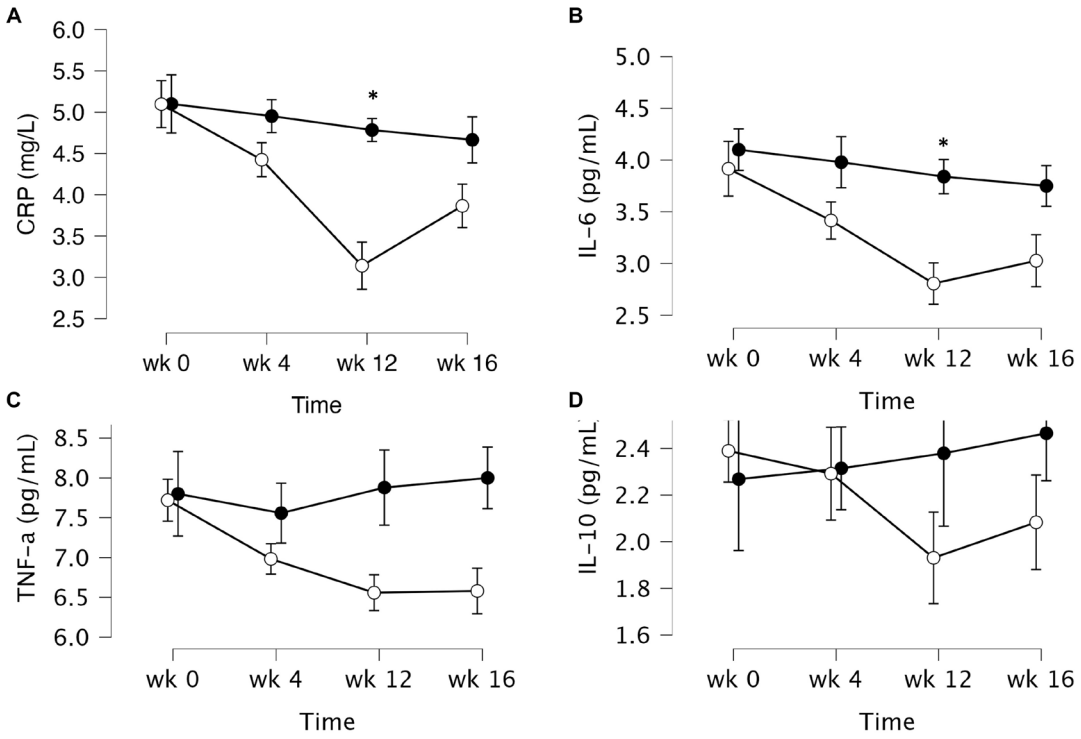

FIGURE 2
Mean ± SD values of fasting plasma levels of select inflammatory markers after 12 weeks of supplementation with a lyophilizate *Bifidobacterium breve* BB091109 postbiotic (VMK223): **(A)** Plasma C-reactive protein (CRP), **(B)** IL-6, **(C)** TNF-α and **(D)** IL-10. Black circles, placebo group ($n = 10$); white circles, VMK223 group ($n = 20$). wk 0, Baseline; wk 4, following 4 weeks of supplementation; end of treatment with wk 12, end of treatment; wk 16, end of follow up period. *Significant difference versus the placebo at the particular time point.

the supplementation period with VMK223, CRP levels were significantly lower than at baseline ($p < 0.001$) and lower than the placebo group at baseline ($p = 0.043$) and end of supplementation ($p < 0.001$) (Figure 2). TNF-a levels at the end of the supplementation period with VMK223 were significantly lower than at baseline ($p < 0.001$), but not significantly different from the placebo group levels at baseline ($p = 0.293$) and end of supplementation ($p = 0.226$) (Figure 2). IL-6 levels at the end of the supplementation period with VMK223 were significantly lower than at baseline ($p < 0.001$) and lower than the placebo group at baseline ($p = 0.001$) and end of supplementation ($p = 0.017$) (Figure 2). Regarding hormone data, progesterone levels at the end of the supplementation period with VMK223 were significantly higher than at baseline ($p < 0.001$) and higher than the placebo group at baseline ($p < 0.001$) and end of supplementation ($p = 0.003$) (Figure 3). Oestradiol and estriol levels were significantly higher at the end of the supplementation period with VMK223 than at baseline ($p < 0.001$ for both), and higher than the placebo group levels at baseline ($p < 0.001$ and $p = 0.002$, respectively) and end of supplementation ($p = 0.003$ and $p = 0.004$, respectively) (Figure 3). HGH levels at the end of the supplementation period with VMK223 were significantly higher than at baseline ($p < 0.001$) but not significantly different from the placebo group levels at baseline ($p = 0.171$) and end of supplementation ($p = 0.118$) (Figure 3). Cortisol levels at the end of the supplementation period with VMK223 were significantly lower than at baseline ($p < 0.001$) and lower than the placebo group levels at baseline ($p = 0.038$) and end of supplementation ($p < 0.001$) (Figure 3). At the end of the follow up period, 4 weeks after the termination of the supplementation with VMK223, the levels of IL-6 ($p = 0.736$), IL-10 ($p = 0.913$), TNF-a ($p = 1.000$), DHEA ($p = 0.203$), oestradiol ($p = 0.452$), DHEA ($p = 0.792$), human growth hormone ($p = 0.661$) and cortisol ($p = 0.824$) were not significantly different from the levels at the end of the supplementation period. However, the levels of CRP were significantly higher ($p < 0.001$) and the levels of estriol were significantly lower ($p < 0.001$) than the levels at the end of the supplementation period.

3.3 Effect of VMK223 on markers of chronic low-grade inflammation

In the test group, CRP levels showed a significant reduction after 4 weeks ($p = 0.002$) and 12 weeks ($p < 0.001$) of supplementation, as well as 4 weeks ($p < 0.001$) after the termination of supplementation, compared to baseline. In contrast, the placebo group exhibited no significant difference in CRP levels from baseline at 4 weeks ($p = 1.000$), 12 weeks ($p = 0.852$) and 4 weeks ($p = 0.531$) post supplementation (Table 3). Between-group comparisons showed that CRP values were significantly lower in the test group than in the placebo group after 12 weeks of supplementation ($p = 0.001$) (Table 3). Figure 4 illustrates that at the end of the 12-week supplementation

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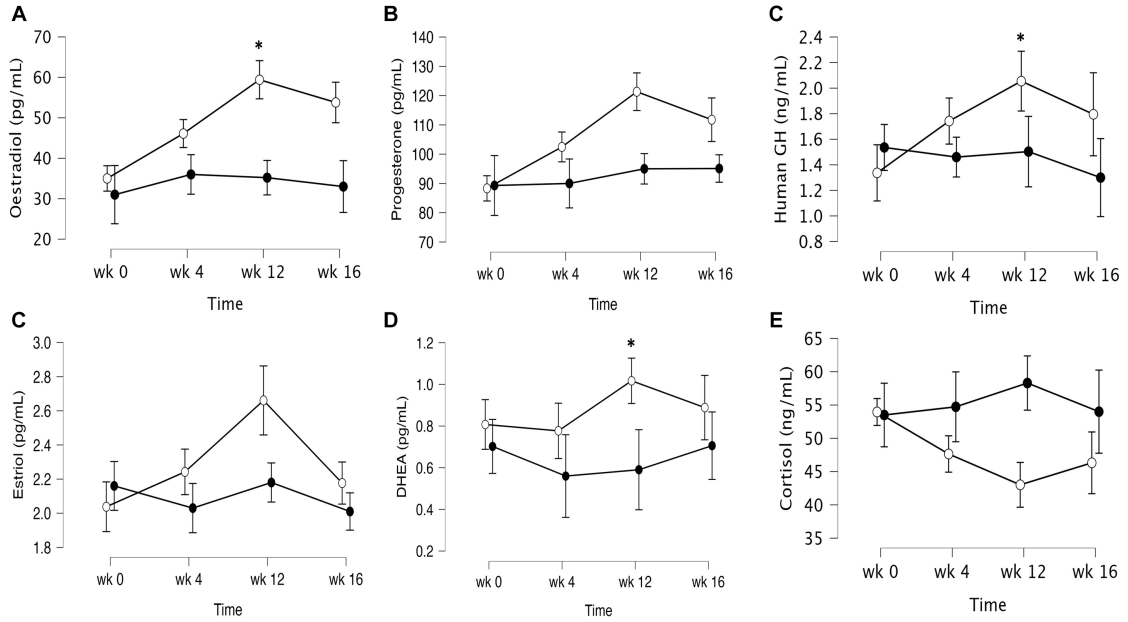

FIGURE 3
Mean ± SD values of fasting levels of selected after 12 weeks of supplementation with a lyophilizate *Bifidobacterium breve* BB091109 postbiotic (VMK223): **(A)** Oestradiol, **(B)** Estriol, **(C)** progesterone **(D)** human growth hormone, **(E)** cortisol, **(F)** DHEA. Black circles, placebo group ($n = 10$); white circles, VMK223 group ($n = 20$). wk 0, Baseline; wk 4, following 4 weeks of supplementation; end of treatment with wk 12, end of treatment; wk 16, end of follow up period. *Significant difference versus the placebo at the particular time point.

TABLE 3 Concentration of fasting plasma CRP and IL-6 before and after supplementation with a *Bifidobacterium breve* BB091109 postbiotic (VMK223) extract (Mean ± SD).

	VMK223 group ($n = 20$)		Placebo group ($n = 20$)		Between group *p*-value*
	Mean ± SD	*p*-value	Mean ± SD	*p*-value	
CRP (mg/L)					
Baseline	5.09 ± 1.80		5.10 ± 1.82		0.997
Week 4	4.42 ± 1.62	0.002*	4.95 ± 1.74	1.000	0.418
End of treatment (12wk)	3.14 ± 0.94	<0.001**	4.78 ± 1.59	0.852	0.001[$]
End of follow up (16wk)	3.87 ± 1.29	<0.001**	4.67 ± 1.62	0.531	0.153
IL-6 (pg/ml)					
Baseline	3.92 ± 0.68		4.09 ± 0.52		0.457
Week 4	3.42 ± 0.69	0.009*	3.98 ± 0.49	0.998	0.031*
End of treatment (12wk)	2.81 ± 0.94	<0.001**	3.84 ± 0.64	0.875	0.004*
End of follow up (16wk)	3.03 ± 0.93	<0.001**	3.75 ± 0.59	0.607	0.034*

p-values for *t*-test comparisons between values in the VMK223 group and the placebo group.
*$p < 0.05$, **$p < 0.001$.

period, the test group had significantly lower CRP levels compared to the placebo group ($p = 0.021$). Similarly, IL-6 levels in the test group demonstrated a significant reduction after 4 weeks ($p = 0.009$) and 12 weeks ($p < 0.001$) of supplementation, as well as 4 weeks ($p < 0.001$) after the termination of supplementation compared to baseline. Conversely, the placebo group exhibited no significant difference in IL-6 levels from baseline at 4 weeks ($p = 0.998$), 12 weeks ($p = 0.875$) and 4 weeks ($p = 0.607$) post supplementation (Table 3). Comparing the two groups, IL-6 values were significantly lower in the test group than in the placebo group after 4 weeks ($p = 0.031$), 12 weeks ($p = 0.004$), as well as 4 weeks ($p = 0.034$) after the termination of supplementation (Table 3). Figure 4 demonstrates that at the end of the 12-week supplementation period, the test group had significantly lower IL-6 levels compared to the placebo group ($p = 0.044$, respectively). At the end of the 12-week supplementation period, the test group demonstrated a substantial reduction in both CRP and IL-6 levels compared to the placebo group, with the reduction being 4-fold greater for CRP and 4.7-fold greater for IL-6.

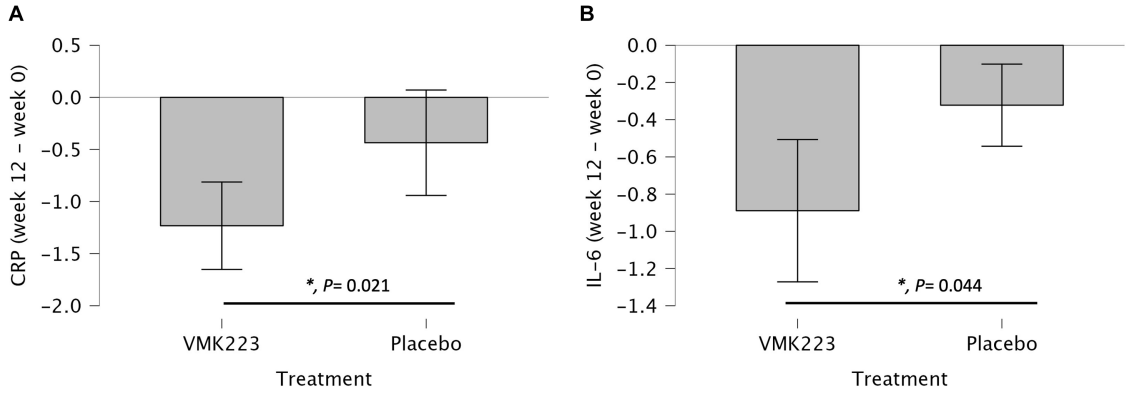

FIGURE 4
Changes in Plasma C-reactive protein (CRP) (mg/L) and IL-6 (pg/mL) in individuals *Bifidobacterium breve* BB091109 postbiotic (VMK223) preparation or placebo. **(A)** Changes in CRP. **(B)** Changes in IL-6. Changes in parameter values from baseline are shown in mg/L (CRP) or pg/mL (IL-6). Data are expressed as Mean ± SD. *p-values for t-test comparisons between values in the test group and the placebo group; *$p < 0.05$.

4 Discussion

In this study, we investigated the effects of VMK223, a postbiotic mixture of exo- and capsular- polysaccharides derived from *Bifidobacterium breve* BB091109, consisting mainly of β-glucan, on plasma markers of systemic inflammation and sex hormones in healthy females over 40 years old who were not receiving hormonal therapy. The daily oral supplementation with VMK223 (500 mg daily) resulted in a significant reduction in markers of systemic inflammation (CRP, IL-6, TNF-α) after 4 weeks of supplementation, which was sustained throughout the 12-weeks treatment period and the 4-week follow-up period. This reduction in systemic inflammatory markers was accompanied by a significant decrease in cortisol levels, a hormone associated with increased energy-expenditure and immune system activation (Roney and Simmons, 2015). Cortisol increase is known to lead to a significant decrease of female sex hormones estrogen, oestradiol, progesterone, and DHEA (Roney and Simmons, 2015). Current data support sexual dimorphism of the gut microbiota in animals (McGee and Huttenhower, 2021). Moreover, mucosal immune function and susceptibility to chronic inflammation differs between sexes (Casimir and Duchateau, 2011). Those distinct differences in the male and female gut microbiota, for both animal and human models, inevitably generate differences in metabolic processes and therefore, differences in dysbiosis and the protection or susceptibility to chronic systemic inflammation (Casimir and Duchateau, 2011). We have therefore selected only female participants to overcome potential sexual dimorphism issues and chosen the perimenopause stage, as the endocrine transition during this life stage has been associated with reduction in the integrity of the intestinal barrier and a rise in chronic low-grade inflammation (Brettle et al., 2022). The pathogenesis of numerous chronic inflammatory diseases (CIDs) involves complex interactions among genetic predisposition, environmental triggers including alterations in the gut microbiome, gut permeability, antigen trafficking and immune activation (Fasano, 2020). At the forefront of this interplay are the biological interfaces that separate our bodies from the external environment. Of these interfaces, the human intestine represents the largest and most critical interface. Through tightly packed epithelial cells, the intestinal mucosa interacts with various environmental factors, such as microorganisms, nutrients, and antigens to regulate molecular traffic between the gut and submucosa, impacting immune responses and tolerance (Yoo et al., 2020; de Vos et al., 2022). The gut microbiota plays a vital role in maintaining the health of the host and it influences various physiological processes, including digestive function, pathogen resistance, intestinal permeability, endocrine function, and immune stimulation. The influence of the gut microbiota on human health extends beyond metabolic activities. Microbial translocation, a process where disruptions in the gut epithelial barrier allow microbes and their products to enter the systemic circulation, plays a role in several diseases (de Vos et al., 2022). The activation of TLRs by MAMPs has been shown to contribute to the development of chronic systemic inflammation. Dysbiosis in the gut microbiome can lead to compromising the epithelial barrier and causing the translocation of luminal contents, triggering an inflammatory response (Salvo-Romero et al., 2020). Depending on the host's genetic makeup, activated T cells may remain in the gastrointestinal tract, leading to CID of the gut, or migrate to other organs, causing systemic CIDs. It is well known that certain bacterial species, such as *Akkermansia muciniphila*, *Bifidobacterium* spp., and *Lactobacillus* spp. have been shown to improve intestinal barrier integrity and reduce inflammation (Jian et al., 2023). Probiotic administration has shown promising results in enhancing intestinal barrier integrity, reducing translocation of LPS, alleviating low-grade systemic inflammation, and improving endocrine function and immune tolerance (Plaza-Diaz et al., 2014; Zheng et al., 2023). Additionally, *B. breve* has been reported to modulate the expression of TLRs, normalizing TLR4 expression, enhancing TLR2 expression, and reducing the expression of pro-inflammatory cytokines, including IL-1 beta, IL-6, and tumor necrosis factor alpha in an animal model of necrotizing enterocolitis (NEC) (Wong et al., 2019). The current findings are also in line with results from experimental models that have shown that the administration of specific *Bifidobacterium* strains, such as *B. breve* M-16 V, reduced the severity of a condition characterized by intestinal inflammation and damage (Satoh et al., 2016; Wong et al., 2019). It has been demonstrated that viability is not a pre-requisite for the health benefits associated with probiotic administration. Postbiotics have emerged as a new class of bacteria derived effector molecules that are either produced by live bacteria or

are released after bacterial cell lysis (Vinderola et al., 2022). Postbiotics must be derived from a well-defined microorganism or combination of microorganisms for which genomic sequences are known and prepared using a delineated technological process of biomass production and inactivation, which can be reliably reproduced (Tsilingiri and Rescigno, 2013; Vinderola et al., 2022). A postbiotic may be inanimate intact cells or may be structural fragments of the microorganisms, such as cell walls. Among the various potential cell wall postbiotics, particular attention is given to CPS and EPS. Recent work has described the unique and potent antigen polysaccharide A (PSA) synthesized by *Bacteroides fragilis* and its involvement in promoting the differentiation of mouse Treg cells, through the expression of TLR2 on both DC and T cells (Mazmanian et al., 2005, 2008). It was also demonstrated that a critical step defining the pro-tolerance phenotype and differentiation of Treg cells was the recognition of PSA by plasmacytoid DCs. Similarly, *Bifidobacterium* spp. derived EPS has been reported to play an immunomodulatory effect (Fanning et al., 2012). Overall, the cell wall polysaccharides, CPS and EPS, from *Bifidobacterium* spp. and some other Gram+ commensal bacteria are recognized by the TLR2 receptor, although they may also bind directly to other PRRs such as C-type lectin receptors, e.g., dectin-1, dectin-2, or mannan receptors, affecting the signaling pathway induced by bacteria. Recent research has also reported that such polysaccharides from Gram+ beneficial commensals can also be recognized by the TLR4 receptor and act as an LPS-antagonist (Zheng et al., 2020). Nevertheless, the chemical composition and structure of EPS defines its immunomodulatory properties and has been shown to depend on genetics (bacterial species) and environmental (growth conditions) factors. Following fermentation of *B. breve* BB091109 under different conditions of stress (pH, bile salts, O2 levels), carbon sources and ethanol extraction, VMK223 was selected for this study following *in vitro* screening with HEK (Human Embryonic Kidney) 293 cell line, based on its increased ability to reduce expression of TLR4 and enhance expression of TLR2, amongst the generated postbiotic mixtures. Regarding female sex hormones, it is well known that estrogens and progesterone are master regulators of the immune system and mucosal barrier in the female reproductive tract (Peters et al., 2022). These hormones may be similarly important in the gastrointestinal tract, with their reduction observed during menopause reducing barrier integrity and increasing microbial translocation in the gut as well. Accumulating evidence has linked TLR function to estrogen and estrogen receptor α (ERα) and β (ERβ), suggesting a possible contribution of different microbial sensing and TLR activation to sex bias in chronic inflammatory diseases, likely linked to gut MAMPs recognition (Maffei et al., 2022; Peters et al., 2022). Experimental evidence indicates that estradiol and progesterone maintain the gut barrier and protect from gut injury. Recent studies have reported that intestines from female rat are more resistant to shock-induced injury than from male rat, with the resilience being attributed to estradiol levels (Maffei et al., 2022). *In vitro* treatment with estradiol protects mucus-producing intestinal epithelial cells against oxidant injury (Homma et al., 2005), while colonic epithelial barrier function is protected through the estrogen receptor-β signaling (Langen et al., 2011; Diebel et al., 2015). Both estradiol and progesterone improve epithelial barrier function by upregulating tight junction proteins (Braniste et al., 2009). Estrogen mRNA has been found reduced in the colon of animal with colitis and patients with inflammatory bowel disease (Collins et al., 2017). Plasma progesterone levels have been inversely correlated with plasma lipopolysaccharide, a marker of reduced gut barrier function (Zhou et al., 2018). The results of this study suggest that VMK223 has the potential to reduce markers of systemic inflammation in healthy females over 40 years old. VMK223 is a mixture of *B. breve* derived MAMPs that act as TLR4 antagonist and TLR2 agonist *in vitro*, and the supplementation of VMK223 is followed by significant improvements in the level of specific sex hormones, the depletion of which during the perimenopause and menopause stage increases gut permeability and systemic inflammation. Taken together the results suggest that VMK223 may protect intestinal epithelial barrier function limiting gut permeability and preventing microbial translocation. When the MAMPs in VMK223 (CPS and EPS from *B. breve*) interact with TLR2 and TLR4 on the intestinal epithelium, they activate certain proteins and transcription factors that enhance the function of the intestinal epithelial barrier, reducing intestinal permeability. This may prevent microbial translocation and gut microbiome metabolites to trigger an immune response, based on the reduction of cortisol levels, preventing thus the accumulation of systemic inflammation that eventually can lead to chronic inflammatory states. The potential mechanism, however, has not been evaluated in this study, and future studies focusing on markers of intestinal epithelial barrier function are warranted. Additionally, given that perimenopause can last 7–15 years with a wide range of impact on immune and endocrine function, future studies should consider adopting a longitudinal approach to monitor changes throughout this period, ideally by recruiting a larger sample.

5 Conclusion

The prospect of restoring a normal microbiota is an appealing hypothesis to manage inflammation mediated conditions associated with a gut microbiota dysbiosis. Restoration of the composition of gut microbiota and its community structure conceivably leads to restoration of its function. Several ways for manipulating gut microbiota composition and function include the use of dietary interventions based on probiotics and prebiotics. However, due to environmental factors such as diet, lifestyle and drugs being major determinants of gut microbiota composition and activity, the effectiveness of such approaches at population level is reduced. Postbiotics on the other hand, offer a promising alternative by delivering the actual bioactives of the probiotic bacteria, without depending on the presence and metabolic activity of the beneficial bacteria in the GI tract. In the current study, VMK223, a probiotic cell wall derived postbiotic, showed to improve immune and endocrine markers of intestinal epithelial barrier function. Further investigation into the immunomodulatory potential of cell wall components and their specific interactions with innate immune cells and TLRs holds promise for developing advanced therapeutics and understanding the precise molecular mechanisms underlying these interactions will contribute to the rational design of targeted interventions for various health conditions.

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

The studies involving humans were approved by University of Roehampton Research Ethics Committee (Ethics reference number: LSC 18/274). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

D-EM: Conceptualization, Investigation, Writing – review & editing, Data curation. BB: Investigation, Writing – review & editing. PH: Writing – review & editing. GT: Writing – review & editing. JV: Writing – review & editing. AC: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Data curation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The corresponding work was supported by veMico Ltd., (Research Agreement Roehampton University and VeMiCo awarded 2022, Ref. LSC/2021/43).

Conflict of interest

JV and GT were employed by veMico 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.2023.1273861/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 05 February 2024
DOI 10.3389/fmicb.2024.1342705

OPEN ACCESS

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RECEIVED 22 November 2023
ACCEPTED 15 January 2024
PUBLISHED 05 February 2024

CITATION
Bu L, Li Y, Wang C, Jiang Y and Suo H (2024) Preventive effect of *Lacticaseibacillus rhamnosus* 2016SWU.05.0601 and its postbiotic elements on dextran sodium sulfate-induced colitis in mice. *Front. Microbiol.* 15:1342705. doi: 10.3389/fmicb.2024.1342705

Preventive effect of *Lacticaseibacillus rhamnosus* 2016SWU.05.0601 and its postbiotic elements on dextran sodium sulfate-induced colitis in mice

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Microbial-based therapies are one of the hotspots in the field of ulcerative colitis research. The lactic acid bacteria and their postbiotics occupy a key position in microbial therapies, however, the mechanism by which they alleviate ulcerative colitis in mice is unknown. We investigated the effects of *Lacticaseibacillus rhamnosus* 2016SWU.05.0601 (Lr-0601) and its postbiotics on male Kunming mice with dextran sulfate sodium salt (DSS)-induced ulcerative colitis (UC). The results showed that Lr-0601 significantly alleviated the deterioration of UC and restored the expression of intestinal mechanical barrier proteins. In addition, Lr-0601 significantly reduced the expression of inflammatory cytokines in the body and regulated the expression of key regulatory genes of the NF-κB-iNOS/COX-2 signaling pathway in colon tissues to a large extent. Our results suggest that supplementation with Lr-0601 and its postbiotics can effectively prevent DSS-induced UC and have a beneficial effect on intestinal health, which also provides new insights and research bases for the prevention as well as the treatment of ulcerative colitis and other diseases related to intestinal barrier dysfunction and other diseases.

KEYWORDS

DSS, NF- kappa B, postbiotic, probiotic, ulcerative colitis

Introduction

Ulcerative colitis (UC), a non-specific chronic intestinal disease, mainly occurs in the sigmoid colon and rectum and is typically accompanied by abdominal pain and bloody diarrhea (Eisenstein, 2016; Ungaro et al., 2017). UC is characterized by a long course of disease, repeated attacks, difficulty healing, and has a strong tendency to become cancerous. The etiology and pathogenesis of UC have not been fully elucidated, but existing studies have concluded that UC results from a combination of genetic susceptibility, environment, infection, and abnormal immune response (Iyer and Corr, 2021). Currently, the treatment

of UC mainly relies on traditional drugs such as amino salicylic acid, corticosteroids, biologics and immunosuppressants. Although they have clinical efficacy, they also have the disadvantages of high side effects and easy to form drug dependence (Yang et al., 2022). Therefore, it is of great practical significance to find new strategies for the prevention and treatment of UC that are safe and effective with few side effects.

Clinical studies and animal experiments have indicated that the occurrence of UC is closely related to intestinal flora imbalance (Yu, 2023). Research has also suggested that the core pathological feature of UC is loss of integrity of the intestinal barrier, resulting in increased bacterial antigen translocation and dysregulation of the intestinal flora. This increase promotes pro-inflammatory and inflammatory substances into the lamina propria of the intestinal mucosa, triggering an uncontrollable vicious cycle of immune response and leading to colonic damage (Capaldo et al., 2017). Intake of probiotics exerts positive effects on human intestinal health. The activities and metabolites of probiotics can improve intestinal health by inhibiting the growth of pathogenic bacteria, maintaining intestinal flora balance, and regulating intestinal barrier function and immune function. Thus, probiotics exhibit potential for the prevention and treatment of UC (Maciel-Fiuza et al., 2023). Whereas, when it comes to intake of probiotics, choosing natural food sources is more beneficial for body absorption and utilization and has a higher safety profile, therefore, *lactobacilli* strains isolated from yak yogurt were chosen for this study. It has been reported that various probiotics such as *Lactobacillus plantarum* HY01 isolated from yak yoghurt and *L. plantarum* CQPC06 isolated from kimchi have exhibited to prevent UC (Chen et al., 2017; Zhang et al., 2018).

Probiotic organisms and metabolites have been defined by the International Society for Probiotics and Prebiotics (ISAPP) in May 2021 as the term "postbiotic," which refers to a substance that is beneficial to the health of the host. It refers to preparations of inanimate microorganisms and/or their components that are beneficial to the health of the host (Salminen et al., 2021). Recent studies have shown that postbiotics show potential in the treatment of UC, with some studies claiming that probiotics and postbiotics have similar abilities to improve disease phenotypes (Liu et al., 2023). For example, a preclinical study found that *Lactobacillus plantarum*-derived postbiotics were able to optimize the composition of the intestinal flora and significantly modulate the levels of short-chain fatty acids and neuroactive molecules (5-hydroxytryptamine, γ-aminobutyric acid) *in vivo*, which in turn ameliorated *Salmonella*-induced neurological dysfunction (Wu et al., 2022). In addition, researchers found that in a mouse model of colitis, postbiotics from *Bifidobacterium adolescentum* B8589 had a stronger gut microbiome-modulating effect than probiotics and were able to effectively attenuate colonic inflammatory cell infiltration, mucosal damage, and crypt loss and alleviate colitis symptoms in mice (Zhang T. et al., 2022). These studies suggest to us that postbiotics have antimicrobial, antioxidant, immunomodulatory and intestinal barrier capabilities, and have a greater potential to improve the health of the organism. However, current research has focused on the use of probiotics to alleviate intestinal diseases, while the *in vivo* application of postbiotics is still in its infancy, and their mechanism of action is still unclear. Therefore, the present study focused on the effects of probiotics and postbiotics on dextran sulfate sodium salt (DSS)-induced colitis. It also preliminarily investigates the mechanism of action of probiotics and postbiotics in improving colitis.

The strain Lr-0601 used in this study is a strain of lactic acid bacteria isolated from yak yogurt collected in Qinghai. Yak yoghurt, a fermented dairy product distributed in the Qinghai-Tibet Plateau, is prepared by local herders using traditional methods (Guo et al., 2013). Due to the unique geographical and climatic characteristics of the Qinghai-Tibet Plateau, the microorganisms in yak yogurt have formed a complex microbiota through natural selection and long-term domestication (Ding et al., 2022; Franceschi et al., 2023). Yak yogurt is richer in nutrients than regular yogurt and is considered a good reservoir of lactic acid bacteria (Wei et al., 2022), of which *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus swissii* are the dominant strains, and have a wide range of beneficial properties such as antioxidant and lipid-lowering (Zhang X. et al., 2022). Therefore, yak yogurt is an ideal source for the isolation and screening of excellent lactic acid bacteria. Lr-0601 is a strain of lactic acid bacteria isolated from yak yoghurt collected in Qinghai.

In the experiments presented in this study, we established a model of colitis by administering DSS and examined the effects of supplementation with Lr-0601 and its postbiotics in alleviating DSS-induced colitis. The findings confirmed that both probiotic and postbiotics alleviated symptoms associated with colitis, which was reflected in improved colon length weight, colon tissue status, *in vivo* inflammation levels, and intestinal barrier integrity in colitis mice. Our findings may help to capitalize on the potential benefits of Lr.0601 and its postbiotics in alleviating colitis, mitigating intestinal disease, and protecting intestinal health, and support that Lr-0601 and its postbiotics are promising and safe adjunctive therapies for colitis.

Materials and methods

Experimental strain

Lacticaseibacillus rhamnosus 2016SWU.05.0601 (Lr-0601) was isolated from traditional fermented yak yoghurt (Hexi Town, Guide County, Tibetan Autonomous Prefecture of Hainan, Qinghai, China), stained with Gram stain, subjected to 16s rDNA sequencing, and biochemical identification, and preserved in the China Center for Type Culture Collection (Wuhan, Hubei, China), with preservation number M2018592.

In vitro tolerance assay of the strain

In simulated artificial gastric juice assay, 10 mL of Lr-0601 culture solution was centrifuged at 3,000 r/min for 15 min at 4°C. The collected pellets were washed with 10 mL of sterile physiological saline and resuspended in the same buffer. The resuspended cells and artificial gastric juice (0.2% NaCl, 0.35% pepsin, pH 3.00) were mixed at a ratio of 1:9 and incubated at 37°C for 3 h. The cultures were coated on MRS broth (BD, Franklin Lakes, NJ, USA) agar plates and incubated for 48 h at 37°C. The colony-forming units (CFU) on each plate were counted. Three parallels were made for each sample.

TABLE 1 Sequences of primers used for qRT-PCR.

Primer name	Forward primer	Reverse primer
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
ZO-1	GCCGCTAAGAGCACAGCAA	GCCCTCCTTTTAACACATCAGA
Occludin	TGAAAGTCCACCTCCTTACAGA	CCGGATAAAAAGAGTACGCTGG
IκB-α	TGAAGGACGAGGAGTACGAGC	TGCAGGAACGAGTCTCCGT
NF-κB	ATGGCAGACGATGATCCCTAC	CGGAATCGAAATCCCCTCTGTT
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
eNOS	TCAGCCATCACAGTGTTCCC	ATAGCCCGCATAGCGTATCAG
nNOS	CCCAACGTCATTTCTGTCCGT	TCTACCAGGGGCCGATCATT
COX-2	GGTGCCTGGTCTGATGATG	TGCTGGTTTGGAATAGTTGCT

In the bile salt tolerance assay, Lr-0601 was inoculated in MRS broth plus 0.2% of sodium thioglycolate containing 0.00, 0.10, 0.20, and 0.30% (w/v) of porcine bile salt. The amount of inoculum was 2% (v/v). The mixture was then incubated at 37°C for 4 h. Bile salt tolerance was then conducted by reading the optical density of the bacterial culture solution at 600 nm. Three parallels were made for each sample.

Animal grouping and administrations

All animal procedures described in this study were reviewed and approved by the Ethics Committee of Chongqing Medical University (Animal Experimentation Ethics: 201804021B). Fifty male mice, aged 6 weeks, from Kunming were purchased from Experimental Animal Center of Chongqing Medical University (Chongqing, China). The mice were exposed to the following conditions: temperature, 25°C ± 2°C; relative humidity, 50% ± 5%; light/dark cycle, 12 h/12 h. In addition, the mice were given free access to a standard chow diet and water. After a week of adaptive feeding, all mice were randomized into five groups (N = 10/group): normal, DSS, high-dose treatment with 1.0×10^{10} CFU/kg body weight (Lr-0601-H), low-dose treatment with 1.0×10^{9} CFU/kg body weight (Lr-0601-L) and the concentration of 1×10^{10} CFU/mL Lr-1601 bacterial suspension was heat-killed at 100°C for 30 min, centrifuged, and finally resuspended in physiological saline (Lr-0601-I). The experimental period was set to 5 weeks. Mice in the Lr-0601-H and Lr-0601-L groups were treated daily with Lr-0601 via gavage, mice in the Lr-0601-I group were treated daily with inactivated Lr-0601 at 1.0×10^{10} CFU/kg body weight via gavage, mice in the normal group and the DSS group were not administrated with lactic acid bacteria. The normal group were given free access to a standard chow diet and water from the beginning to the end of the experiment. The other mice were fed a normal diet but were given water with 2% (w/v) dextran sulfate sodium (DSS: 40 kDa; MP Biomedicals, Santa Ana, CA, USA) in Week 3 and 4% DSS in Week 5. The body weight of the mice was recorded daily during administration and the gavage volume was adjusted according to the body weight.

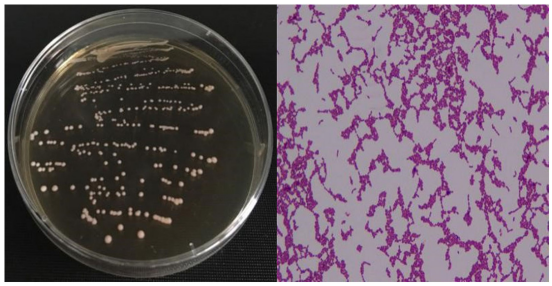

FIGURE 1
Colony morphology and Gram staining results of the strain.

Blood sampling collection and colon collection

Five weeks after administration, mice were fasted without water for 12 h. Blood samples were collected, transferred to 1.5 mL EP tubes, and then centrifuged at 3,000 rpm for 10 min at 4°C before serum was collected and stored in a −80°C refrigerator for further study. After obtaining blood samples, all mice were euthanised and the samples disposed of. Colon tissue was collected and the length and weight of the colon were quickly measured. About 1 cm of colon was taken, fixed in 10% formalin solution for 24 h, washed with water, dehydrated by alcohol gradient and embedded in paraffin, the tissue was cut into standard sections and stained with hematoxylin-eosin (H&E) for histological examination. The remaining colon tissues were stored in −80°C refrigerator for further study.

Histological observations

After mice were executed, colon tissue samples were rapidly dissected and immersed in 10% neutral formalin solution, dehydrated with a gradient of 75, 85, 95, and 100% ethanol and embedded in paraffin wax for embedding. Then the wax blocks were cut into thin slices of about 5 μm and dried, then dewaxed

10.3389/fmicb.2024.1342705

TABLE 2 API 50 CH fermentation results of Lr-0601.

Carbohydrate name	Reaction result	Carbohydrate name	Reaction result
Negative control	–	Aesculus ferric citrate	+
Glycerol	–	Salicin	+
Erythritol	–	D-Cellobiose	+
D-Arabinose	–	D-Maltose	+
L-Arabinose	–	D-Lactose	+
D-Ribose	+	D-Melibiose	–
D-Xylose	–	D-Sucrose	+
L-Xylose	–	D-Trehalose	+
D-Ribitol	–	Inulin	–
Methyl-β-D Xylopyranoside	–	D-Matsutose	+
D-Galactose	+	D-Raffinose	–
D-Glucose	+	Modified starch	–
D-Fructose	+	Glycogen	–
D-Mannose	+	Xylitol	–
L-Sorbitose	+	D-Gentiobiose	+
L-Rhamnose	+	D-Toron sugar	+
Dulcitol	–	D-Lyxose	–
Inositol	–	D-Tagatose	+
Mannitol	+	D-Salalose	–
Sorbitol	+	L-Salalose	–
Methyl-α-D-Mannopyranoside	–	D-Arabinol	–
Methyl-α-D-Glucopyranoside	+	L-Arabinol	–
N-Acetyl Glucosamine	+	Potassium Gluconate	+
Amygdalin	+	Potassium 2-ketogluconate	–
Arbutin	+	Potassium 5-ketogluconate	–

by xylene, rinsed with ethanol and distilled water and stained with hematoxylin and eosin, and the stained sections were dehydrated by ethanol and transparent by xylene, then sealed with gum, and then observed and photographed under a microscope (BX43, Olympus Corp., Tokyo, Japan).

Determination of cytokine levels in serum

Serum cytokine levels (IL-1β, IL-6, IL-8, and TNF-α) were determined using the enzyme-linked immunosorbent kit (Cloud-Clone Corp, Wuhan, Hubei Province, China).

Quantitative real-time polymerase chain reaction

Total RNA in the colon tissue was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and then transcribed into cDNA by using RevertAid First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The mixed PCR system was placed in a gene amplification instrument (A200, Langji Scientific Instrument Co., Hangzhou, Zhejiang Province, China) and run at 65°C for 5 min, 42°C for 60 min, and 70°C for 5 min for 40 cycles. Moreover, qRT-PCRs combining 1 μL cDNA, 1 μL Master Mix (Invitrogen, Carlsbad, CA, USA), 1 μL of forward primer and reverse primer (10 mmol/mL), and 7 μL ddH_2O were conducted in an automatic thermocycler (StepOnePlus Real-Time PCR System; Thermo Fisher Scientific, Waltham, MA, USA), run for 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 50 s and then at 75°C for 10 min. Relative mRNA transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in this study are provided in Table 1.

TABLE 3 Effect of simulated gastrointestinal environment on viability of Lr-0601.

Strain	Survival rate in artificial gastric juice pH 3.0(%)	Growth rate in bile salts(%)		
		0.10%	0.20%	0.30%
Lr-0601	119.53 ± 1.12	77.97 ± 1.56	60.78 ± 1.39	41.64 ± 0.06

Results are shown as means ± SD. Lr-0601, *Lacticaseibacillus rhamnosus* 2016SWU.05.0601.

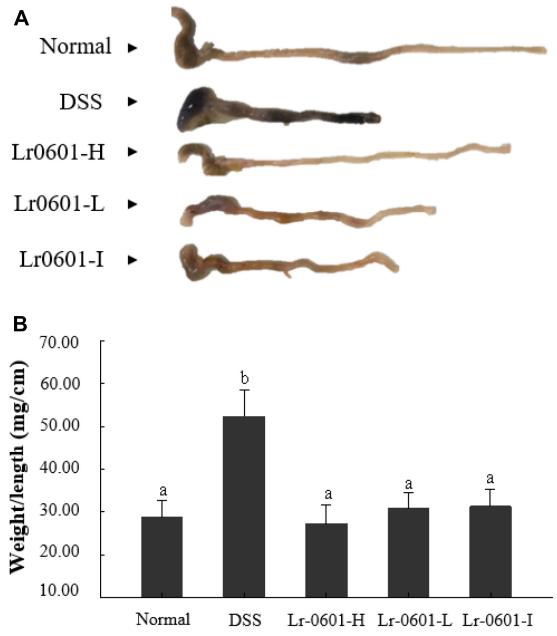

FIGURE 2
Morphological observation of colon in mice. **(A)** Colon length; **(B)** colon weight/length ratio. According to the Duncan's multiple range tests, significant ($p < 0.05$) was found between groups labeled with different lowercase letters (a, b). There was no significant difference ($p > 0.05$) between the groups marked with the same lowercase letters (a, b). Normal, normal mice; DSS, mice treated with DSS; Lr-0601-H, high dose of *L. rhamnosus* 2016SWU.05.0601 (1.0 × 1010 CFU/kg body weight (bw)/day); Lr-0601-L, low dose of *L. rhamnosus* 2016SWU.05.0601 (1.0 × 109 CFU/kg bw/day); Lr-0601-I: heat-inactivated *L. rhamnosus* 2016SWU.05.0601.

Statistical analysis

Data are presented in the form of means ± standard deviations. The statistical significance of differences among means was evaluated using ANOVA and Duncan's multiple range tests; $p < 0.05$ was considered statistically significant. The analysis was performed using SPSS 25.0 (IBM, Armonk, NY, USA).

Results

Strain identification results

The strain Lr-0601 isolated from yak yoghurt was observed by Gram staining, and 16s rDNA sequencing and biochemical identification were performed. As shown in Figure 1, strain Lr-0601 formed single colonies with consistent morphology on De Man, Rogosa, and Sharpe (MRS) medium agar plates, which were mostly white and round in morphology, with neat edges, and with moist and smooth surfaces. In addition, Gram staining of the strain revealed a short blue-purple rod with uniform cell morphology, which was determined to be a pure Gram-positive bacterium. Further 16s rDNA sequencing and biochemical identification showed that strain Lr-0601 was *Lacticaseibacillus rhamnosus* (Table 2).

Acid and bile salt resistance

Gastric juice is considered one of the primary physiological challenges to be faced by probiotic strains due to the low pH and antimicrobial effects of pepsin, while the survival of probiotics in the small intestine is considered another challenge due to the presence of bile salts and pancreatic enzymes in the small intestine. Thus the ability of strains to tolerate bile salts of gastric juice can reflect whether the strains can survive the passage through the upper gastrointestinal tract after entering the host and thus reach the intestine to exert beneficial effects. Lr-0601 exhibited a considerably strong tolerance *in vitro* in simulated gastrointestinal environment (Table 3). Lr-0601 was still vigorously grown in the

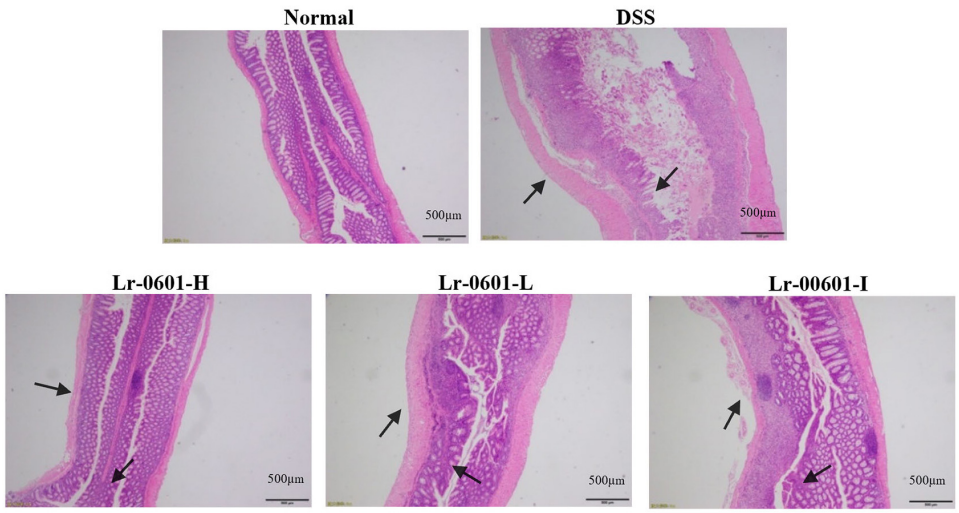

FIGURE 3
Histopathology observation of colon tissues in mice (magnification 100 ×). Representative inflammatory lesions and tissue damage in the colon are shown by arrows.

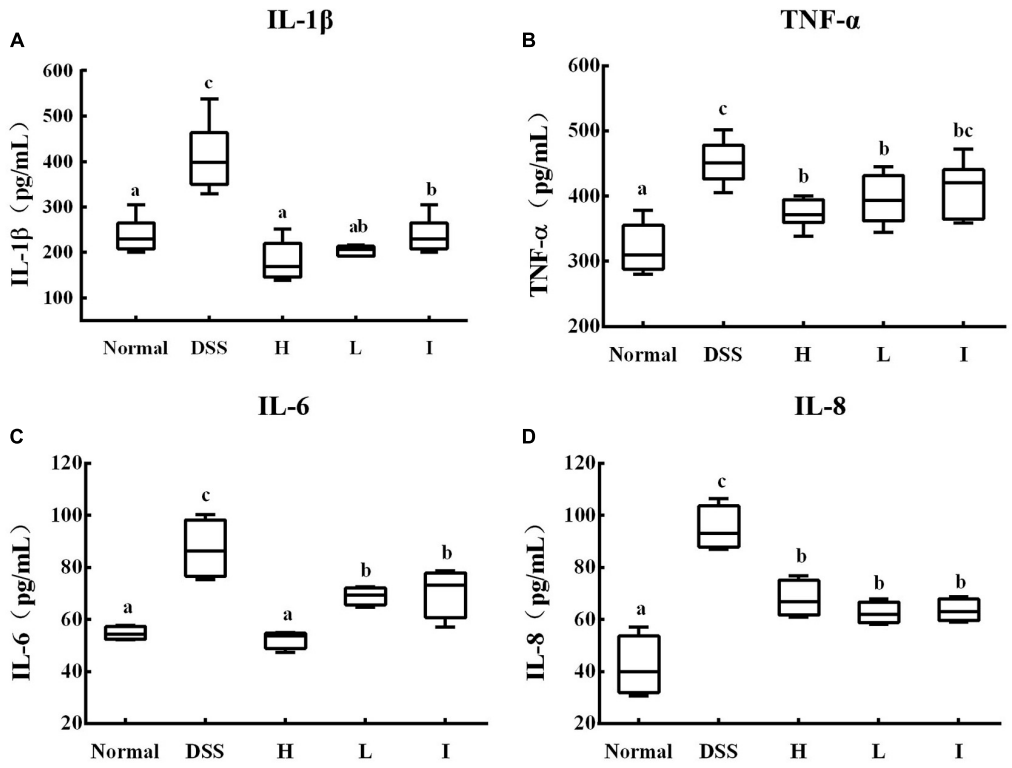

FIGURE 4
Levels of cytokines in serum of different experimental groups. **(A)** IL-1β cytokine level in serum; **(B)** IL-6 cytokine level in serum; **(C)** IL-8 cytokine level in serum; **(D)** TNF-α cytokine level in serum. a-c Mean values with different letters over the bars are significantly different ($p < 0.05$). Normal, normal mice; DSS, mice treated with DSS; H, high dose of *L. rhamnosus* 2016SWU.05.0601 (1.0×10^{10} CFU/kg body weight (bw)/day); L, low dose of *L. rhamnosus* 2016SWU.05.0601 (1.0×10^{9} CFU/kg bw/day); I, heat-inactivated *L. rhamnosus* 2016SWU.05.0601.

artificial gastric juice (pH 3.0), and growth rate reached 119%. The growth rate of Lr-0601 in bile salt solution decreased with increasing concentration but exhibited a higher growth ability in 0.3% bile salt solution.

Effect of *L. rhamnosus* 2016SWU.05.0601 on colon length and weight in mice with UC

DSS-induced colitis caused congestion and edema in the colon of mice, resulting in a significant increase in colon weight and a significant shortening of colon length (Figure 2A). The colon weight-length ratio is an important indicator of the severity of colitis, and in general, the higher the ratio is, the more pronounced the shortening and edema of the colon in the mouse, and the more severe the condition is. Compared with the normal group, the colon weight-length ratio of mice in the DSS group was significantly increased ($p < 0.05$), whereas after supplementation with different doses of Lr-0601 treatment, the shortening of the colon, edema and congestion were alleviated, and the colon weight-length ratio of mice was significantly decreased ($p < 0.05$). It is noteworthy that the colon weight-length ratio of mice in the Lr-0601-H group, was closer to that of the normal group (Figure 2B). Overall, it was demonstrated that Lr-0601 was effective in alleviating the adverse effects of DSS-induced colitis on the colon of mice.

Effect of *L. rhamnosus* 2016SWU.05.0601 on colon histology in mice with ulcerative colitis

Tissue damage can be visualized by H&E staining. Upon observation of pathological histological sections of the mouse colon, we found that DSS mice exhibited significant inflammatory cell infiltration, mucosal damage and crypt loss, and significant oedema and ulceration of the colon were observed (Figure 3). In contrast, colonic damage was significantly reduced in mice receiving Lr-0601 intervention, and all three intervention conditions showed positive improvement.

Effect of *L. rhamnosus* 2016SWU.05.0601 on serum cytokine levels in mice with ulcerative colitis

Serum inflammatory factor levels can reflect the level of body inflammation to a certain extent, so we detected several typical pro-inflammatory factors in serum, including IL-1β, IL-6, IL-8, and TNF-α. The results showed that compared with the normal group, the serum levels of IL-1β, IL-6, IL-8, and TNF-α in the DSS group of mice were significantly elevated ($p < 0.05$; Figures 4A-D), whereas supplementation with Lr-0601 was able to significantly reduce the

levels of these inflammatory factors and effectively alleviate the inflammation of the organism.

Effect of *L. rhamnosus* 2016SWU.05.0601 on mRNA expression of inflammatory regulatory genes and tight junction proteins in the colon of mice with ulcerative colitis

The intestinal barrier is the first line of defense to prevent harmful substances from entering the bloodstream, so the integrity of the intestinal barrier helps to maintain a normal intestinal micro-ecological balance and prevent the invasion of pathogens, which in turn serves to reduce inflammation and slow down disease progression. While the disruption of the intestinal barrier and body inflammation may be important triggers of ulcerative colitis, it was found that the expression of endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), IκB-α, ZO-1 and Occludin was significantly reduced in the DSS group, while the expression of iNOS, COX-2, and NF-κB was significantly increased when compared with that of the normal group ($p < 0.05$) (**Figure 5**). In contrast, supplementation with Lr-0601 effectively reversed this phenomenon. Overall, the administration of Lr-0601 was able to repair the intestinal barrier damage and alleviate the disease by modulating the expression of key genes in the NF-κB-iNOS/COX-2 signalling pathway to reduce the body inflammation.

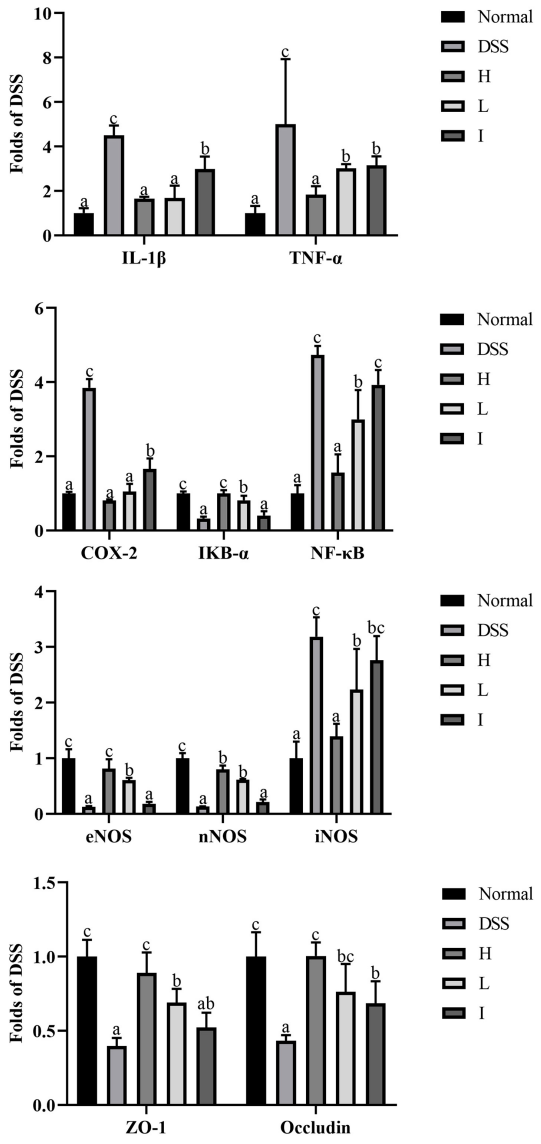

FIGURE 5
mRNA expression levels of TNF-α, IL-1β, eNOS, nNOS, iNOS, COX-2, IκB-α, NF-κB, ZO-1, and Occludin in colon tissues. a-c Mean values with different letters over the bars are significantly different ($p < 0.05$).

Discussion

DSS model is widely used in animal models of UC because this method because of its simplicity and reproducibility. Pathological changes similar to UC in the human colon occurred when mice ingest DSS (Zhang et al., 2018). DSS-sensitized intestinal epithelial cells can directly contact immune cells, promote the production of reactive oxygen species, impair the intestinal barrier integrity, as well as increase the recruitment of immune cells and the production of inflammatory cytokines (Shin and Kim, 2018). In the animal experiments, DSS-treated mice showed intestinal flora imbalance, decreased microbial diversity and abundance, and changes in specific bacterial species (Taylor et al., 2022). As the role of gut flora in the etiology of ulcerative colitis is well established, microbiota modulation is an attractive area for applying methods such as probiotic supplementation to reduce inflammation and induce intestinal homeostasis. Lactic acid bacteria, comprising a common type of probiotics, have been used for the treatment of colitis with beneficial effects on intestinal health. However, the beneficial effects and mechanisms involved in different probiotics vary (Astó et al., 2019), so it is important to explore different probiotics for the alleviation of UC in order for them to facilitate their clinical use and increase the availability of treatments for UC patients.

In addition, probiotics are generally considered as living microorganisms that are beneficial to human health after appropriate doses are administered to humans (Astó et al., 2019). Therefore, the prerequisite for probiotics to function is to enter the human body in the form of live bacteria and a certain degree of colonization in the intestine. However, it has been found that the metabolites or cellular components of probiotics can also have a positive impact on the body, and this part is defined by ISAPP as postbiotics (Cai et al., 2022; Liu et al., 2022). Whereas previous studies have focused more on probiotics application to ameliorate UC and less on the role of postbiotics, in this study we investigated the effects of both probiotic and postbiotic applications for the treatment of UC. In this study, we evaluated the beneficial effects of live bacteria of strain Lr-0601 and its inactivated substances on DSS-induced UC mice.

We observed that Lr-0601 exerted a positive colon injury-relieving effect in mice after entering the body in a live bacterial state, with high-concentration Lr-0601 having a better effect. Interestingly, the inactivated form of Lr-0601 also showed beneficial effects on DSS-induced colitis, suggesting that postbiotics show

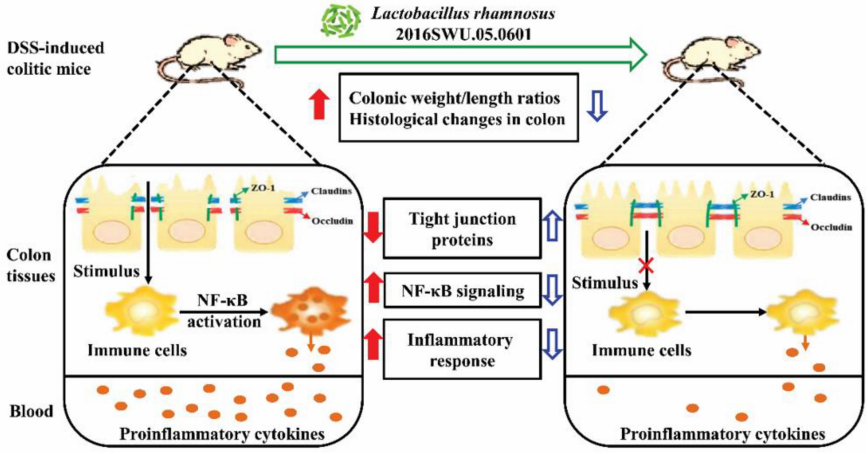

FIGURE 6
Protective effects of *L. rhamnosus* 2016SWU.05.0601 (Lr-0601) on dextran sulfate sodium (DSS)-induced colitis in mice. Lr-0601 increased the expression levels of tight junction proteins, which contributed to inhibit the activation of nuclear factor (NF)-κB signaling and inflammatory response in DSS-treated mice. These beneficial effects ultimately attenuated DSS-induced colitis. Red filled arrows represented changes in response to DSS; Blue unfilled arrows represented changes in response to DSS plus Lr-0601.

similar potential to probiotics in alleviating colitis symptoms. Although the present study did not analyze the specific substances responsible for the beneficial effects of postbiotics, previous studies have shown that a variety of substances such as cell surface and other types of proteins, peptides, endo- and exopolysaccharides, extracellular vesicles, short-chain fatty acids, bacteriocins, enzymes, phospholipidic acids, peptidoglycan-derived polypeptides, vitamins, acetylated phospholipids, and organic acids, or their free supernatants (CFS) and many others, are beneficial for the body's health, and that they have antimicrobial and immunomodulatory properties, and that they do not contain live microorganisms (Nataraj et al., 2020; Vallejo-Cordoba et al., 2020). These substances have antimicrobial, antioxidant and immunomodulatory effects, and their absence of live microorganisms, thus circumventing the risk of undesirable infections, are recommended for use in the treatment of UC.

Bacteriocins, cell wall and extracellular proteins from heat-inactivated probiotics were shown to reduce pro-inflammatory responses and inhibit cytokine-induced apoptosis in intestinal epithelial cells. In an animal study (Quévrain et al., 2016), a 15 kDa protein produced by Fusarium pratensis was identified to prevent UC by inhibiting the NF-κB signalling pathway in intestinal epithelial cells. Furthermore, in another cellular assay looking at the effects of heat-inactivated probiotics on UC, postbiotics were found to exert anti-inflammatory effects by inducing the secretion of IL-10 production by peripheral blood mononuclear cells from patients with UC, and inhibiting the secretion of IL-8 by HT-29 cells, thereby alleviating the effects of heat-inactivated probiotics (Imaoka et al., 2008). These substances such as bacteriocins, cell wall and extracellular proteins may also be the key components of Lr-0601 inactivator acting in this study and ameliorating UC by reducing inflammation.

Our results indicate that Lr-0601 and its postbiotic elements administered orally reduces the serum IL-1β, TNF-α, IL-6, and IL-8 levels in the DSS-induced UC model. These inflammatory cytokines play an important role in the development of UC. IL-1β and TNF-α can promote the infiltration of inflammatory substances by increasing the permeability of epithelial cells and endothelial cells, resulting in congestion, edema, and erosion of the colonic mucosa. In addition, aggregation of IL-1β and TNF-α stimulates the secretion of IL-6 and then activates the NF-κB signaling pathway (Neurath, 2022). IL-8 is the strongest chemokine, which aggravates inflammatory response by accumulating inflammatory cells to the colonic lesion (Chen et al., 2017). As a key transcription factor, NF-κB is widely distributed in various tissue cells and plays a key role in the occurrence and development of UC. In healthy state, NF-κB binds to its inhibitory protein IκB to form an inactive complex. In UC, IKK kinase induces phosphorylation of IκB and releases NF-κB from the complex. Free NF-κB then induces the production of inflammatory substances such as nitric oxide and prostaglandins (Li et al., 2020).

Simultaneously, NF-κB promotes the expression of downstream pro-inflammatory genes, such as IL-1β and TNF-α, which in turn further activate NF-κB, forming positive feedback regulation and expanding the inflammatory cascade (Shao et al., 2015; Dou et al., 2023). NO is an important inflammatory mediator in UC, and its synthesis is regulated by NOS. iNOS can continuously produce NO by catalyzing arginine, and eNOS and nNOS maintain NO synthesis balance (Hibiya et al., 2016). The inducible cyclooxygenase COX-2 is a key rate-limiting enzyme that induces arachidonic acid metabolism to produce prostaglandins (Hegazi et al., 2006). iNOS and COX-2 have binding sites for NF-κB; thus, their transcriptional expression is regulated by NF-κB. When NF-κB is overactivated in colon tissue, the activities of iNOS and COX-2 are correspondingly increased, inducing the production of large amounts of NO and PGE2. Excessive amounts of NO and PGE2 can dilate blood vessels and increase blood flow, causing congestion and edema of colon tissue. In the present study, Lr-0601 significantly inhibited the mRNA expression of NF-κB, iNOS, and COX-2 in the colon tissue of mice with UC as well as promoted the expression of IκB-α.

A complete intestinal barrier helps maintain intestinal homeostasis, and impaired intestinal barrier may contribute to

intestinal disease. Tight junctions are the structural basis for the formation of a cell bypass seal, regulating intercellular molecular transport and maintaining selective permeability of intestinal epithelial cells. Therefore, tight junctions are key to protecting the intestinal mucosa from harmful substances in the external environment (Wan et al., 2019). ZO-1 is a peripheral membrane protein in tight junction proteins that links transmembrane proteins to the cytoskeleton. And Occludin is a transmembrane protein in tight junction proteins that regulate substance transport and seal cell by pass (Capaldo et al., 2017). In the UC animal model, DSS accumulated in the intestinal tract of mice impairs intestinal barrier integrity, improves intestinal permeability, and prompts the recruitment of immune cells and inflammatory cytokine production, disrupting intestinal homeostasis (Shin and Kim, 2018). We confirmed these results by measuring the mRNA expression of ZO-1 and Occludin. It was also observed that supplementation with Lr-0601 and its postbiotics significantly upregulated these tight junction proteins and protected the intestinal barrier function, which plays an important role in the prevention of UC.

Overall, our study demonstrated that both Lr.0601 and its prepared postbiotics showed positive protection against UC, but the mechanism of their preventive effects on UC needs to be explored more deeply (**Figure 6**). In addition, we demonstrated the research and application potential of postbiotics in UC prevention, and compared with live probiotics, postbiotics are stable and biologically safe, they do not need to colonize the organism to exert beneficial effects after entering the organism, and it requires lower storage conditions than live bacteria, which makes it more suitable for diversified applications in different industries, but large-scale preclinical animal models and high-quality clinical studies in human are still needed to validate the safety and health effects of postbiotics. In conclusion, our findings highlight the beneficial effects of probiotics and postbiotics in UC remission and provide insights into the application of probiotic and postbiotic products in the prevention of UC, especially the multiple applications of postbiotics may be an effective complement to probiotics, which has a great potential as an extension of the direction of probiotics to provide a promising therapeutic strategy for live adjuvant therapies in the treatment of UC.

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

The animal study was approved by the Animal Care and Use Committee of Southwest University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LB: Data curation, Investigation, Writing – original draft, Writing – review & editing. YL: Data curation, Investigation, Writing – original draft, Writing – review & editing. CW: Resources, Writing – review & editing. YJ: Resources, Writing – review & editing. HS: Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was partly supported by the National Key Research and Development Plan (2018YFD0502404), Fundamental Research Funds for the Central Universities (SWU019026), Research Project of Sichuan Key Laboratory of Bamboo Diseases and Insect Pests Prevention and Control, and Resource Development (17ZZ016), China.

Conflict of interest

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

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frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 21 February 2024
DOI 10.3389/fmicb.2024.1360225

OPEN ACCESS

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RECEIVED 22 December 2023
ACCEPTED 06 February 2024
PUBLISHED 21 February 2024

CITATION
Xie W, Zhong Y-S, Li X-J, Kang Y-K, Peng Q-Y and Ying H-Z (2024) Postbiotics in colorectal cancer: intervention mechanisms and perspectives.
Front. Microbiol. 15:1360225.
doi: 10.3389/fmicb.2024.1360225

Postbiotics in colorectal cancer: intervention mechanisms and perspectives

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Colorectal cancer (CRC) is a common malignancy affecting the gastrointestinal tract worldwide. The etiology and progression of CRC are related to factors such as environmental influences, dietary structure, and genetic susceptibility. Intestinal microbiota can influence the integrity of the intestinal mucosal barrier and modulate intestinal immunity by secreting various metabolites. Dysbiosis of the intestinal microbiota can affect the metabolites of the microbial, leading to the accumulation of toxic metabolites, which can trigger chronic inflammation or DNA damage and ultimately lead to cellular carcinogenesis and the development of CRC. Postbiotics are preparations of inanimate microorganisms or their components that are beneficial to the health of the host, with the main components including bacterial components (e.g., exopolysaccharides, teichoic acids, surface layer protein) and metabolites (e.g., short-chain fatty acids, tryptophan metabolite, bile acids, vitamins and enzymes). Compared with traditional probiotics, it has a more stable chemical structure and higher safety. In recent years, it has been demonstrated that postbiotics are involved in regulating intestinal microecology and improving the progression of CRC, which provides new ideas for the prevention and diagnosis of CRC. In this article, we review the changes in intestinal microbiota in different states of the gut and the mechanisms of anti-tumor activity of postbiotic-related components, and discuss the potential significance of postbiotics in the diagnosis and treatment of CRC. This reviews the changes and pathogenesis of intestinal microbiota in the development of CRC, and summarizes the relevant mechanisms of postbiotics in resisting the development of CRC in recent years, as well as the advantages and limitations of postbiotics in the treatment process of CRC.

KEYWORDS

postbiotics, colorectal cancer, intestinal microbiota, metabolites, intestinal immunity

1 Introduction

Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract, ranking third worldwide in terms of both incidence and mortality rates (Bray et al., 2018). The etiology and progression of CRC is influenced by genetic factors, chronic inflammatory bowel disease, dietary habits, and environmental factors (Lund et al., 2011). The intestinal microbiota, as an important component of the gut, has been increasingly demonstrated to be involved in the development of CRC. The intestinal microbiota is a complex community

of bacteria, fungi, archaea, protozoa, and viruses (Costello et al., 2012). The intestinal microbiota plays a crucial role in the host's nutritional, metabolic, and immune functions, as well as other physiological processes by secreting a variety of metabolites (Dzutsev et al., 2015). Dysbiosis of intestinal microbiota is often manifested as a decrease in beneficial bacteria and an increase in harmful bacteria, and the disruption of this balance will result in the disturbance of microbial metabolites, leading to the accumulation of toxic metabolites, which in turn will lead to the destruction of the intestinal mucosal barrier, resulting in chronic inflammation and DNA damage, and ultimately triggering the cellular carcinogenesis and the development of CRC (Clinger and Hao, 2021).

Probiotics are defined as "live, non-pathogenic microorganisms that, when given in sufficient amounts, can be beneficial to the health of the host" and are mainly Bifidobacteria, *Lactobacillus* and other acid-producing bacteria, including *Streptococcus*, *Enterococcus*, and *Lactococcus*. Probiotics can play a role in maintaining a healthy intestinal microbiota, preventing the invasion of pathogenic microorganisms, and stabilizing and strengthening the intestinal barrier function through the secretion of anticancer or anti-inflammatory substances, such as short-chain fatty acids (SCFAs), vitamins K or B, and others. Prebiotics are mostly composed of non-starch polysaccharides and oligosaccharides that are difficult to be digested by enzymes in the body, which can provide nutritional support for beneficial bacteria in the intestinal tract, such as resistant starch, lactulose, inulin, oligofructose, oligogalactose and so on. And synbiotics are a mixture of probiotics and prebiotics, which can play the roles of both probiotics and prebiotics at the same time. NGP can be used as a preventive and therapeutic potential application for CRC, in contrast to traditional probiotics, which are based on macrogenomics studies that analyze microbiota differences between healthy and diseased individuals, identify live microorganisms and administer them in a strain-specific and dose-dependent manner, resulting in health benefits for the host (Martín and Langella, 2019). In addition, FMT is the most direct way to regulate the intestinal microbiota, and FMT can intervene and treat intestinal diseases by proposing specific flora from the flora of healthy donors, mating and culturing them and colonizing them in the patient's intestinal tract to rebuild the patient's intestinal microcosm, thus achieving intervention and treatment of intestinal diseases. More and more studies now show that oral administration of probiotics, NGP, or FMT can restore the balance of intestinal microbiota and thus achieve improvement in CRC progression (Eslami et al., 2019; Chen et al., 2022). However, being live microorganisms and the uncertainty of their growth during processing, there are potential biosafety risks associated with the use of live bacteria as a therapeutic strategy for CRC.

Postbiotics are preparations of inanimate microorganisms or their components that are beneficial to the health of the host, with the main components including bacterial components and metabolites. As a new type of biological agent, Postbiotic shows good benefits in regulating the balance of intestinal microbiota (Guéniche et al., 2010). It has a relatively stable chemical structure and higher biosafety than traditional probiotics, NGP and FMT. In recent years, there has been increasing evidence that oral administration of postbiotics can regulate intestinal microbiota, improve immunity and reduce the incidence of diarrhea (Yeom et al., 2021; Jung et al., 2023). Therefore, postbiotics has been emphasized as a complementary therapeutic strategy for CRC. This reviews the changes and pathogenesis of intestinal microbiota in the development of CRC, and summarizes the relevant mechanisms of postbiotics in resisting the development of CRC in recent years, and discusses the advantages and limitations of postbiotics in the treatment process of CRC.

2 The role of intestinal microbiota dysbiosis in the development of CRC

The human body harbors a wide variety of microorganisms, primarily located in the oral cavity, intestines, respiratory tract, skin, vagina, and other mucosal surfaces, creating a highly intricate microbial ecosystem. The intestinal tract, in particular, hosts a significant population of commensal bacteria, making it the largest reservoir of commensal microbiota. This bacterial community comprises approximately 800 species, collectively weighing around 1 to 2 kilograms (Zhang et al., 2018). The intestinal tract is considered the most abundant, diverse, and functionally significant microbial community in the human body (Meng et al., 2018). The intestinal microbiota demonstrates characteristics such as diversity, stability, resistance to drugs and antiretrovirals, and plays a crucial role in maintaining normal physiological functions and disease development. Dysbiosis of intestinal microbiota is primarily linked to changes in bacterial diversity, which leads to the proliferation of harmful bacteria in the gut. This can result in the release of virulence factors, suppression of the immune system, and stimulation of inflammation, ultimately contributing to the onset of CRC.

Microorganisms parasitizing the human gut are classified in the phylum *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Fusobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Spirochaetes*. Studies have shown that the composition of the intestinal microbiota differs between the healthy and CRC states, and these changes are summarized in **Figure 1** (Kvakova et al., 2021).

Under normal conditions, *Anaplasma*, *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Clostridium*, and *Escherichia coli* are the predominant species in the human intestinal tract, which participate in the formation of the intestinal mucosal barrier to inhibit the growth and invasion of harmful bacteria, and at the same time, they secrete metabolites, such as short-chain fatty acids, indole, etc., which affect the intestinal immunity and participate in the metabolism of the human body (Hooper and Gordon, 2001).

Whereas in disease states it is often characterized by a decrease in beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* and an increase in hamful bacteria such as enterotoxin producing *Bacteroides*, *Escherichia coli*, and *Clostridium difficile*. A study suggests that intestinal microbial species are strongly associated with the development of CRC, showing increased abundance of pro-inflammatory opportunistic bacteria such as *Bacteroides fragilis*, *Escherichia coli*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, and *Streptococcus gallolyticus* in patients with CRC. Meanwhile, the abundances of *Bifidobacterium*, *Clostridium*, *Faecalibacterium*, and *Roseburia declined* (Janney et al., 2020). The mechanisms by which intestinal microbiota affects CRC progression include destroying the intestinal mucosal

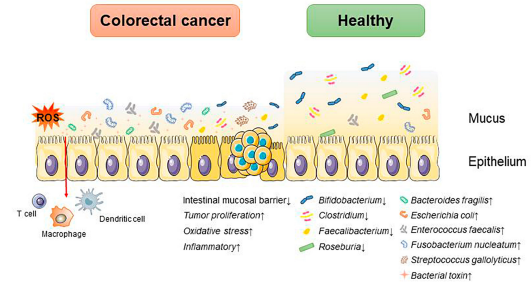

FIGURE 1
Dysbiosis of intestinal microbiota accelerates the progression of CRC. An increase in associated pathogenic bacteria promotes CRC progression by disrupting the intestinal mucosal barrier, triggering inflammatory responses and oxidative stress, and altering the tumor proliferation cycle.

barrier; regulating the cell cycle of CRC tumor cells, promoting CRC proliferation and metabolism; reprogramming the tumor immune microenvironment; causing DNA damage; triggering inflammatory responses; inducing gene mutations and altering the resistance to tumor chemotherapy, and so on. These mechanisms are summarized in Table 1.

3 Application of postbiotics in colorectal cancer

Past studies have revealed the use of microbial agents such as probiotics in CRC control. However, the biosafety of the products is somewhat controversial due to numerous uncertainties in the production, transportation and storage of these products. There is growing evidence that the health benefits of intestinal microbes may not require intact microbes, and that their inactivated by-products (including bacterial fragments and extracts) can still be useful. This is the category later associated with postbiotics. Postbiotics are preparations of inanimate microorganisms and their components that are beneficial to the host (Salminen et al., 2022). The main components of postbiotics include inactivated bacteria, bacterial fractions (cytosolic polypeptides, phosphoglycolic acids, intracellular and extracellular polysaccharides (EPSs), and surface proteins), and their metabolites (short-chain fatty acids (SCFAs), organic acids, bacteriocins, and enzymes) (Homayouni Rad et al., 2021). The mechanism of action of postbiotics to improve CRC progression is similar to that of prebiotics. It includes regulating intestinal microbiota, enhancing intestinal mucosal barrier function, regulating immune response and regulating systemic metabolism (Figure 2). However, compared to probiotics, postbiotics have a higher safety profile, better generalizability, longer shelf life and faster biological activity. The mechanism of improvement of CRC process by postnatal meta-related components is shown in Figures 3 and 4.

3.1 SCFAs

Firmicutes, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* all create SCFAs, which can give host intestinal cells energy. SCFAs play an important role in gut microenvironmental homeostasis as essential components of the glycolytic process, act as protective molecules in the development

TABLE 1 Mechanisms associated with CRC progression induced by intestinal microbes.

Organism	The role for CRC	Mechanism	References
Fusobacterium nucleatum	Promote	• Regulates the E-cadherin/β-catenin signaling pathway and promotes tumor cell proliferation. • Activation of NF-κB pathway/RAS-MAPK pathway via MYD88 enhances CRC cell proliferation. • Increased H3K27ac histone modification in CRC cells, which activates glycolysis and carcinogenesis in CRC. • Activation of the NF-κB pathway/Recruits bone marrow-derived immune cells for CRC development. • Inhibits NK cell and T cell activity/Promotes M2-like polarization of macrophages to promote tumor immune escape. • Increased levels of *Fusobacterium nucleatum* promoted microsatellite instability (MSI) with CpG island methylation phenotype (CIMP) in CRC. • Activation of the TLR4/AKT/Keap1/NRF2 signaling pathway increases the production of 12 and 13-EpOME and promotes CRC metastasis. • Activation of autophagy to induce chemoresistance.	Kostic et al., 2013; Tahara et al., 2014; Gur et al., 2015; Sivan et al., 2015; Wang and Huycke, 2015; Abed et al., 2016; Mima et al., 2017; Yu et al., 2017; Chen et al., 2018, 2020; Rubinstein et al., 2019; Hong et al., 2021; Hu et al., 2021; Zheng et al., 2021
Escherichia coli	Promote	• DNA damage • Increased IL-17c expression and inhibited tumor cell apoptosis by increasing bcl-2 and bcl-xl expression. • Pro-inflammatory infiltrate	Cuevas-Ramos et al., 2010; Song et al., 2014; Yu and Fang, 2015; Veziant et al., 2016; Bertocchi et al., 2021; Dalal et al., 2021; Lee et al., 2021
ETBF	Promote	• Release of BFT, leading to E-cadherin cleavage with intestinal epithelial cell detachment and disruption of the intestinal epithelial barrier. • Th17 immune response, promotes IL-6, TNF-α production, activates STAT3, NF-kB signaling pathway and promotes tumorigenesis. • DNA damage.	Destefano Shields et al., 2011; Goodwin et al., 2011; DeDecker et al., 2021
Escherichia coli	Promote	• It leads to CIN in epithelial progenitor cells, resulting in gene mutations.	Wang et al., 2015; Wang and Huycke, 2015

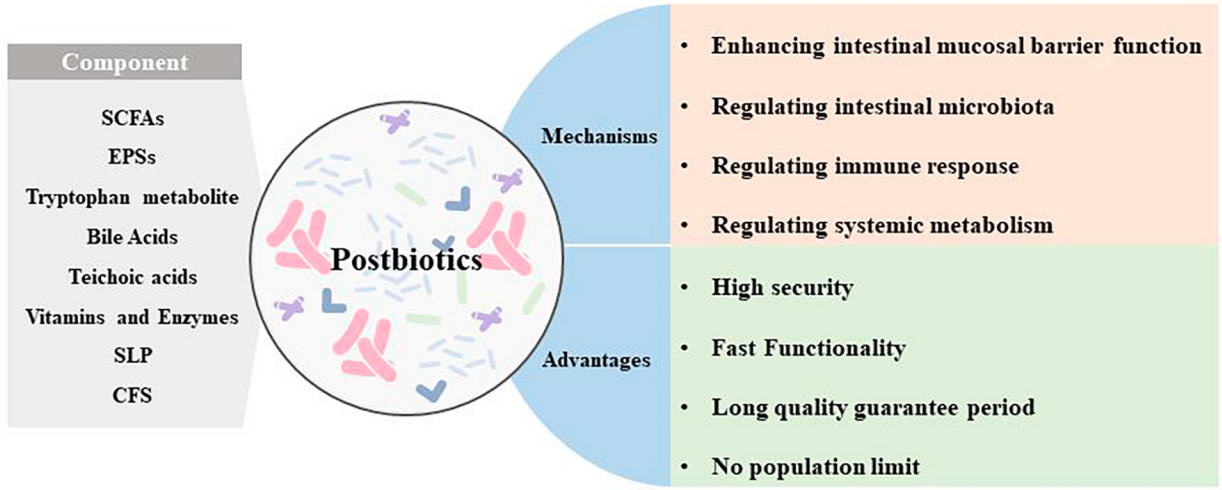

FIGURE 2
Main components, mechanism of action, and advantages of postbiotics.

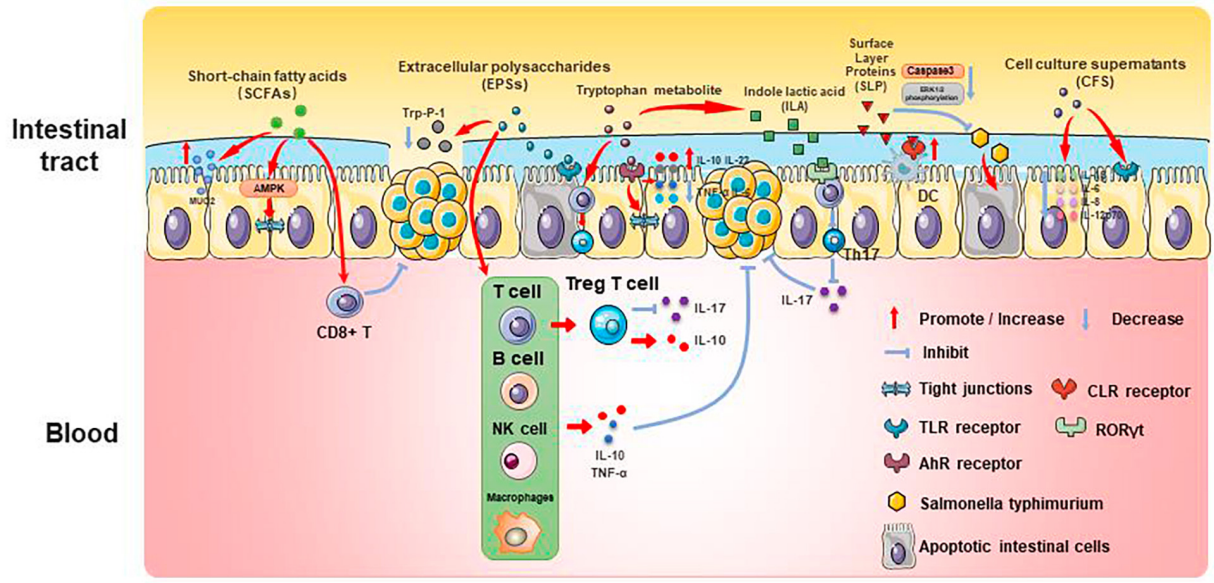

FIGURE 3
Mechanisms associated with postbiotic-related components protecting the gut and delaying CRC progression.

of CRC, and change in response to changes in the composition of the intestinal microbiota. Therefore, SCFAs are closely associated with the development of CRC.

Butyric acid is produced by anaerobic bacteria in the intestinal tract, supplying energy to intestinal epithelial cells and inhibiting inflammatory responses and tumorigenesis. Butyric acid maintains the tight junctions of intestinal epithelial cells by activating the AMPK pathway and protects the intestinal mucosal barrier by inducing MUC2 secretion (Chen and Vitetta, 2021). Butyric acid regulates endogenous nervous system excitability and promotes intestinal motility, thereby preventing the development of CRC. Butyric acid also inhibits HDAC deacetylation and promotes apoptosis in CRC cells (Donohoe et al., 2014). In addition, butyric acid modulates the neuropilin-1/vascular endothelial growth factor pathway to inhibit CRC angiogenesis (Yu et al., 2010). It induces apoptosis in CRC tumor cells by inhibiting the Wnt/β-catenin signaling pathway (Wu et al., 2018). Butyric acid also promotes anti-tumor effects by modulating CD8 + T cell activity (He et al., 2021).

Acetic acid can be converted to butyric acid by bacteria such as *Roseburia* spp., *F. prausnitzii* and *Coprococcus* sp. to exert CRC inhibitory effects. Propionic acid can also act as an HDAC inhibitor to promote CRC apoptosis, but it is less effective than butyric acid. Formate secreted by *Fusobacterium nucleatum* can set off the AhR pathway, thus promoting CRC tumor invasion, and can serve as a tumor metabolite associated with CRC progression (Ryu et al., 2022; Ternes et al., 2022).

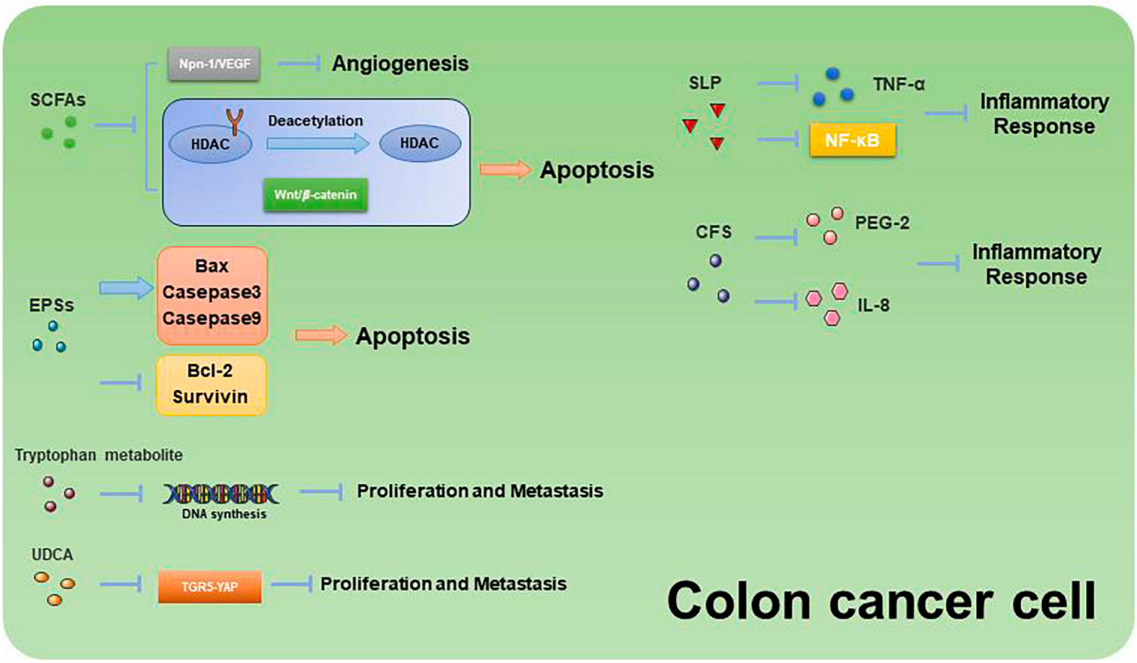

FIGURE 4
Mechanisms related to the promotion of apoptosis and inhibition of proliferation and metastasis in CRC cells by postbiotic-related components.

3.2 EPSs

Extracellular polysaccharides are bacterial surface macromolecules in the form of pods or pericellular mucus, which have a variety of biological functions such as immunomodulation, antitumor, and antioxidant. Previous studies have shown that EPSs inactivate the oncogenic component Trp-P-1 and alleviate the cancer process (Tsuda et al., 2008). In addition, EPSs can play an anti-inflammatory and anti-tumor role by enhancing the activity of T cells, B cells, NK cells and macrophages, promoting the expression of cytokines such as TNF-α and IL-10, and inducing apoptosis of tumor cells and scavenging of free radicals. For example, EPSs can inhibit the development of inflammation by binding to the TLR2 receptor of intestinal epithelial cells, inducing apoptosis and stimulating Treg T cells, inhibiting IL-17 production and promoting IL-10 expression (Levy et al., 2017; Zhou et al., 2017). In a study of the effect of EPSs on HT-29 proliferation, EPSs produced by *Lactobacillus lactis* promoted apoptosis and inhibited the proliferation of HT-29 cells by promoting the expression of Bax, Caspase3, and Caspase9, while decreasing the expression of Bcl-2 and Survivin genes (Tukenmez et al., 2019).

Extracellular polysaccharides can also affect the content of SCFAs to play an anti-inflammatory role. β-Glucan fermentation produces SCFAs, which activate macrophage Dectin-1 receptor, as well as T cells and NK cells, and enhances the immune response (Jayachandran et al., 2018; Chen and Li, 2020).

3.3 Tryptophan metabolite

Tryptophan is an essential amino acid that can be consumed from foods such as chicken, eggs, cheese and chocolate. It can be metabolized by bacteria (mainly *Lactobacillus reuteri* and *Clostridium sporogenes*) in the human intestinal tract to a variety of products such as 5-hydroxytryptamine (5-HT), kynurenine and indole, etc. These metabolites can be used as ligands for aromatic hydrocarbon receptors (AhR), and play an important role in the regulation of intestinal immune homeostasis, mucosal barrier function, inflammatory response and neural function.

Several studies have found that indole derivatives can inhibit inflammatory responses and improve the intestinal mucosal barrier by activating AhR, promoting the secretion of IL-22 and IL-10, decreasing the expression of TNF-α and IL-6, and increasing the production of tight junction proteins and mucins in intestinal epithelial tissue (Lanis et al., 2017; Shi et al., 2020). In addition, indole derivatives can reprogram CD4 + T cells in the intestinal epithelium to Treg T cells, street tumor immunity (Cervantes-Barragan et al., 2017). Another study showed that the tryptophan metabolic end product 8-hydroxyquinaldic acid could inhibit DNA synthesis and suppress proliferation and metastasis of colon cancer cells HT-29 and LS-180 (Walczak et al., 2020). A recent study of the chemopreventive effects of statins on CRC found that in a mouse model of CRC, atorvastatin inhibited tryptophan consumption by inhibiting the expression of indole derivatives in the intestinal epithelium, which in turn inhibited the consumption of tryptophan by intestinal epithelial cells, resulting in increased tryptophan concentration in the gut, increased abundance of *Lactobacillus reuteri*, whose catabolism of tryptophan produces the indole-3 -lactic acid (ILA) targets the transcription factor RORγt, which inhibits Th17 differentiation, decreases IL-17 expression, and inhibits CRC development (Han et al., 2023). This study demonstrates the role of tryptophan metabolites in mediating pharmacologic prevention of CRC.

3.4 Bile acids (BAs)

Primary bile acids include cholic acid (CA) and goose deoxycholic acid (CDCA), and there is increasing evidence that BA metabolism is strongly associated with CRC. CA and CDCA can promote CRC development by activating the NF-kB and JAK2/STAT3 pathways (Guan et al., 2022). In addition, a study showed that CA with CDCA was given to *N*-methyl-N′nitro-N-nitrosoguanidine-treated mice, which had a higher tumorigenicity than germ-free mice, demonstrating the critical role of BAs in CRC (Ridlon et al., 2016a).

Deoxycholic acid (DCA) is one of the secondary bile acids produced by *Clostridium*. DCA levels were found to be significantly elevated in CRC patients and correlated with intestinal mucosal hyperplasia. Studies have shown that the main mechanism by which DCA causes CRC is the activation of COX-2 and lipoxygenase, which catabolizes arachidonic acid to produce prostaglandins and ROS, triggering inflammation, angiogenesis, DNA damage, and inhibition of DNA damage repair (Cai et al., 2022). In addition, DCA stimulates the ERK signaling pathway and regulates p53 expression, thereby promoting the development of CRC.

Lithocholic acid (LCA) is another secondary bile acid produced by *Clostridium*. Similar to DCA, LCA can induce CRC by damaging the intestinal mucosa through the production of ROS, reducing apoptosis, enhancing cell proliferation, contributing to DNA damage, and stimulating inflammatory responses (Sinha et al., 2020). In addition, LCA regulates muscarinic 3 receptor and Wnt/β-catenin pathways to promote tumor stem cells in CRC (Farhana et al., 2016). LCA also induced the expression of MMP-1, MMP-2, and MMP-7 genes and stimulated the urokinase plasminogen activator receptor, promoting invasion and metastasis of CRC cells (Rossi et al., 2020). Another study found that LCA elevated the expression of the cytokine IL-8, which activated the ERK/MAPK signaling pathway, thereby inhibiting STAT3, stimulating CRC angiogenesis, and promoting CRC development (Nguyen et al., 2017).

Ursodeoxycholic acid (UDCA) has been reported to be protective against digestive disorders. However, its effect on CRC remains controversial. Studies have shown that UDCA can play a therapeutic role in reducing intestinal inflammation by modulating the epidermal growth factor receptor/ERK pathway and reducing harmful secondary bile acids (DCA and LCA) (Ridlon et al., 2016b). In addition, UDCA inhibited the activation of COX-2 by DCA and suppressed CRC progression through the TGR5-YAP pathway (Zhang et al., 2021). However, it has also been reported that UDCA can be converted by microorganisms into harmful secondary bile acids that promote the development of CRC. Meanwhile, the study found no significant effect of UCDA in reducing cancer risk (Sorbara and Pamer, 2022). These studies suggest that modulation of bile acid production, especially DCA with LCA, may have a positive effect on the prevention and treatment of CRC.

3.5 Teichoic acids (TA)

Teichoic acids (TA) are the main components of the cell walls of Gram-positive bacteria, and studies have shown that TA and lipoteichoic acid (LTA), obtained by extraction with butanol and phenol, can mediate immune responses by inhibiting the production of certain inflammatory factors (e.g., IL-12 and IL-10) (Kaji et al., 2010) and modulating the function of Treg T cells to suppress intestinal inflammation and maintains intestinal homeostasis, and exerts antitumor and antioxidant effects (Tomkovich and Jobin, 2016). However, some studies have found that LTA not only fails to reduce the inflammatory response, but also causes damage to the intestinal mucosa (Zadeh et al., 2012). Therefore, further studies on the protective effects of TA and LTA on the intestinal tract are needed.

3.6 Vitamins and enzymes

In addition to obtaining vitamins from food, animals synthesize some vitamins through gut microbes, which play an important role in the storage and conversion of vitamins in the body. Vitamin D can regulate intestinal immunity by influencing T cell activation. Vitamin A plays an important role in the functional integrity of the cuprocytes. Deficiency of vitamin A and its receptors can lead to disruption of the intestinal mucosal barrier and disruption of the intestinal immune system, increasing the risk of intestinal infections and injuries.

Gut microbes also produce antioxidant enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase and reduced coenzyme I oxidase to protect against oxidative damage caused by ROS.

3.7 SLP

Surface Layer Proteins (SLP) are bioactive macromolecules encapsulated on the surface of the cell wall of many bacteria and archaea, and are involved in the regulation of various cellular physiological and biochemical processes. Studies have shown that SLP from lactic acid bacteria modulate intestinal inflammation by resisting the adhesion and invasion of pathogenic bacteria, regulating Th1 and Th17 activities, and influencing the immune response.

Normal apoptosis mediates clearance of invading bacteria and facilitates repair of the intestinal epithelial barrier. In contrast, abnormal apoptosis induced by pathogenic bacteria leads to inflammation. SLP of various *Lactobacillus* species play important roles in anti-inflammatory and immunomodulatory aspects. SLP of *Lactobacillus acidophilus* antagonizes Salmonella typhimurium-induced apoptosis by inhibiting caspase-3. SLP of *Lactobacillus acidophilus* ATCC-4356 induced cell proliferation and differentiation by inhibiting Salmonella typhimurium-induced apoptosis in Caco-2 cells and decreasing ERK1/2 phosphorylation (Li et al., 2011). In addition, SLP of *Lactobacillus acidophilus* NCFM binds to C-type lectin receptors (CLRs) on the surface of dendritic cells, regulates intestinal immunity, enhances intestinal barrier function, and slows down inflammation (Konstantinov et al., 2008).

Surface Layer Proteins isolated from Propionibacterium fischeri reduced TNF-α levels in HT-29 cells (Rabah et al., 2018). SLP isolated from *Lactobacillus helveticus* MMLh5 inhibited the NF-κB pathway in Caco-2 cells and exerted anti-inflammatory effects (Taverniti et al., 2013). SLP from *Lactobacillus plantarum*

reversed pathogenic E. coli-induced intestinal epithelial cell damage (Liu et al., 2011).

3.8 CFS

Probiotic cell culture supernatants contain organic acids, short-chain fatty acids, bacteriocins, and other active substances, and studies have shown that the probiotic CFS has a health-promoting effect on human health. In a study in which dendritic cells attacked by E. coli were treated with *Lactobacillus rhamnosus* and its CFS, the results showed a significant decrease in the levels of pro-inflammatory factors (IL-1β, IL-6, IL-8, and IL-12p70) in CFS-treated cells as compared to *Lactobacillus rhamnosus*, indicating that in the presence of E. coli CFS is more effective than probiotics in reducing the secretion of pro-inflammatory factors (Bermudez-Brito et al., 2014). CFS of *Bifidobacterium shortum* CNCM?-4035 down-regulates pro-inflammatory pathways by reducing pro-inflammatory factors and chemokines in dendritic cells attacked by Salmonella typhi through activation of the TLR and protects the body against highly infectious pathogens such as Salmonella typhi (Bermudez-Brito et al., 2013). CFS of probiotics such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactococcus lactis*, *Lactobacillus rohita*, and *Saccharomyces boulardii* down-regulate the expression of PGE-2 and IL-8 in HT-29, as well as regulating the expression of IL-1β, IL-6, TNF-α, and IL-10 in macrophages, exerting anti-inflammatory effects (De Marco et al., 2018). In addition, CFS of *Lactobacillus plantarum* not only reverses the resistance of tumor cells to 5-FU and reduces CRC stem cells, but also exerts specific antiproliferative and apoptosis-inducing effects on tumor cells, while having no effect on normal cells (Chuah et al., 2019). This suggests that CFS of *Lactobacillus plantarum* can be used as a complementary and adjuvant therapy for CRC.

4 Advantages

Compared with traditional probiotics, NGP and FMT, which regulate intestinal microbiota and protect intestinal function, postbiotics have higher safety, faster functionality, longer quality guarantee period and no population limit, etc. First of all, postbiotics are the bacterial components and bacterial metabolites obtained by non-heat inactivation technology, with a clear chemical structure and composition, so it can avoid the serious safety hazards of traditional probiotics therapy such as bacterial translocation, increasing the host's resistance to antibiotics, transmission of drug-resistant genes or contamination of pathogenic bacteria, etc., and it has a higher level of safety. Secondly, traditional probiotics therapy often need to pass through gastric acid and bile salts, colonize in the intestines and finally participate in intestinal metabolism before they can exert their biological activities. As a direct metabolite of intestinal microbiota, probiotics can be directly absorbed by the intestines after drinking and participate in intestinal metabolism to exert their biological activity, which has a faster onset of action. During the production and storage of traditional probiotics, the characteristics of the bacteria themselves and the environment (including temperature, humidity, pressure and oxygen content, etc.) may lead to a reduction in the biological activity or death of the bacteria, thus affecting the quality and efficacy of the product, and may even lead to adverse reactions. In fact, there have been reports of discrepancies between the actual probiotic content and the standardized content of commercial probiotic products. Postbiotics, on the other hand, after being processed by inactivation technology, exhibit heat-resistant, acid-resistant and durable properties, and are not easily interfered with by antibiotics and other substances, providing a Longer and more stable shelf life with higher therapeutic benefit. Finally, for special groups such as children and sensitive people, postbiotics do not have the risks associated with the application of traditional probiotics, and there is no restriction on the number of people, so they have a wider range of applications.

However, of the 7 randomized controlled trials of 1,740 children younger than 5 years of age, 3 assessed adverse reactions in subjects. In 1 trial investigating *Lactobacillus*-origin postbiotics on acute diarrhea in children, 36 of the 40 children in the trial group experienced symptomatic relief, 1 experienced an adverse reaction (severe dehydration), and the remaining 2 studies showed no adverse reactions in subjects (Malagón-Rojas et al., 2020). This suggests that there may still be some adverse reactions to postbiotic, but the incidence is low. Subsequent studies should pay attention to improving the handling and preservation of postbiotic products, improving the safety of postbiotic products, and decreasing the incidence of adverse reactions to postbiotic products.

5 Conclusion and outlook

Intestinal microbiota and its metabolites are closely related to the progression of CRC. Intestinal microbiota can influence the development of CRC by altering the intestinal microenvironmental homeostasis, influencing the intestinal immune response, and secreting a variety of metabolites. Since certain bacteria are more likely to affect the development of CRC, together with the differences in the composition and abundance of the flora in CRC patients have been reported in this study.

More and more microbial agents protect the intestinal barrier function by regulating the composition and function of intestinal microorganisms, and Postbiotic stands out among many microbial agents due to its unique safety and stability. Numerous studies have shown that postbiotics exert anti-tumor effects by regulating the composition of intestinal microorganisms, immunomodulation, inhibiting proliferation and inducing apoptosis, enhancing the intestinal mucosal barrier, and regulating systemic metabolism. Therefore, the prospect of postbiotic as an adjuvant and supplemental agent for tumor treatment is wide-ranging. In addition, certain postnatal metabolic components, such as DCA, are significantly elevated in CRC patients and can be used as an indicator for early diagnosis and screening for CRC.

Despite the unique advantages of postbiotics in microbial preparations, there are still many challenges in the application of postbiotics in the prevention and treatment of CRC. Firstly, the complexity of the components and the non-uniformity of the production process of postbiotic formulations have led to some ambiguity in the definitional scope of postbiotics; therefore, more in-depth studies are needed to investigate the mechanism of action of postbiotic-related components and to determine a reasonable

range of components as well as a uniform production process. Secondly, in the research and development of postbiotics, it is recommended to prioritize the selection of microorganisms that have completed safety evaluations and the safety evaluations of the prepared postbiotic components, rather than relying solely on the safety of the original strains of bacteria used in the preparation of postbiotics to judge the safety of postbiotics. Thirdly, traditional probiotics have clear dosage standards, while the qualitative and quantitative analysis methods have not been established for postbiotics, and there are certain problems in the quality regulation and use of postbiotics, so it is necessary to construct a quantitative and effective relationship evaluation method of the role of postbiotics to provide theoretical support for the development of the postbiotics industry. In addition, the interaction with food components should be considered during the use of postbiotic elements. Finally, although recent studies on postbiotics have demonstrated the potential for CRC prevention and treatment, the related mechanism of action still requires further research, and there is still a lack of more clinical studies on the effective treatment of CRC. In conclusion, as an emerging field, the advantages and potential of postbiotics in CRC diagnosis and treatment still deserve attention and expectation.

Author contributions

WX: Writing – original draft, Writing – review and editing. Y-SZ: Funding acquisition, Writing – review and editing. X-JL: Writing – original draft, Writing – review and editing. Y-KK: Writing – original draft, Writing – review and editing. Q-YP: Writing – review and editing. H-ZY: Funding acquisition, Writing – review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by specific project of Zhejiang Academy of Medical Sciences (Hangzhou Medical College) (No. 2006Y).

Conflict of interest

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

Publisher's note

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TYPE Review
PUBLISHED 04 March 2024
DOI 10.3389/fmicb.2024.1359949

OPEN ACCESS

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RECEIVED 22 December 2023
ACCEPTED 21 February 2024
PUBLISHED 04 March 2024

CITATION
Chen Q, Li X-J, Xie W, Su Z-A, Qin G-M and Yu C-H (2024) Postbiotics: emerging therapeutic approach in diabetic retinopathy. *Front. Microbiol.* 15:1359949. doi: 10.3389/fmicb.2024.1359949

Postbiotics: emerging therapeutic approach in diabetic retinopathy

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Diabetic retinopathy (DR) is a prevalent microvascular complication in diabetic patients that poses a serious risk as it can cause substantial visual impairment and even vision loss. Due to the prolonged onset of DR, lengthy treatment duration, and limited therapeutic effectiveness, it is extremely important to find a new strategy for the treatment of DR. Postbiotic is an emerging dietary supplement which consists of the inactivate microbiota and its metabolites. Numerous animal experiments have demonstrated that intervention with postbiotics reduces hyperglycemia, attenuates retinal peripapillary and endothelial cell damage, improves retinal microcirculatory dysfunction, and consequently delays the progression of DR. More strikingly, unlike conventional probiotics and prebiotics, postbiotics with small molecules can directly colonize the intestinal epithelial cells, and exert heat-resistant, acid-resistant, and durable for storage. Despite few clinical significance, oral administration with postbiotics might become the effective management for the prevention and treatment of DR. In this review, we summarized the basic conception, classification, molecular mechanisms, and the advances in the therapeutic implications of postbiotics in the pathogenesis of DR. Postbiotics present great potential as a viable adjunctive therapy for DR.

KEYWORDS

postbiotic, gut-retinal axis, short chain fatty acids, secondary bile acids, safety

1 Introduction

Diabetes mellitus (DM) is a chronic disease that is widely prevalent worldwide, with an extremely high incidence, leading to a variety of complications such as macrovascular or microvascular lesions. The number of adults with this disease has more than tripled in the last two decades. According to the IDF Diabetes Atlas, there are currently 537 million people living with diabetes globally, a number that is expected to rise to 784 million by 2045 (Sun et al., 2022). Diabetic retinopathy (DR) is the most common microvascular complication in DM patients with poor glycemic control. Its pathogenesis is complex, involving a variety of causative factors. Currently, it is believed that chronic hyperglycemia is the primary basis of its pathogenesis, leading to microvascular damage and retinal dysfunction (Song et al., 2021). It has been reported that approximately 35% of diabetic patients have varying degrees of retinopathy, with nearly 10% at risk of progressing to blindness (Lin et al., 2021). It is widely recognized that DR is the leading cause of DM-related visual impairment or blindness in

working-age and elderly individuals worldwide (Teo et al., 2021). However, the underlying mechanisms of DR pathogenesis remain unclear, and there are no specific drugs for its treatment.

Recently, gut dysbiosis has been demonstrated to be one of the important causes of DR (Huang et al., 2021; Bai et al., 2022; Liu et al., 2022). Numerous studies have shown that the intestinal microbiota in the patients with type 2 diabetes mellitus (T2DM) is often altered; these changes can not only affect intestinal glucose metabolism, but also cause insulin resistance, inflammation, oxidative stress, vascular endothelial dysfunction, and other pathological damage by altering various pathways, potentially leading to serious ocular complications (Liu et al., 2022, 2023). Therefore, regulating gut microbiota has become a novel strategy for treating DR. Microecological agents, such as probiotics, prebiotics, and synbiotics, play a crucial role in maintaining the balance of gut microbiota and enhancing blood glucose levels. However, their use is limited during certain special circumstances, such as pregnancy, immunodeficiency, and severe infections.

Postbiotics are defined as "inactivated bacteria and bacterial components that have a beneficial effect on the host." They include cellular components, secreted materials, metabolites, and non-viable microorganisms, which play the vital roles in restoring gut microbiota and alleviating microangiopathy. Given their unique biological activity and potential to replace antibiotics, postbiotics have been widely used in general food, health food, and gastrointestinal therapeutic drugs (Tsai et al., 2019). Especially, it can lower blood glucose levels, improve insulin sensitivity, and shorten DR duration. Therefore, non-viable postbiotics have recently been used as a better alternative for the treatment and prevention of metabolic diseases and their complications (Abdelazez et al., 2022; Mishra et al., 2023). This paper summarizes the various types of postbiotics and their potential benefits in preventing DR and highlights recent advancements in their clinical applications.

2 Gut dysbiosis and its relation to DR

Gut dysbiosis is a common characteristic of patients with DR, leading to a remarkably reduction in the bacterial abundance and species (Figure 1). The gut microbiome of patients with DR was more distinct than that of patients with T2DM and healthy controls (HC). Compared with HC, there was a reduced abundance of anti-inflammatory genera such as *Roseburia*, *Lachnospira*, and *Blautia* in T2DM patients. In addition to those 3 genera, other genera like *Faecalibacterium*, *Bifidobacterium*, *Ruminococcus*, *Mitsuokella*, *Streptococcus*, *Lactobacillus*, and *Butyrivibrio* were also found to be decreased in DR patients (Das et al., 2021). The absence of these microbiota would reduce the production of organic acids, such as butyric acid and lactic acid, as well as the secretion of anti-inflammatory mediators, such as IL-10 and anti-inflammatory protein MAM, in intestine. This reduction affected the intestinal barrier function, leading to a systemic inflammatory response. In particular, elevated serum endotoxin directly accelerated damage to diabetic retinal endothelial cells and promoted the progression of DR (Tian et al., 2021; Huang et al., 2023). But in another clinic trail, four pro-inflammatory pathogens (*Aspergillus*, *Diutina*, *Pseudogymnoascus* and *Cladorrhinum*) were sharply decreased in DR patients compared with HC (Jayasudha et al., 2020). Additionally, gut dysbiosis could compromise the intestinal mucosal barrier by producing metabolic products like trimethylamine oxide (TMAO), lipopolysaccharides (LPS), choline, and amino acid metabolites (Liu et al., 2022; Xue et al., 2023). This can lead to increased intestinal permeability and temporary disruption of the blood-retinal barrier, allowing these substances to enter the eye and trigger host immune response, thereby contributing to the development and advancement of ocular diseases.

Although multiple mechanisms for changes in microvascular and glial components have been identified, the functional and structural mechanisms of cellular damage and cellular adaptive changes in the retina remain unknown due to the complex pathogenesis of DR. It had been reported that intermittent fasting alters gut microbiota composition and/or circulating bile acid levels, resulting in the increase of tauroursodeoxycholic acid (TUDCA), a secondary bile acid with neuroprotective properties. This bile acid subsequently prevented retinopathy by TGR5 activation in retinal neural cells (Haluzík and Mráz, 2018). However, there is not any direct association between these alterations and DR; instead, they are part of an intermediate process. Currently available studies have only emphasized the involvement of gut dysbiosis in the pathogenesis of T2DM. The connection between gut microbiota composition and DR is still far from being fully understood.

3 The conception and classification of postbiotics

Probiotics and synbiotics are commonly used as food supplements in the food industry to promote a balanced diet and enhance the health of the intestinal microbiota. However, with the gradual increase in research, more and more evidence has showed that the use of probiotics or synbiotics in the treatment of certain diseases has not achieved the expected effectiveness (Piqué et al., 2019). This is because probiotics need to colonize, compete for intestinal adherence, and balance the intestinal microbiota to promote good health. New scientific evidence indicates that probiotics are beneficial to the body's health. It is not necessarily the live bacteria that have a direct relationship, but rather the metabolites or bacterial components of live bacteria that promote health. As a result, probiotics have been gradually recognized to exert beneficial effects based on their bacteria themselves, metabolites, or lysate products, which are formally referred to as postbiotics by International Scientific Association of Probiotics and Prebiotics (Salminen et al., 2021).

The definition emphasizes that postbiotics must be components (e.g., cell walls, lipoteichoic acid and exopolysaccharides) and metabolites (e.g., short-chain fatty acids, bacteriocins, tryptophan catabolites and vitamins) of inactive microorganisms that, in certain doses, can produce health benefits for the host. Their beneficial functions include, but are not limited to, antimicrobial and antioxidant properties, as well as modulation of intestinal barrier function and immune response. Much of the current postbiotic research is focused on *Lactobacillus* and *Bifidobacterium*, similar to probiotic organisms (Magryś and Pawlik, 2023; Motei et al., 2023). Overall, postbiotics offer advantages that probiotics cannot match, including a well-defined chemical structure, safe dosage parameters, and a longer shelf life of up to 5 years as a nutritional additive. Furthermore, postbiotics are effectively absorbed, metabolized, and distributed throughout the body. The use of postbiotics can provide probiotic-like benefits while

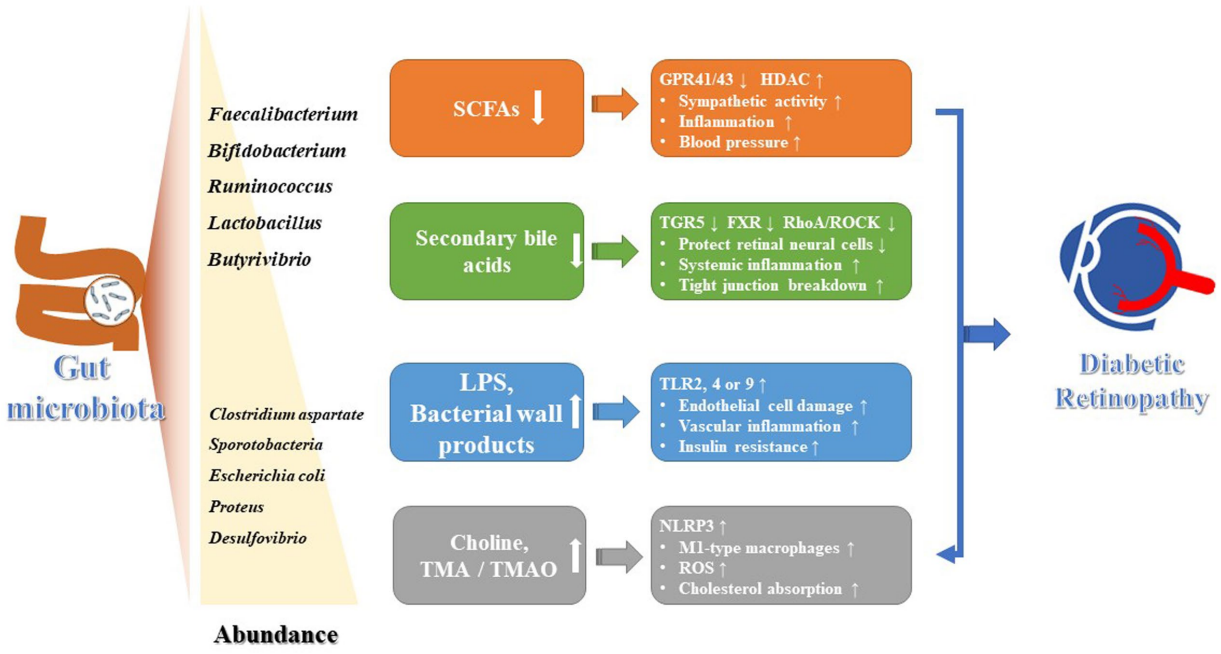

FIGURE 1
The roles of gut microbiota and its metabolites on the development of DR.

avoiding issues such as low bioavailability of live bacteria, unstable effects, and the potential for resistance gene transmission (Suthar et al., 2023). This will mark a new direction for future research in the field of probiotics.

4 Function and application of postbiotics in DR treatment

Postbiotics can regulate body metabolism through systemic reactions *in vivo* and *in situ* reactions in the intestinal lumen. *In vivo* systemic reaction refers to the ability of postbiotics to act more rapidly and directly on the human body after heat inactivation, thanks to their small molecule characteristics. This allows them to enhance human immunity, balance intestinal microbiota, and regulate physiological functions by crossing the intestinal barrier. The luminal *in situ* reaction refers to the ability of compounds like fatty acids to inhibit the growth of harmful bacteria in the intestinal lumen, decrease the aggregation of harmful bacteria, and maintain a balanced ratio of beneficial and harmful bacteria in the intestinal tract (Mosca et al., 2022). This process contributes to the overall health of the human intestine. Thus, the health benefits offered by postbiotics may be driven by various mechanisms (Figure 2). Recently, scientists have identified various forms of postbiotics. The postbiotic components produced by probiotic cells can beneficially regulate the body's metabolism through different pathways. Some postbiotics exhibit probiotic effects like those of probiotics, and in some cases, their mechanisms may also be like the known probiotic mechanisms. Notably, postbiotics are not reliant on bacterial activity, and the probiotic mechanisms of these postbiotics can also function independently or in combination (Salminen et al., 2021). However, up to now, the mechanism of action of postbiotics for their beneficial effects in humans or animals has not been fully understood.

4.1 Short-chain fatty acids (SCFAs)

SCFAs are metabolites produced by intestinal microbiota that have significant importance for the human body. When dietary fiber is fermented by intestinal microbiota, SCFAs like acetic acid, propionic acid, and butyric acid are generated. Some SCFAs enter the bloodstream through the portal vein, while others serve as an energy source for the epithelial cells of the colonic mucosa, providing calories for the host (Fang et al., 2022). SCFAs play a crucial role in mitochondrial energy metabolism, as well as regulating glucose and lipid metabolism, immune responses, and inflammation levels. They exert their biological functions through two main pathways: binding to free fatty acid receptors (FFAR2/FFAR3) as ligands, and inhibiting histone deacetylases (HDACs) to regulate gene expression (Mirzaei et al., 2021; Zhang et al., 2022b). However, the effects of SCFAs on host function remain inconsistent due to their complex biological functions, especially when interacting with signaling pathways in the host. In terms of glucose metabolism, high-fat foods rich in butyrate have been found to promote thermogenesis and energy expenditure in mice, while acetate injections in rats improved their glucose tolerance capacity. SCFAs can also cross the blood–brain barrier and play a role in the central nervous system, potentially entering the retina through the retinal inner blood-retinal barrier (iBRB) and regulating inflammation and oxidative damage (Schaefer et al., 2022). Additionally, as SCFAs act as inhibitors of HDACs, they may play a role in regulating the development of DR through epigenetic pathways, given the multiple ways in which epigenetically related histones are modified in DR disease development (Tawarayama et al., 2023).

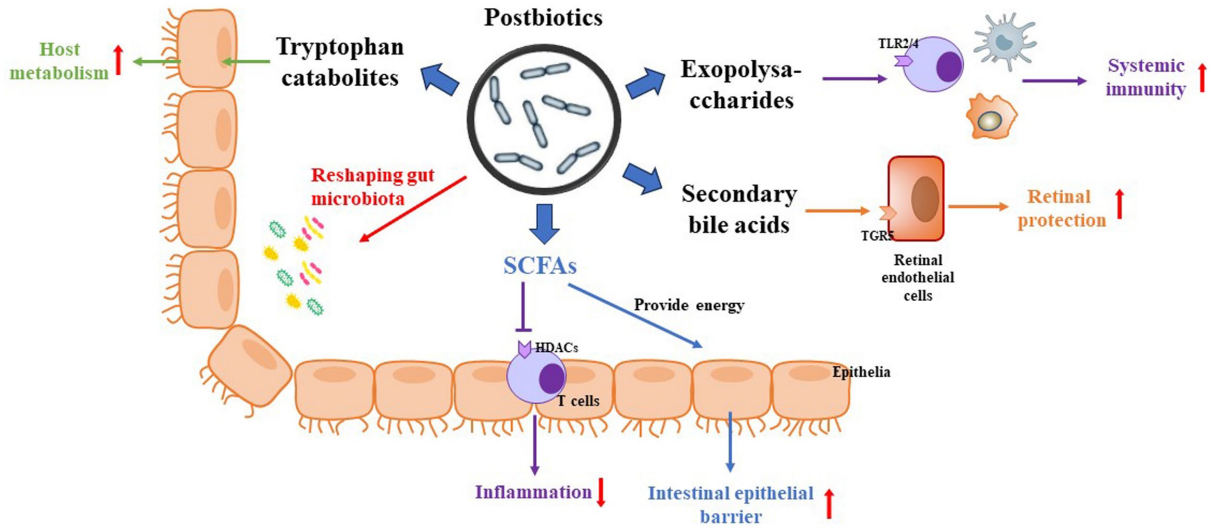

FIGURE 2
Potential mechanism of postbiotics on DR. (1) Postbiotics reshape the populations of gut microbiota. (2) SFCAs not only provide calories for the host but also inhibit HDACs via activation of inflammatory signaling. (3) Exopolysaccharides regulate host immunity via activation of TLR2/4 in immune cells. (4) Secondary bile acids regulate host metabolism and present neuroprotection via activation of TGR5. (5) Tryptophan catabolites regulate host metabolism and inhibit inflammation.

4.2 Secondary bile acids (SBAs)

Primary bile acids, derived from cholesterol and combined with taurine or glycine in the liver, are stored in the gallbladder and released into the duodenum during feeding to aid in the emulsification of dietary lipids. While most of these primary bile acids are reabsorbed in the intestine, a small percentage is degraded by anaerobic bacteria in the gut, resulting in the production of secondary bile acids such as TUDCA and ursodeoxycholic acid (UDCA) (Grüner and Mattner, 2021). These hydrophobic secondary bile acids are mainly excreted in feces, with a small portion entering circulation.

Secondary bile acids, known as active metabolites, possess bioregulatory activity and act as signaling molecules within the human body. They play a role in regulating host metabolism by binding to the nuclear receptor FXR and the G-coupled membrane protein 5 (TGR5) receptor (Win et al., 2021). Study has displayed that intermittent fasting prevents diabetic retinopathy in db/db diabetic mice by reshaping the microbiota to favor species that produce TUDCA, leading to subsequent retinal protection through TGR5 activation (Beli et al., 2018). As a result, secondary bile acids, such as TUDCA, contribute to reducing the incidence of DR. TUDCA, a neuroprotective BA, has been found to decrease the levels of nitric oxide (NO) and down-regulate the expression of proteins such as ICAM-1, NOS, NF-κB, and VEGF, thereby slowing down the progression of DR (Fu et al., 2021; Lenin et al., 2023). Similarly, UDCA has shown the ability to reduce retinal inflammation in a mouse model of STZ-induced diabetes (Ouyang et al., 2018). It achieves this by attenuating endoplasmic reticulum stress-associated peripapillary retinal cell loss through the inhibition of ionized calcium-binding adapter molecule 1 (Iba-1) expression (Chung et al., 2017). It is important to note that a weakened bile acid signaling pathway can exacerbate DR pathology, while an upregulated or activated TGR5 pathway can slow down its progression by inhibiting RhoA/ROCK and PKCδ/Drp1-HK2 pathways (Zhu et al., 2020; Zhang et al., 2022a).

4.3 Exopolysaccharides (EPSs)

EPSs are carbohydrate polymers found on the surface of most bacteria in the form of pods or pericellular mucus. Recently, EPSs had a wide range of applications in food and medical practice, due to their distinguished immunomodulatory functions, including preventing the formation of bacterial biofilms and maintaining the balance of the intestinal microenvironment (Zhang et al., 2023). EPS derived from *Lactobacillus paracasei* DG exhibited immunostimulatory properties by upregulating the expression of TNF-α, IL-6, IL-8 and CCL20 genes in the human monocyte THP-1 cells (Balzaretti et al., 2017). Additionally, EPSs regulate innate and adaptive immune responses, stimulating T cells, B cells, NK cells, and macrophages to eliminate pathogen and scavenge free radicals (Rusinova-Videva et al., 2022; Niu et al., 2023). The polysaccharide produced by *Bacillus fragilis* can bind to the TLR2 receptor, upregulate regulatory T cells, inhibit the production of IL-17, and promote the expression of IL-10, thereby inhibiting inflammation (Sittipo et al., 2018). An EPS from *Bacillus subtilus* reduced the serum levels of intercellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM) levels in STZ-induced diabetic rats, and improved hyperglycemia-microvascular endothelial cell injury (Ghoneim et al., 2016).

4.4 Amino acid metabolites

Gut microbiota can produce a variety of amino acids and their intermediates, which protect against high glucose-induced microvascular endothelial damage and are the rate-limiting materials

for glutathione synthesis (Mardinoglu et al., 2015; Beaumont and Blachier, 2020). A 12-year follow-up study by metabolomics analysis showed that N-lactoyl isoleucine, N-lactoyl valine, N-lactoyl tyrosine, and N-lactoyl phenylalanine, N-(2-furoyl) glycine, and 5-hydroxylysine were associated with an increased risk of DR, while citrulline was associated with a decreased risk of DR (Fernandes Silva et al., 2023). Increased oxidative stress in the retinal iBRB is partly due to the production of reduced glutathione.

Notably, among the amino acids studied, tryptophan is an essential amino acid, which can be metabolized primarily to kynurenine by indoleamine 2,2-dioxygenase of *Lactobacillus reuteri* and *Clostridium sporogenes*. The tryptophan derivative indole activates the aromatic hydrocarbon receptor of $CD4^+$ T cells in the mouse intestine, induces their differentiation into $CD4^+CD8\alpha\alpha^+$ double-positive intraepithelial lymphocytes, which would promote the secretion of IL-22 and IL-10, and then inhibit inflammatory responses (Cervantes-Barragan et al., 2017). Clinical trial demonstrated that the level of plasma tryptophan was significantly decreased whereas kynurenine was increased in patients and mice with DR, indicating that the altered tryptophan–kynurenine metabolism pathway plays a key role in the pathogenesis of DR (Wang et al., 2022). Moreover, kynurenic acid, the final catalysate of kynurenine, serves as anti-inflammatory mediator and can cross various endothelial barriers to access the central and peripheral nervous systems, including the visual nervous system. Thus, kynurenic acid administration potentially contributes to the treatment of retinal aging and neurodegeneration (Fiedorowicz et al., 2019).

On the other hand, intestinal microbiota can modulate neuroendocrine and intestinal immune responses by regulating tryptophan metabolism, leading to the production of serotonin, kynurenine, tryptophan, indole, and their derivatives (Yano et al., 2015). Among these, serotonin is a crucial monoamine neurotransmitter that regulates central neurotransmission and intestinal physiological functions. Given that serotonin levels in patients with proliferative diabetic retinopathy (PDR) are significantly lower than in healthy subjects, and that the incidence of DR is lower in diabetic patients taking serotonin reuptake inhibitors compared to controls, it is possible that gut microbiota influences DR by regulating the production of tryptophan and serotonin (Kern et al., 2021).

4.5 Other metabolites

Gut microbiota can synthesize a variety of small molecules with signaling effects, such as hydrogen sulfide, which influences the development of DR. It was found that the levels of hydrogen sulfide in the vitreous and plasma of patients with PDR was significantly higher than those in the healthy individuals (Han et al., 2020). Administration with exogenous hydrogen sulfide also protects the retina by reducing oxidative stress in streptozotocin-induced diabetic DR rats (Si et al., 2013). Furthermore, different postbiotic fractions from *Lactobacillus rhamnosus* GG exert significant immunomodulatory effects via inactivation of TLR4/7, MAPK, ERK, and NF-κB signaling pathways in LPS-stimulated mouse RAW264.7 cells (Qi et al., 2020).

Extracellular vesicles (EVs) derived from gut microbiota mediate the communication between microorganisms and their host to the maintenance of intestinal homeostasis and may ultimately be implicated in the regulation of various metabolic diseases (Chen et al., 2022; Díez-Sainz et al., 2022). Several studies have demonstrated a direct relationship among EVs and gut barrier integrity and metabolic status in high fat diet-induced diabetics mice; oral administration of EVs from *Akkermansia muciniphila* (AKK) could reduce gut barrier permeability and improve glucose tolerance in diabetic mice (Chelakkot et al., 2018; Nah et al., 2019; Moosavi et al., 2020). But the roles of EVs on high glucose-induced DR should be investigated by more experiment and clinical verification.

5 Security issues

Compared to conventional probiotics and prebiotics, postbiotics as novel food supplements can directly colonize the intestinal epithelial cells for controlling the microbial population, and exert heat-resistant, acid-resistant, and durable for storage. Several clinical studies have investigated the potential for absorption, metabolism, and distribution of postbiotics (Kim et al., 2023; Sato et al., 2023). The benefits of postbiotics, including their defined chemical structure, low toxicity, minimal storage requirements, long shelf-life, and stability, offer significant advantages in microbe-related products. However, in a randomized controlled trial involving 40 children under 5 years of age, 36 of 40 children experienced relief from symptoms, and one child experienced severe dehydration (Malagón-Rojas et al., 2020), suggesting the potential adverse effects of postbiotics. Therefore, further studies should prioritize enhancing the safety of postbiotics during use and minimizing adverse reactions.

6 Future challenges and prospects

As byproducts of the intestinal microbiota, postbiotics can serve a wide range of functions in the intestinal tract and throughout the body. Up to now, the anti-inflammatory, antimicrobial, antitumor, hypoglycemic, and hypolipidemic effects of postbiotics have been substantiated. These effects have led to the manufacturing and marketing of postbiotics as health care products for the prevention and treatment of diseases. Recently, the most common application is still adding postbiotic elements to dairy products to boost immunity and regulate intestinal function. Especially in livestock feed supplementation, it can replace the use of some antibiotics, demonstrating its significant practical value. Emerging studies have confirmed the potential benefits of postbiotics in treating and alleviating obesity and diabetes (Osman et al., 2021; Balaguer et al., 2022). The main strains involved are AKK, Bifidobacterium, and Lactobacillus. The diversification of postbiotics in various forms indicates a promising future for their development in preventing and treating metabolic diseases. However, there is still a gap in its clinical applicability, long-term and short-term toxicity, and bioavailability, which are urgent issues for current research.

In addition to preventing and treating certain diseases, some of the characteristics of postbiotics also offer clear advantages compared to probiotics. As mentioned earlier, they can mitigate some of the health risks associated with probiotics. Additionally, postbiotics offer greater convenience in terms of storage and transportation. However, there are still many limitations in the current research on postbiotics. Firstly, there is a lack of understanding regarding how various

metabolites or bacterial components interact with intestinal cells and influence downstream pathways. Secondly, the relationship between bacterial components and their effects, as well as the conformational relationship resulting from the combination of different types of postbiotics, remains unclear. Thirdly, short-chain fatty acids can be obtained through the fermentation of plant polysaccharides by intestinal microorganisms, extraction of cell-free supernatant via centrifugation and filtration, and production of bacterial lysates through chemical or mechanical degradation. As a result, there is still no unified standard and process for the mass production of postbiotics technology. Additionally, safety and regulatory concerns must be addressed, and consistent regulatory standards need to be established. Despite its faults, the potential use of postbiotics in medical and healthcare fields deserves attention. Furthermore, it is worth exploring the combination of postbiotics and prebiotics, as well as the potential application value of different postbiotic combinations.

Author contributions

QC: Writing – original draft, Writing – review & editing. X-JL: Conceptualization, Writing – original draft. WX: Conceptualization, Writing – original draft. Z-AS: Visualization, Writing – review & editing. G-MQ: Visualization, Writing – review & editing. C-HY: Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 09 April 2024
DOI 10.3389/fmicb.2024.1362266

OPEN ACCESS

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RECEIVED 28 December 2023
ACCEPTED 12 March 2024
PUBLISHED 09 April 2024

CITATION
Nealon NJ, Worcester CR, Boyer SM, Haberecht HB and Ryan EP (2024) Metabolite profiling and bioactivity guided fractionation of *Lactobacillaceae* and rice bran postbiotics for antimicrobial-resistant *Salmonella* Typhimurium growth suppression. *Front. Microbiol.* 15:1362266. doi: 10.3389/fmicb.2024.1362266

Metabolite profiling and bioactivity guided fractionation of *Lactobacillaceae* and rice bran postbiotics for antimicrobial-resistant *Salmonella* Typhimurium growth suppression

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Probiotic-fermented supplements (postbiotics) are becoming increasingly explored for their activity against antibiotic-resistant enteropathogens. Prebiotics are often incorporated into postbiotics to enhance their efficacy, but due to strain differences in probiotic activity, postbiotic antimicrobial effects are poorly understood. To improve postbiotic antimicrobial efficacy, we investigated and compared metabolite profiles of postbiotics prepared with three lactic acid bacteria strains (*L. fermentum*, *L. paracasei*, and *L. rhamnosus*) cultured with and without rice bran, a globally abundant, rich source of prebiotics. At their minimum inhibitory dose, *L. fermentum* and *L. paracasei* postbiotics + rice bran suppressed *S.* Typhimurium growth 42–55% more versus their respective probiotic-alone postbiotics. The global, non-targeted metabolome of these postbiotics identified 109 metabolites increased in *L. fermentum* and *L. paracasei* rice bran postbiotics, including 49 amino acids, 20 lipids, and 12 phytochemicals metabolites. To identify key metabolite contributors to postbiotic antimicrobial activity, bioactivity-guided fractionation was applied to *L. fermentum* and *L. paracasei* rice bran-fermented postbiotics. Fractionation resulted in four *L. fermentum* and seven *L. paracasei* fractions capable of suppressing *S.* Typhimurium growth more effectively versus the negative control. These fractions were enriched in 15 metabolites that were significantly increased in the global metabolome of postbiotics prepared with rice bran versus postbiotic alone. These metabolites included imidazole propionate (enriched in *L. fermentum* + rice bran, 1.61-fold increase; *L. paracasei* + rice bran 1.28-fold increase), dihydroferulate (*L. fermentum* + rice bran, 5.18-fold increase), and linoleate (*L. fermentum* + rice bran, 1.82-fold increase; *L. paracasei* + rice bran, 3.19-fold increase), suggesting that they may be key metabolite drivers of *S.* Typhimurium growth suppression. Here, we show distinct mechanisms by which postbiotics prepared with lactic acid bacteria and rice bran produce metabolites with antimicrobial activity capable of suppressing *S.* Typhimurium growth. Probiotic strain differences contributing to postbiotic antimicrobial activity attract attention as adjunctive treatments against pathogens.

KEYWORDS

postbiotic, rice bran, metabolomics, Lactobacillaceae, bioactivity-guided fractionation, *Salmonella enterica* serovar Typhimurium, antimicrobial resistance

1 Introduction

Salmonella enterica serovar Typhimurium is a leading global cause of infectious diarrhea. Escalating levels of antimicrobial resistance across non-typhoidal *Salmonella* isolates complicates containment and treatment efforts, and consequently, alternative methods of microbial control are needed (CDC, 2019). A rapidly growing body of research examines probiotics as widespread gut health-promoting dietary supplements and native gut commensal microorganisms that may prevent and reduce *Salmonella* outbreaks (Luoma et al., 2017; Nealon et al., 2017a). More recently, probiotics have been combined with prebiotics, including purified carbohydrates and whole foods. Among whole food prebiotics, rice bran, milled from brown rice, is a phytochemically diverse source of lipids, amino acids, and vitamins/cofactors and contains prebiotic carbohydrates (Salmeron, 2017; Zarei et al., 2017; Nealon and Ryan, 2019). Dietary supplementation with rice bran was shown to enhance the growth of probiotic Lactobacillaceae in people, mice, pigs, and broiler chickens and reduce *Salmonella* shedding (Rubinelli et al., 2017; Sheflin et al., 2017; Zarei et al., 2017; Nealon et al., 2017a; Zambrana et al., 2019).

An emerging area of dietary supplement research includes postbiotics, which are the cell-free products of probiotics, including small molecules, cell wall components, microbial proteins, and extracellular polysaccharides (Cuevas-González et al., 2020). Postbiotics have been explored for their roles in immune modulation, as antioxidants, chemopreventive agents, and antimicrobial adjuncts and alternatives (Kumbhare et al., 2023). Specifically, postbiotics, prepared with lactic acid bacteria, a diverse group of probiotic strains native to human and animal microbiotas, have been increasingly explored for their roles in antimicrobial activity, including as anti-biofilm agents for interference with quorum sensing, for enhancing the growth of other beneficial microbial symbionts, as applications to food products or used, and in animal production systems to attenuate antimicrobial use (Ali et al., 2023; Chuah et al., 2023; Kudra et al., 2023; Kumbhare et al., 2023; Penchuk et al., 2023; Sepordeh et al., 2023; Sharafi et al., 2023; Tong et al., 2023). Across postbiotic studies, there remains a knowledge gap in understanding postbiotic mechanisms of action, including incomplete characterization of the bioactive small molecules driving these diverse functions (Kumbhare et al., 2023). This includes how both probiotic strain and prebiotic selection impact the function of postbiotics (Ali et al., 2023; Kumbhare et al., 2023).

Metabolomics, the systematic evaluation of small, bioactive molecules in living systems, is a tool that can improve our understanding of postbiotic functionality. When applied to microbial products and other biologics, metabolomics has expanded the suite of small molecules (metabolites) that we can detect and identify from microbial products and fermented foods (Shaffer et al., 2017). Specifically, a postbiotic prepared with probiotic *Lacticaseibacillus paracasei,* an established lactic acid probiotic strain, and rice bran enhanced *S.* Typhimurium growth suppression compared with an *L. paracasei* postbiotic alone, and this was associated with increases in 84 metabolites, predominantly lipid, amino acid, and phytochemical compounds, which had previously reported antimicrobial functions (Nealon et al., 2017a). In a second study, a postbiotic prepared with *Lacticaseibacillus rhamnosus* suppressed the growth of antimicrobial-resistant *S.* Typhimurium, *Escherichia coli*, and *Klebsiella oxytoca*, and *in silico* metabolic flux analysis of the postbiotic global metabolome revealed that amino acid metabolites were key contributors to its antimicrobial activity (Hove et al., 2023). Given that non-targeted metabolomics often identify numerous potential functional compounds, bioactivity-guided fractionation, which chromatographically subsets complex, functional, and natural substances into smaller suites of metabolites, can additionally be applied to postbiotics to subset and identify major contributors to their antimicrobial activity (Najmi et al., 2022). In these instances, fractionated postbiotics can be applied to a target of interest, such as *S.* Typhimurium, and modulations in *S.* Typhimurium growth with different postbiotic fractions can be identified and further profiled using metabolomics to identify the metabolite subsets in these fractions. These high-throughput and sensitive tools were applied herein to systematically characterize postbiotics that have functional bioactivity against antimicrobial-resistant *S.* Typhimurium.

The objective of this study was to functionally compare the bioactive small molecules present in postbiotics produced by three strains of Lactobacillaceae and rice bran. The overarching hypothesis of this study is that Lactobacillaceae + rice bran postbiotics produce distinct suites of small molecules that suppress multidrug-resistant *S.* Typhimurium growth. Targeted postbiotic preparations may become promising treatments and strategic preventive agents against antimicrobial-resistant pathogen outbreaks.

2 Materials and methods

2.1 Bacterial strains and culture preparation

Three Lactobacillaceae strains isolated from human fecal and colon tissue samples were purchased from ATCC (Manassas, VA): *Limosilactobacillus fermentum* ATCC 23271, *Lacticaseibacillus paracasei* ATCC 27092, and *Lacticaseibacillus rhamnosus* ATCC 21052. *Salmonella enterica* subsp. *enterica* serovar Typhimurium was provided by Dr. Sangeeta Rao from Colorado State University. The antimicrobial-resistance phenotype of this isolate is shown in Supplementary File S1 and was established using broth microdilution assay methods, which were standardized by the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (CLSI, 2010). Before use, all bacteria were stored at −80°C as 1 mL aliquots with 20% glycerol (Avantor, Radnor, PA) with autoclaved de Man Rogosa Sharpe (MRS) broth (Becton, Dickinson and Company, Difco Laboratories, Franklin Lakes, NJ) for Lactobacillaceae strains or Luria–Bertani (LB) broth (MOBIO Laboratories Inc., Carlsbad, CA) for *S.* Typhimurium.

2.2 Rice bran extraction and media preparation

Rice bran extract was prepared using heat-stabilized Calrose rice bran (USDA-ARS Rice Research Unit, Stuttgart, AK), as previously described (Forster et al., 2013). Heat stabilization of rice bran was completed in a commercial dryer for 30 min at 110°C. In total, 4 g of rice bran was extracted in 42.6 mL of 80% aqueous solution of ice-cold (−80°C) methanol, vigorously vortexed for 5 min (232 Vortexer Fisher Scientific, Pittsburgh, PA, USA), incubated overnight at −80°C, and centrifuged at 4,000 *g* for 5 min (Beckman Coulter Allegra X-14R). The supernatant was collected and dried in a speedvac concentrator (SPD1010, Thermo Scientific, Pittsburgh, PA) at 45°C for approximately 48 h. To prepare MRS + rice bran extract, 100 μg of rice bran extract was added to 1 mL of MRS broth and autoclaved using a sterilization time of 45 min. Broth was stored at 4°C until use. The concentration of MRS + rice bran extract was the same as previous dose response studies of MRS + rice bran extract broth on *S.* Typhimurium growth (Nealon et al., 2017a).

2.3 Preparation of Lactobacillaceae postbiotics in the presence and absence of rice bran

To create postbiotics for all Lactobacillaceae and Lactobacillaceae + rice bran treatments, cell-free supernatant was prepared as described previously (Nealon et al., 2017a). In brief, 1×10^6 CFU ml^{-1} (colony forming units) of *L. fermentum*, *L. paracasei*, or *L. rhamnosus* was grown for 24 h to mid/late logarithmic phase, added to 15 mL of MRS broth or 15 mL of MRS broth +100 μg ml^{-1} rice bran, and incubated at 37°C for 24 h. A, 100 μg ml^{-1} dose of rice bran extract was selected from previous studies, whereby 100 μg ml^{-1} of rice bran extract significantly suppressed *S.* Typhimurium growth compared with a rice bran extract-free control (Nealon et al., 2017a). Following the 24 h incubation, when all cultures were in the stationary growth phase, cultures underwent two rounds of centrifugation at 4,000 *g* for 10 min to separate the supernatant from the remaining bacterial pellet. The supernatant was adjusted to a pH of 4.5, which approximates the lower limit of acidity tolerated by *S.* Typhimurium (Chung and Goepfert, 1970), to control the effect of pH-dependent changes on *S.* Typhimurium growth. The supernatant was filter-sterilized through a 0.22-μm pore (Pall Corporation Life Sciences Acrodisc syringe filters, Port Washington, NY) and stored as 1 mL aliquots at −80°C until use. Supernatant sterility was confirmed prior to use by screening it for the absence of any growth at 37°C with repeated OD600 reads every 20 min for 24 h. A minimum of three replicates of each Lactobacillaceae and Lactobacillaceae + rice bran postbiotic were used to conduct each experiment described herein.

2.4 *Salmonella* Typhimurium growth suppression assay

The assay for *S.* Typhimurium growth suppression with Lactobacillaceae +/− rice bran postbiotics, including the selected dose range, was adapted from previously reported methods (Nealon et al., 2017a). *S.* Typhimurium was grown in LB broth at 37°C, until it reached early/mid logarithmic growth phase, as determined by repeated optical density reads at 600 nm. Lactobacillaceae and Lactobacillaceae + rice bran postbiotic supernatants were tested for dose-dependent growth suppression effects on *S.* Typhimurium. Approximately 2×10^6 *S.* Typhimurium were added to sterile LB in a 96-well plate, and different concentrations of supernatant (12–25% per volume of LB) were added to each well. This range of concentrations was selected to identify a range of doses over which postbiotic supernatants exhibited no enhanced growth suppression of *S.* Typhimurium versus the vehicle controls (12%) to a dose, where all treatments showed an equivalent minimal growth of *S.* Typhimurium (25%) through the 16 h of the assay. The vehicle controls were either MRS (for Lactobacillaceae) or MRS + 100 μg ml^{-1} rice bran extract (for Lactobacillaceae + rice bran) that were added to LB broth. The negative control was *S.* Typhimurium inoculated into equivalent volumes of LB. All controls were adjusted to a pH of 4.5, which approximates the lower limit of acidity tolerated by *S.* Typhimurium (Chung and Goepfert, 1970).

To measure *S.* Typhimurium growth over time, OD600 was measured at 37°C every 20 min for 16 h. To quantify *S.* Typhimurium growth suppression, the percentage difference in growth suppression was calculated between pairs of treatments at 16 h using the following formula:

$$\frac{\left(OD600\ Treatment\ 1 - OD600\ Treatment\ 2\right)}{\left(OD600\ Treatment\ 2\right)} * 100\%$$

Each experiment contained a minimum of two technical replicates per treatment dose, and each experiment was repeated a minimum of three times.

2.5 Fractionation of Lactobacillaceae + rice bran cell-free supernatant

Given the increased efficacy of *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics against *S.* Typhimurium versus their respective probiotic-alone postbiotics, each of these treatments underwent further chromatographic separation for elucidation of key metabolites driving their antimicrobial activity. In total, 5 ml of *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics were each separated into 24 fractions using reverse-phase flash chromatography on a Combiflash® RF+ Flash Chromatography Purification System (Teledyne ISCO, Thousand Oak, California). The 5-ml starting volume was determined following a range-finding analysis that optimized sample injection volume for metabolite recovery during non-targeted metabolomics analysis (data not shown). The stationary phase column was a C18-aq, 15.5 g-Gold Redisep column (Teledyne ISCO, Thousand Oaks, California), and the mobile phase gradient consisted of a water:methanol solution that increased in hydrophobicity over the course of the separation. To account for machine and batch variability in postbiotic antimicrobial activity, each supernatant was fractionated three times using a minimum of three batches collected on different days. UV absorbance detected at 214 and 254 nm wavelengths was compared across each

fractionation run, to confirm consistency in chromatographs between biological and technical replicates. *L. fermentum* and *L. paracasei* cell-free supernatants were fractionated using the same column and run conditions. Following separation, fractions were dried at 55°C under a sterile fume hood and then re-constituted in 5 mL LB broth titrated to a pH of 4.5. All re-constituted fractions were stored at −80°C until use.

2.6 *Salmonella* Typhimurium growth suppression with postbiotic fractions

In total, 1×10^6 CFU of each *S.* Typhimurium was added to LB broth on a 96-well plate and treated with each re-constituted fraction to create a 22% v/v concentration in LB, which was a Lactobacillaceae + rice bran postbiotic dose that previously exhibited growth suppression for this *S.* Typhimurium isolates (Nealon et al., 2017a). In each assay, *S.* Typhimurium inoculated in 4.5 pH-adjusted LB was used as the negative control. To adjust for starting differences in fraction optical densities and confirm media sterility over the course of the assay, blank LB and blank fraction + LB were included as controls. Each assay contained a minimum of two technical replicates for each treatment and was repeated three times for each *S.* Typhimurium isolate incubated with either *L. fermentum* + rice bran or *L. paracasei* + rice bran postbiotic supernatants.

2.7 Quantification and statistical analysis of *Salmonella* Typhimurium growth suppression assays with postbiotics

For analysis of unfractionated supernatants, a repeated-measures two-way analysis of variance was used to examine treatment and time-dependent differences in supernatant growth suppression when comparing Lactobacillaceae + rice bran with their respective Lactobacillaceae postbiotic treatments. For analysis of bioactive supernatant fractions, the OD600 of each fraction at each timepoint was compared with that of the negative control. In both analyses, significance was defined as $p < 0.05$ following p-value adjustment with a Tukey's post-hoc test. All statistical analyses for these assays were performed using GraphPad Prism Version 10.1.1 (La Jolla, CA).

2.8 Postbiotic and postbiotic fraction metabolomics processing

The global, non-targeted metabolite profiles of each Lactobacillaceae supernatant, Lactobacillaceae + rice bran supernatant, vehicle control, and vehicle control + rice bran were performed by Metabolon Inc© (Durham, NC). Selected fractions for postbiotic *L. fermentum* + rice bran (fractions 18, 21, and 22) and *L. paracasei* + rice bran (fractions 18, 21, 22, and 24) additionally underwent non-targeted metabolomics profiling. These fractions were selected for profiling because they exhibited differential magnitudes of *S.* Typhimurium growth suppression compared with the vehicle control treatment. In brief, all samples were shipped on dry ice to Metabolon and frozen at −80°C until sample processing with ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Samples were re-solubilized in methanol, centrifuged at room temperature, and separated into five aliquots for downstream analysis: two aliquots for reverse-phase chromatography coupled with positive ion mode electrospray ionization (ESI), one aliquot for reverse phase chromatography coupled with negative ion mode ESI, a fourth aliquot for hydrophilic-interaction UPLC-MS/MS coupled with negative ion mode ESI, and the fifth aliquot saved as a back-up sample. Quality control samples were prepared by pooling similar aliquots across all fraction samples to account for chromatographic drift across subsequent UPLC-MS/MS runs.

Before injection, each aliquot was dried using an automated evaporation system (TurboVap®, LV Automated Evaporation System, Thermo Scientific, Pittsburgh, PA). Each dried sample was re-constituted, mixed with internal standard compounds of known concentration, and processed for the following UPLC-MS/MS workflows: Acidic positive ion mode conditions optimized for hydrophilic metabolite extraction with a C18 column (Waters UPLC BEH C18-2.1×100 mm, 1.7 µm) stationary phase and a mobile phase solution of water and methanol with 0.05% v/v perfluoropentanoic acid and 0.1% v/v formic acid; acidic positive ion mode conditions optimized for hydrophobic metabolite extraction with the same C18 column stationary phase as the previous condition and a mobile phase solution of methanol, acetonitrile, and water with 0.05% perfluoropentanoic acid and 0.01% formic acid; basic negative ion mode conditions with a C18 column (Waters UPLC BEH C18-2×100 mm, 1.7 µm) stationary phase and a mobile phase solution of methanol and water adjusted to a pH of 8 with ammonium bicarbonate; negative ESI coupled with a hydrophilic interaction stationary phase column (Waters UPLC BEH Amide 2.1×150 mm, 1.7 µm) and a mobile phase solution of water and acetonitrile adjusted to a pH of 10.8 with ammonium formate. All workflows used Waters AQUITY ultra-performance liquid chromatography columns coupled to a Thermo Scientific Q-Exactive high resolution mass spectrometers equipped with a heated ESI source and an Orbitrap mass analyzer set to a 35,000 mass:charge (m/z) resolution, with a tandem mass spectrometry setup that fluxed between dynamic exclusion MS and data-dependent MS^n scans covering 70–1000 m/z.

Raw mass spectral data were extracted using software developed by Metabolon where data were peak-extracted and normalized using area under the curve abundances with reference to quality control samples and internal standard recoveries. Mass spectral features were identified to known compounds using their retention indices, accurate masses (+/− 10 parts per million), and their MS/MS forward and reverse scores compared with Metabolon's internal compound library containing ~3,300 purified chemical standards. Metabolite identities were cross-validated using the online mass spectral databases (Human Metabolome Database, "HMDB"; Kyoto Encyclopedia of Genes and Genomes, "KEGG," and PubChem) (UniProt Consortium, 2018; Wishart et al., 2018; Kanehisa et al., 2019). For compounds that did not have a matching internal standard with the Metabolon library, identifies were directly made using these public databases.

2.9 Statistical analysis and data visualization of postbiotic and postbiotic fraction metabolomes

To examine the differences in metabolite abundance across Lactobacillaceae and Lactobacillaceae + rice bran postbiotics, raw metabolite abundances for each sample were median-scaled across the dataset and used for downstream statistical analysis. A Welch's *t*-test with a Benjamini–Hochberg false discovery rate correction was used to identify metabolites that were differentially abundant between the following pairs of treatments: *L. fermentum* + rice bran versus *L. fermentum, L. paracasei* + rice bran versus *L. paracasei*, and *L. rhamnosus* + rice bran versus *L. rhamnosus*. Statistical significance was defined as $p < 0.05$ following false discovery rate (q-value) adjustment of p-values. Treatment fold differences were calculated by dividing the average median-scaled abundance of one treatment by the second treatment. Data visualization was performed using GraphPad Prism (version 10.1.1) and Metaboanalyst version 5.0 (Pang et al., 2021). To show treatment differences between the global, non-targeted metabolomes of postbiotics with and without rice bran, a partial least squares discriminant analysis (PLS-DA) plot was generated using median-scaled metabolite abundances across the top three components with a 5-fold cross-validation error rate. An integrated heat map and unsupervised hierarchical clustering analysis were generated to visualize the top 50 metabolites with the highest variable importance scores from the PLS-DA model. Hierarchical clustering analysis branch points were calculated using Euclidean distances and ward scaling. Supplementary File S2 provides the R script used for Metaboanalyst visualization.

For metabolite profiles of bioactive fractions, the metabolite raw abundances for each fraction were median-scaled across all *L. fermentum* samples or *L. paracasei* samples. Z-scores for each metabolite were calculated as previously described (Borresen et al., 2017; Zarei et al., 2017). In brief, Z-scores were obtained by subtracting the metabolite median-scaled abundance across all samples from the median-scaled abundance of each fraction, and this difference was then divided by the standard deviation of all *L. fermentum* or *L. paracasei* samples. Metabolites with a Z-score of ≥1.00 in each fraction were defined as enriched in these fractions relative to unfractionated supernatant. Data visualization for postbiotic fractionation metabolome data was completed using GraphPad Prism.

3 Results

3.1 Lactobacillaceae + rice bran postbiotics differentially suppress the growth of antimicrobial-resistant *Salmonella* Typhimurium

Lactobacillaceae and Lactobacillaceae + rice bran postbiotics exhibited concentration-dependent growth suppression on *S.* Typhimurium when applied over a concentration range of 12 to 25% v/v. Table 1 shows the relative percent efficacy of each Lactobacillaceae + rice bran versus Lactobacillaceae postbiotic for each of the three tested probiotic strains. Supplementary Table S3 shows growth suppression levels of each postbiotic versus the control treatment. There was a dose-dependent effect of postbiotics on *S.* Typhimurium growth suppression for all treatments (Lactobacillaceae alone or in combination with rice bran) compared with the vehicle controls and negative controls ($p < 0.05$) (Supplementary Table S4).

The results are presented in Figure 1 for the 18% supernatant concentration, as it was the lowest concentration at which any postbiotic produced significantly enhanced 16 h growth suppression compared with the vehicle controls. At 16 h, the 18% *L. paracasei* + rice bran postbiotic was 55.21% more effective at suppressing *S.* Typhimurium growth compared with the *L. paracasei*-only postbiotic ($p < 0.0001$). *L. fermentum* + rice bran significantly suppressed *S.* Typhimurium growth by 42.47% more than the *L. fermentum*-only postbiotic ($p < 0.0001$). At all tested doses, the *L. rhamnosus* + rice bran postbiotic did not enhance *S.* Typhimurium growth suppression at any point during the assay when compared with the *L. rhamnosus* postbiotic.

3.2 Rice bran differentially modulates the metabolism of Lactobacillaceae strains

Global non-targeted metabolomics identified 381 metabolites in Lactobacillaceae and Lactobacillaceae + rice bran postbiotics, including 325 metabolites that were differentially abundant when comparing each postbiotic prepared with rice bran with its probiotic-only treatment (Supplementary File S4). Differentially abundant

TABLE 1 Percent difference in antimicrobial-resistant *Salmonella* Typhimurium growth suppression when comparing Lactobacillaceae and Lactobacillaceae + rice bran postbiotics at different treatment concentrations.

	S. Typhimurium percent growth suppression			
Postbiotic supernatant concentration (% volume)	12%	18%	22%	25%
L. fermentum + Rice Bran / *L. fermentum*	**↑23.82% ± 7.71%**	**↑42.47% ± 10.93%**	30.04% ± 19.57%	18.21% ± 6.81%
L. paracasei + Rice Bran / *L. paracasei*	12.22% ± 1.87%	**↑55.21% ± 9.72%**	27.41 ± 9.24%	21.87 ± 2.29%
L. rhamnosus + Rice Bran / *L. rhamnosus*	13.94% ± 9.10%	5.76% ± 2.82%	29.15% ± 13.94%	**↓65.45% ± 72.95**

Postbiotic cell-free supernatant treatment concentrations were selected to span a range over which growth suppression was observable by Lactobacillaceae and/or Lactobacillaceae + rice bran treatments (12% volume of *S.* Typhimurium incubation media) versus the vehicle control treatments (de Man Rogosa Sharpe broth with and without rice bran extract) to which no difference between treatments could be readily observed (25% volume of *S.* Typhimurium incubation media). Growth suppression values for each supernatant dose represent the average value for a minimum of three independent experiments ± standard deviation measured at 16 h post-incubation (end point for the assay). Bold treatments indicate statistically significant ($p < 0.05$) growth suppression differences between treatments at 16 h following two-way analysis of variance with Tukey's *post-hoc* test. ↑ indicates treatment in the numerator exhibited increased *S.* Typhimurium growth suppression compared with the treatment in the denominator; ↓ indicates treatment in the numerator exhibited decreased *S.* Typhimurium growth suppression compared with the treatment in the denominator.

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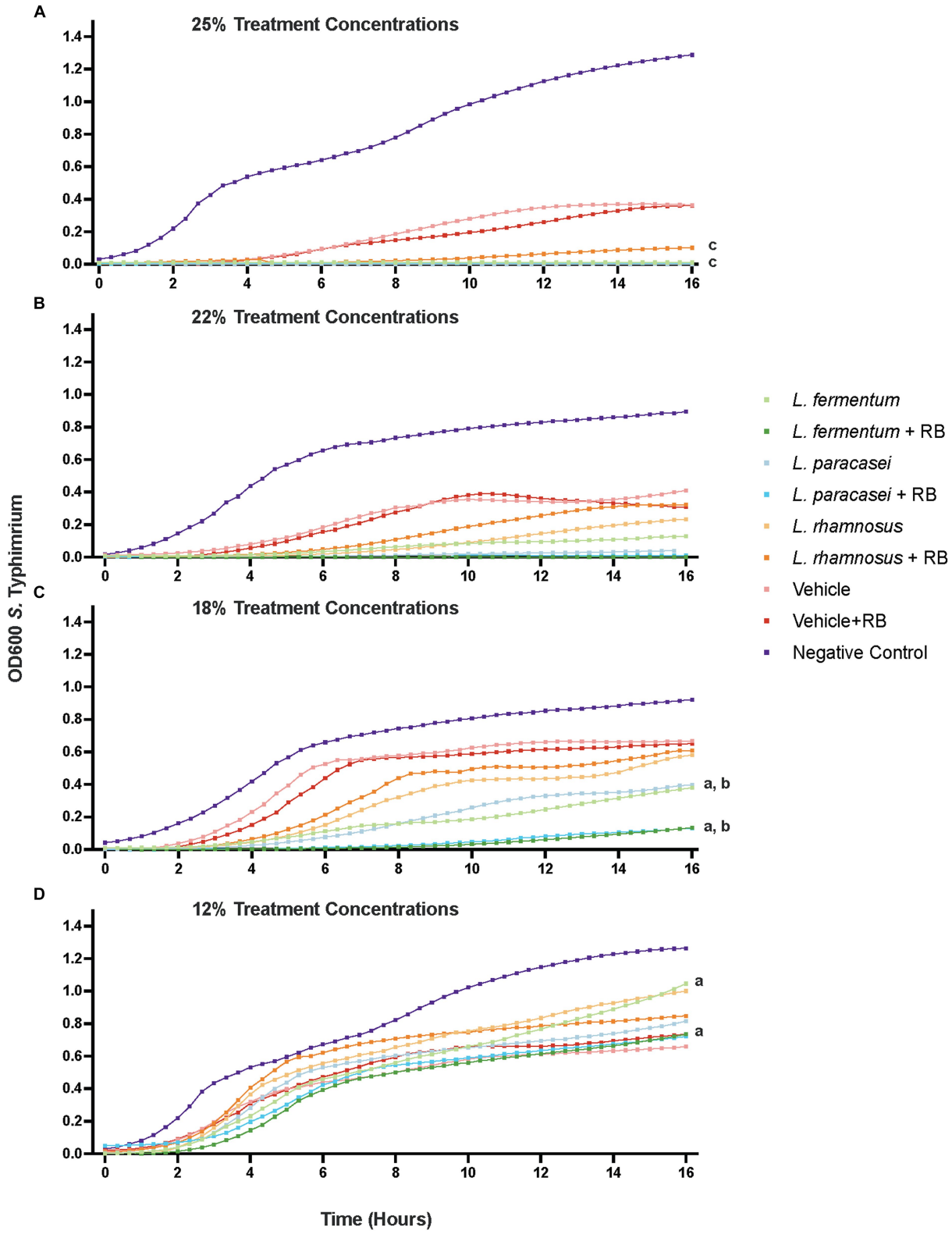

FIGURE 1
Probiotic and probiotic + rice bran postbiotics differentially suppress the growth of antimicrobial-resistant *S*. Typhimurium in a dose-dependent manner. Growth suppression by postbiotic supernatants are depicted at a 25% v/v concentration **(A)**, 22% concentration **(B)**, 18% concentration **(C)**, and 12% concentration **(D)**. Optical density (OD600) at each time point represents the mean of at least three independent experiments with a minimum of three technical replicates per experiment. Letters denote treatments that are significantly different ($p < 0.05$) for 16 h following a two-way repeated measures analysis of variance with a Tukey's post-hoc correction: a. *L. fermentum* + rice bran versus *L. fermentum*; b. *L. paracasei* + rice bran versus *L. paracasei*; c. *L. rhamnosus* + rice bran versus *L. rhamnosus*. RB, Rice Bran.

metabolites included 122 amino acids, 32 peptides, 29 carbohydrates, 9 energy metabolites, 42 lipids, 48 nucleotides, 27 phytochemical/other, and 17 vitamins/cofactors. PLS-DA analysis (Figure 2A) indicated that the largest variation in metabolite profiles, with separation along Component 1 (83.2% of the variation), occurred primarily between postbiotic treatments versus the vehicle control and secondarily between postbiotic strains relative to each other. Component 2 (11.0% of the variation) primarily separated Lactobacillaceae from Lactobacillaceae + rice bran postbiotics for each treatment group. The *L. rhamnosus* versus *L. rhamnosus* + rice bran postbiotic treatment groups exhibited the largest distance from each other along both components. To identify metabolites contributing to treatment differences, unsupervised hierarchical clustering analysis compared Lactobacillaceae + rice bran and Lactobacillaceae postbiotics, where the 50 metabolites with the highest PLS-DA VIP scores across treatments are shown in Figure 2B. Clear separation between postbiotic treatments prepared with different postbiotic strains were identified, including when comparing each postbiotic with its postbiotic + rice bran treatment. Amino acid metabolites were the most highly represented across postbiotics and accounted for ~48% of these visualized metabolites. Lipids (~18%) and carbohydrates (~14%) were the second and third most abundant chemical classes represented. Other metabolite classes contributing to postbiotic differences included energy metabolites (~12%), vitamins/cofactors (~4%), and nucleotides (~4%).

Given the enhanced antimicrobial activity of *L. paracasei* + rice bran and *L. fermentum* + rice bran postbiotics against *S.* Typhimurium, there was particular interest in postbiotic metabolites increased during rice bran fermentation. Supplementary File S5 shows metabolites that were significantly increased in the global, non-targeted metabolome of *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics relative to their probiotic-only postbiotic treatments. For the *L. fermentum* + rice bran postbiotic, 148 metabolites were significantly increased relative to the *L. fermentum* postbiotic, including the carbohydrate glucose (7.75-fold increase, $p = 0.0074$), fatty acids azelate (1.24-fold increase, $p = 0.0220$), and linoleate (1.82-fold increase, $p = 0.0084$) and the rice bran phytochemical dihydroferulic acid (5.18-fold increase, $p = 0.0024$). Multiple methionine metabolites exhibited large increases in *L. fermentum* + rice bran versus *L. fermentum* postbiotics including methionine (89.76-fold increase, $p = 8.94E-05$), N-formylmethionine (15.06-fold increase, $p = 0.0005$), and N-acetylmethionine (9.72-fold increase, $p = 0.0010$). In the *L. paracasei* + rice bran postbiotic, 32 metabolites were significantly increased relative to the *L. paracasei* postbiotic. These metabolites included histidine metabolite imidazole propionate (1.28-fold increase, $p = 0.0400$), carbohydrate sucrose (7.39-fold increase, $p = 0.0001$), fatty acid linoleate (3.19-fold increase, $p = 0.0482$), and rice bran phytochemical 4-hydroxybenzoate (1.41-fold increase, $p = 0.0147$).

3.3 Postbiotic fractions prepared from *Lacticaseibacillus paracasei* + rice bran and *Limosilactobacillus fermentum* + rice bran exhibited growth suppressive activity against *Salmonella* Typhimurium

Given the large number of metabolites that could potentially be contributing to the enhanced antimicrobial activity of the *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics relative to their probiotic-alone postbiotics, these postbiotics underwent fractionation to subset metabolites. Each of the 24 fractions created from these postbiotics were subsequently applied to *S.* Typhimurium and screened for growth suppression activity. Figure 3 shows the maximal percent growth suppression achieved for each *L. fermentum* + rice bran postbiotic fraction (Figure 3A) and *L. paracasei* + rice bran postbiotic fraction (Figure 3B). Table 2 summarizes the maximal percent differences for all fractions that suppressed *S.* Typhimurium compared with the negative control. Four fractions of the *L. fermentum* + rice bran postbiotic exhibited *S.* Typhimurium growth suppression: fraction 18 (11.39% more effective versus negative control, $p < 0.0001$), fraction 21 (7.83%, $p < 0.005$), fraction 22 (12.79%, $p < 0.0001$), and fraction 23 (8.01%, $p < 0.001$). Seven *L. paracasei* + rice bran postbiotic fractions suppressed *S.* Typhimurium growth: fraction 18 (15.95% more effective, $p < 0.0001$), fraction 19 (16.74%, $p < 0.0001$), fraction 20 (17.70%, $p < 0.0001$), fraction 21 (22.30%, $p < 0.0001$), fraction 22 (19.90%, $p < 0.0001$), fraction 23 (17.05%, $p < 0.0001$), and fraction 24 (10.96%, $p < 0.01$). Collectively, *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotic fractions with growth inhibition activity achieved maximal *S.* Typhimurium suppression between 10 and 12 h post-incubation, approximately during the late exponential growth phase of *S.* Typhimurium.

3.4 Metabolite composition for the bioactive postbiotic *Lactobacillaceae* + rice bran fractions

Non-targeted metabolite profiling was completed on selected postbiotic fractions exhibiting *S.* Typhimurium growth suppression in both *L. fermentum* + rice bran (fractions 18, 21, and 22) and *L. paracasei* + rice bran (fractions 18, 21, 22, and 24) and was selected to represent different portions of the mobile phase extraction gradient (different % water versus % methanol solvent ratio). A total of 196 distinct metabolites were identified in the *L. fermentum* + rice bran postbiotic fractions, including 182 metabolites in fraction 18, 138 metabolites in fraction 21, and 123 metabolites in fraction 22. In the *L. paracasei* + rice bran postbiotic fractions, 222 total metabolites were identified, including 167 metabolites in fraction 18, 158 metabolites in fraction 21, 162 metabolites in fraction 22, and 137 metabolites in fraction 24.

To identify metabolites that had increased the abundance in postbiotic fractions relative to unfractionated postbiotics, the metabolite abundances in *S.* Typhimurium suppressing fractions were compared with the complete (un-fractionated) metabolomes of *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics. A metabolite was defined as enriched its relative abundance in a fraction had a Z-score ≥ 1.0 when compared with the metabolite abundance in the respective unfractionated postbiotic. Figure 4 and these metabolites enriched in *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotic fractions and these are additionally detailed with Z-scores and by fraction in Supplementary File S6. *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotic bioactive fractions were enriched in 43 and 106 total metabolites, respectively (Figure 4A). For *L. fermentum* + rice bran, this included 38 metabolites in fraction 18, 1 metabolite in fraction 21, and 4 metabolites in fraction 22. For *L. paracasei* + rice bran, this included 91 metabolites in fraction 18, 4 metabolites in fraction 21, 8 metabolites in fraction 22, and 3 metabolites in fraction 24. For both postbiotics, lipids contributed the

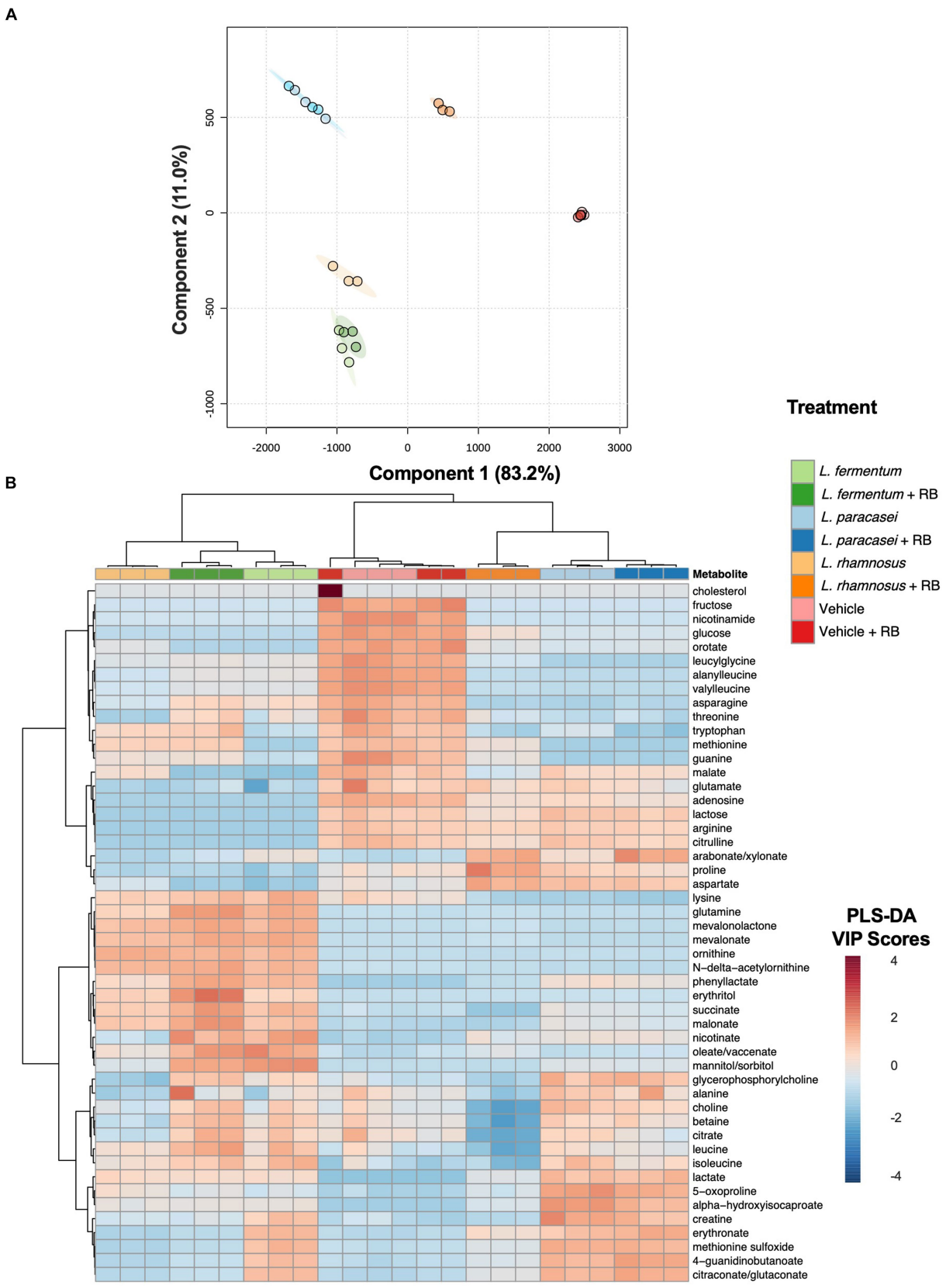

FIGURE 2
Probiotic metabolomes are differentially modulated during rice bran fermentation. Partial least squares discriminant analysis (PLS-DA) projection for components 1 and 2. Each circle represents an individual sample, and ellipses indicate the 95% confidence interval around each treatment group **(A)**. Heat map + unsupervised hierarchical clustering analysis of postbiotic treatments prepared with and without rice bran and vehicle controls (de Man Rogosa Sharpe broth with and without rice bran extract) **(B)**. Metabolites with the 50 largest variable importance (VIP) scores from the partial least squares discriminant analysis model are visualized. Red cells indicate a higher average VIP score for the metabolite in the respective sample when compared with other samples. Blue cells indicate a lower average VIP score for the metabolite when compared with other samples. Branch points are calculated using Euclidean distances and are shown with Ward scaling. All visualizations use median-scaled metabolites.

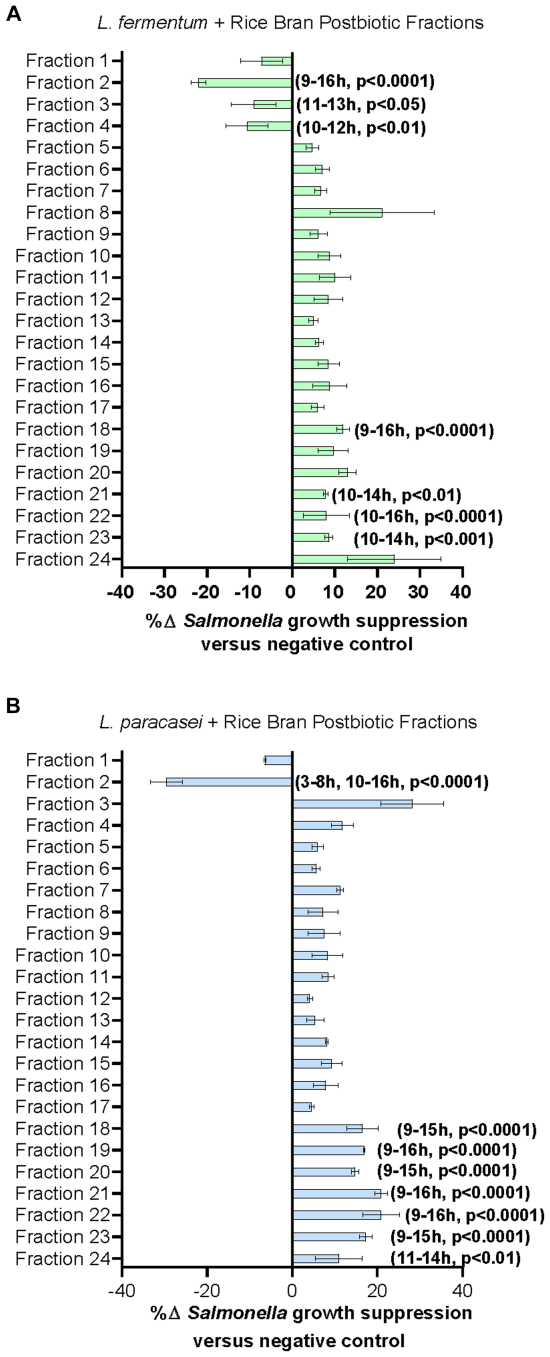

FIGURE 3
Lactobacillaceae+ rice bran supernatant postbiotic fractions suppress *S.* Typhimurium growth. **(A)** *L. fermentum*+rice bran postbiotic and **(B)**: *L. paracasei*+rice bran postbiotic fraction metabolite profiles and fraction percent *S.* Typhimurium growth suppression. Percent growth suppression of fractions is relative to the negative control treatment (Luria–Bertani broth). Values reflect maximal percent difference achieved by the fraction over the time interval where it was significantly different in growth compared with the negative control. Time intervals are rounded to the nearest hour. Parentheses indicate the time interval when the fraction exhibited significantly different growth, where positive values indicate increased *S.* Typhimurium growth suppression relative to the negative control, and negative values reflect decreased *S.* Typhimurium growth suppression relative to the negative control.

largest number enriched metabolites (~48% of metabolites in *L. fermentum* + rice bran and ~ 40% in *L. paracasei* + rice bran). A total of 2 enriched lipid metabolites were distinct to *L. fermentum* + rice bran, 19 lipid metabolites were distinct to *L. paracasei* + rice bran, and 24 lipid metabolites were shared by both postbiotics. These lipids included the fatty acids, such as azelate (Z-score of 1.24 in *L. fermentum* + rice bran fraction 18 and 3.34 in *L. paracasei* + rice bran fraction 18), oleate/vaccenate (Z-score of 34.52 in *L. paracasei* + rice bran fraction 18), and linoleate (Z-score of 1.24 in *L. fermentum* + rice bran fraction 18, 2.47 in *L. paracasei* + rice bran fraction 18). In addition, it is noteworthy that the mevalonate lipid, 3-hydroxy-3-methylglutarate, was enriched in *L. paracasei* + rice bran fraction 18 (Z-score of 8.54) (Figure 4B).

Among other enriched metabolites, amino acids contributed to 12% of the *L. fermentum* + rice bran-enriched metabolites, ~34% of *L. paracasei* + rice bran-enriched metabolites, phytochemicals to ~21% of *L. fermentum* + rice bran, and ~ 14% to *L. paracasei* + rice bran-enriched metabolites (Figure 4 and Supplementary File S6). Enriched amino acid metabolites included the histidine metabolite imidazole propionate (Z-score of 9.23 in *L. paracasei* + rice bran fraction 18), the glycine metabolite dimethylglycine (Z-score of 1.11 in *L. fermentum* + rice bran fraction 18), and the amino acid threonine (Z-score of 2.21 in *L. paracasei* + rice bran fraction 18, 1.25 in *L. paracasei* + rice bran fraction 22). Enriched phytochemical metabolites included the rice bran-derived dihydroferulate (Z-score 1.27 in *L. fermentum* + rice bran fraction 18, 2.35 in *L. paracasei* + rice bran fraction 18) and salicylate (Z-score of 1.27 in *L. fermentum* + rice bran fraction 18, 1.98 in *L. paracasei* + rice bran fraction 18).

4 Discussion

The overarching goal of this study was to examine the antimicrobial activity of three postbiotic preparations prepared with and without rice bran, a prebiotic source, on antimicrobial-resistant *S.* Typhimurium, which is the leading global cause of diarrhea in people and animals (CDC, 2019). This study identified two postbiotic preparations of Lactobacillaceae + rice bran that showed enhanced growth suppression of antimicrobial-resistant *S.* Typhimurium versus their respective probiotic-only postbiotic treatments (Figure 1 and Table 1). Among these treatments, postbiotics produced by *L. fermentum* + rice bran and *L. paracasei* + rice bran demonstrated ~42–55% enhanced *S.* Typhimurium growth suppression compared with their respective Lactobacillaceae postbiotics at a minimum dose of 18% supernatant/v (Table 1 and Figure 1).

Although *L. rhamnosus* + rice bran and *L. rhamnosus* postbiotics were both effective at suppressing *S.* Typhimurium growth relative to their vehicle controls (Supplementary File S2), the *L. rhamnosus* + rice bran postbiotic did not exhibit enhanced *S.* Typhimurium growth suppression versus *L. rhamnosus* postbiotic at any tested dose (Figure 1 and Table 1), highlighting that prebiotics differentially act on probiotic strains to modulate their functionality. While rice bran did not enhance the antimicrobial activity of the *L. rhamnosus* postbiotic against *S.* Typhimurium, it may have enhanced *L. rhamnosus* functions in other ways not captured in this study, as evidenced by the considerable non-targeted metabolome changes observed when comparing the *L. rhamnosus* and the

TABLE 2 Growth suppression of antimicrobial-resistant *Salmonella* Typhimurium by postbiotic supernatant fractions compared with the negative control.

Bioactive fraction	Mobile phase solvent		*S.* Typhimurium percent growth suppression	
	% Water	% Methanol	*L. fermentum* + rice bran postbiotic	*L. paracasei* + rice bran postbiotic
18	12.5%	87.5%	**↑ 11.39% ± 2.82%(12 h)**	**↑ 15.95% ± 6.99%(12 h)**
19	0%	100%	9.11% ± 5.32%*(4 h)*	**↑ 16.74% ± 0.80%(12 h)**
20	0%	100%	8.70% ± 10.52%*(2 h)*	**↑ 17.70% ± 5.45%(12 h)**
21	0%	100%	**↑ 7.83% ± 0.97%***(12 h)*	**↑ 22.30% ± 3.88%(12 h)**
22	0%	100%	**↑ 12.79% ± 8.68%(12 h)**	**↑ 19.90% ± 5.62%(12 h)**
23	100%	0%	**↑ 8.01% ± 4.83%(10 h)**	**↑ 17.05% ± 2.08%(10 h)**
24	100%	0%	23.28% ± 19.92%*(12 h)*	**↑ 10.96% ± 9.53%(12 h)**

Percent differences show maximum percent difference in *S.* Typhimurium growth for fractions versus the negative control (Luria–Bertani broth). Times in parentheses reflect the hour at which the fraction achieved maximal growth difference compared with the negative control, rounded to the nearest hour. The results reflect three independent experiments for each *S.* Typhimurium isolate with each treatment mean ± standard deviation. Treatments are bolded when growth was significantly different ($p < 0.05$), following a two-way analysis of variance test with Tukey's post-hoc correction. ↑ indicates that fraction had significantly more *S.* Typhimurium growth suppression compared with the negative control. Fraction numbers not shown did not have significantly increased *S.* Typhimurium growth suppression relative to the negative control for either the *L. fermentum* + rice bran or *L. paracasei* + rice bran postbiotics.

L. rhamnosus + rice bran postbiotic metabolite profiles (Figure 2). These mechanisms include the impacts on pathogen colonization and interactions with the immune system, which have been demonstrated in previous studies using combinations of *L. rhamnosus* and rice bran (Yang et al., 2015; Lei et al., 2016; Nealon et al., 2017b).

A role for metabolites in the enhanced antimicrobial activity of *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics was supported by the 109 metabolites that were significantly increased when comparing these postbiotics with their respective Lactobacillaceae-only treatments (Figure 2 and Supplementary Files S4, S5). The large number of differentially abundant amino acid and lipid metabolite compounds is consistent with previous studies, which concluded that these chemical classes contributed to the largest metabolomic changes during microbial fermentation of rice bran (Sheflin et al., 2015; Nealon et al., 2017a,b, 2019; Demissie et al., 2020; Seyoum et al., 2021). The substantial enhancement of methionine metabolites in the *L. fermentum* + rice bran postbiotic relative to the *L. fermentum* postbiotic has not been previously explored for its roles in antimicrobial activity. However, strain-dependent uptake and metabolism of environmental methionine have been reported for *L. fermentum* strains (Hossain, 2022), where various lactic acid bacteria strains utilize methionine and its derivatives to produce antimicrobial peptides and/or convert methionine into various downstream fatty acids or other metabolites with potential bioactivity (Rubinelli et al., 2017; Bindu and Lakshmidevi, 2021; Heredia-Castro et al., 2021; Hossain, 2022; Meng et al., 2022). Given these similarities across studies and the large number of differentially abundant metabolites potentially contributing to their antimicrobial activity, these two postbiotics were chromatographically fractionated and further evaluated for key metabolites, driving their antimicrobial activity against *S.* Typhimurium.

Bioactivity-guided fractionation identified four *L. fermentum* + rice bran and seven *L. paracasei* + rice bran postbiotic fractions that exhibited between ~8 and 22% enhanced *S.* Typhimurium growth suppression relative to the negative control (Figure 3 and Table 2), suggesting that they share some of the antimicrobial features of the un-fractionated postbiotic + rice bran supernatants. To investigate this further, the metabolite profiles of selected postbiotic fractions were examined and contained 43 enriched metabolites in *L. fermentum* postbiotic fractions and 106 metabolites in *L. paracasei* + rice bran postbiotic fractions (Figure 4 and Supplementary File S6). Similar to the global metabolite profile of these postbiotics, lipid, amino acid, and phytochemical metabolites comprised the majority of these enriched metabolites, including those metabolites that were dually significantly increased in the *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics versus their respective probiotic-only postbiotic. Specifically, significant increases in the fatty acids, including azelate (*L. fermentum* + rice bran postbiotic) and linoleate (both *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics), amino acids (methionine, *L. fermentum* + rice bran postbiotic), the phytochemicals ferulate and salicylate (enriched in both *L. fermentum* + rice bran and *L. paracasei* + rice bran postbiotics) have been demonstrated to increase during lactic acid bacteria metabolism of rice bran (Nealon et al., 2017a,b, 2019; Demissie et al., 2020; Seyoum et al., 2021) and are associated with enhanced antimicrobial and antiviral activity of these postbiotics when applied to *S.* Typhimurium and other enteropathogens (Rubinelli et al., 2017; Nealon et al., 2017a,b). The dual increase in these metabolites in the non-targeted global metabolome and enrichment in bioactive fraction metabolomes of postbiotics herein support the central roles of these metabolites and chemical classes in driving probiotic + rice bran postbiotic antimicrobial activity.

Limitations of this investigation include incomplete characterization and comparison of volatile organic compounds and short chain fatty acids such as lactate, acetate, propionate, and butyrate, as well as antimicrobial peptides, which are all known contributors to the antimicrobial activity of Lactobacillaceae (Pithva et al., 2011; Ibrahim et al., 2021). These chemical classes were not explored because the UPLC-MS/MS approach used herein was not optimized to capture these metabolites. Future experiments can include fermentation systems that better capture volatile organic compounds, before they partition out of each postbiotic, and couple these with gas chromatography approaches (Meredith and Tfaily, 2022). Proteomics approaches can additionally be applied to characterize antimicrobial peptides derived from probiotics and

10.3389/fmicb.2024.1362266

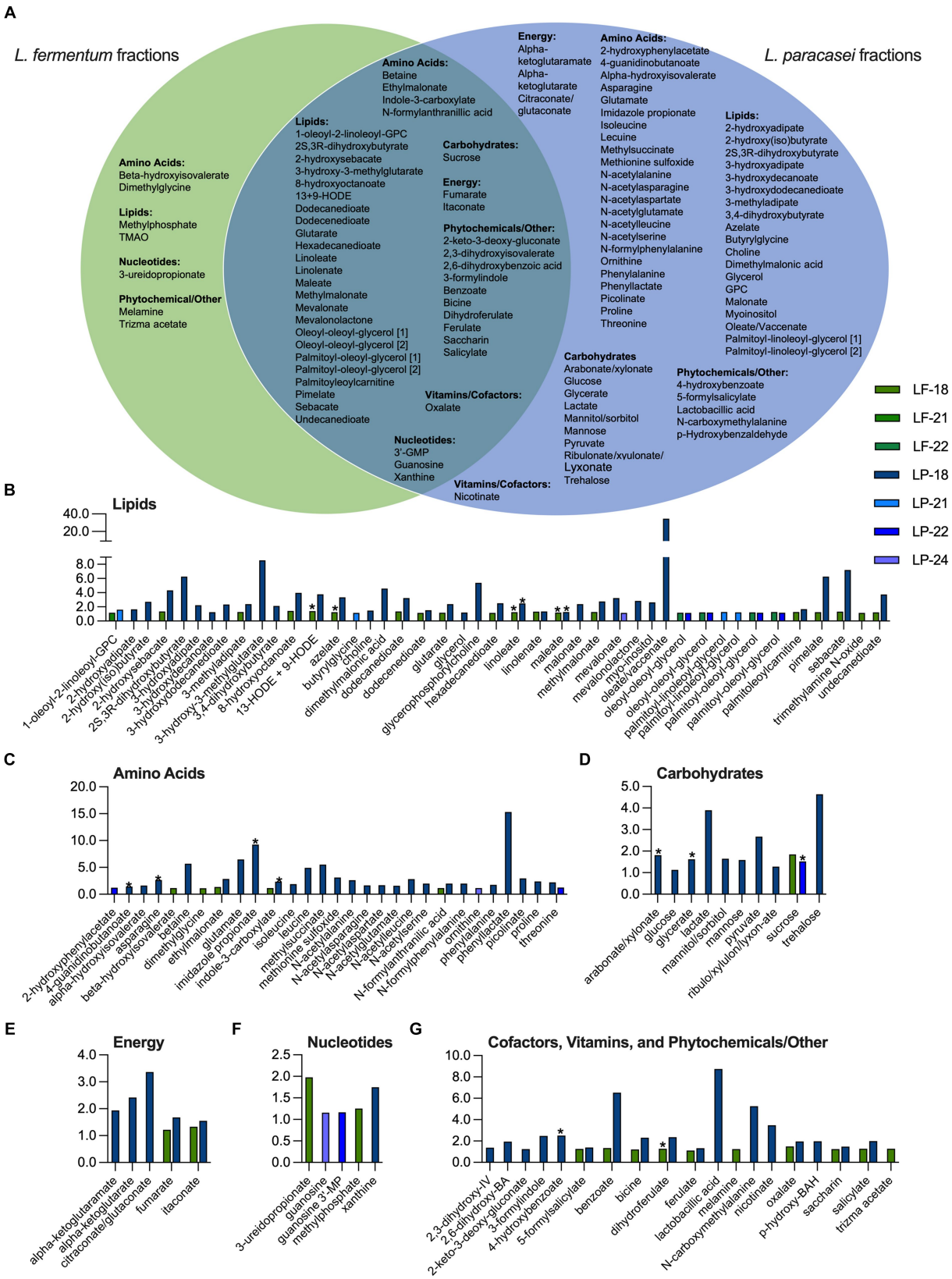

FIGURE 4
L. fermentum + rice bran and *L. paracasei* + rice bran postbiotics produce *S.* Typhimurium growth-suppressive fractions with distinct profiles of enriched metabolites. **(A)** Metabolites that were enriched Z-score ≥ 1.00 in *S.* Typhimurium-suppressive fractions for *L. fermentum* + rice bran (fractions 18, 21, and 22) and the *L. paracasei* + rice bran (fractions 18, 21, 22, and 24) postbiotics that were distinct to each postbiotic versus enriched in both postbiotic treatments. **(B–G)** Metabolite Z-scores for enriched metabolites in the *L. fermentum* + rice bran (green bars) and *L. paracasei* + rice bran (blue bars) postbiotics. * indicates metabolite that was significantly increased in the global, non-targeted metabolome of *L. fermentum* + rice bran versus *L. fermentum* and/or *L. paracasei* + rice bran versus *L. paracasei*. BA, benzoate/benzoic acid; BAH, benzaldehyde; GMP, guanosine monophosphate; IV, isovalerate; LF, *L. fermentum*; LP, *L. paracasei*; MP, monophosphate; GPC, glycerophosphorylcholine; HODE, hydroxyoctadecadienoic acid; RB, rice bran; TMAO, trimethylamine N-oxide; [1] and [2] identify metabolites with isomers.

postbiotics, and they can be integrated with metabolic network analysis and metaboloics analysis to understand the relationships between the proteome and metabolome in conferring antimicrobial activity (Hove et al., 2023). Ultimately, incorporating these metabolite classes will allow for more robust characterization of postbiotic antimicrobial activity, including examination how their production changes with the addition of rice bran to each postbiotic preparation.

Another limitation includes the examination of only a subset of all bioactive fractions with demonstrated antimicrobial activity against *S.* Typhimurium. It is possible that with metabolomic characterization of additional bioactive fractions, a more comprehensive understanding of postbiotic strain differences will develop. For example, additional bioactive fractions may contain enriched levels of methionine metabolites in the *L. fermentum* + rice bran postbiotic that was significantly increased in the global, non-targeted metabolome but not identified among enriched metabolites in *L. fermentum* + bioactive fractions profiled herein. With a more thorough understanding of the enriched bioactive metabolites in these other fractions, future studies can consequently apply targeted metabolomics to identify the concentrations of these compounds to use in downstream testing as purified cocktails of postbiotic compounds for targeted antimicrobial therapy. While no postbiotic fractions were capable of producing *S.* Typhimurium growth suppression levels similar to un-fractionated postbiotic + rice bran supernatants, it is additionally possible that combinations of bioactive fractions and/or their enriched metabolites will provide antimicrobial synergy that mirror the unfractionated postbiotic preparations if screened together in future assays. Furthermore, comparisons between postbiotics with and without rice bran in this study were limited to metabolites with known identities. While characterized metabolites provided robust numbers of treatment differences herein, it is widely reported that many metabolites in non-targeted datasets, including microbial metabolites, are still uncharacterized (Bauermeister et al., 2022; Zhou et al., 2022). In addition to the known metabolites identified herein, future evaluations using these methods may identify different and/or additional antimicrobial metabolites in postbiotics, including explaining strain differences in postbiotic antimicrobial activity. It should be noted that adjustment of all postbiotic treatments to a pH of 4.5, to control the impact of pH on *S.* Typhimurium growth, may have impacted the bioactivity of each postbiotic, such that the antimicrobial activity of any metabolite could be pH-dependent. For example, the metabolites salicylate and acetate have been shown to differentially impact the susceptibility of *E. coli* to the antibiotic kanamycin depending on the culture pH, and the bioactivity of probiotic-derived antimicrobial peptides has been shown to be pH-dependent when applied to various pathogens (Aumercier et al., 1990; Amiri et al., 2022). Future experiments could examine antimicrobial metabolites and peptides over different pH ranges to better characterize their full spectrum of *S.* Typhimurium growth suppressive activity.

The antimicrobial metabolites identified in preparations of Lactobacillaceae + rice bran postbiotics have targeted functional potential for use in industrial, environmental, medical, and veterinary settings. Given the rise in antimicrobial resistance pressures in the environment, alternative and novel sources of safe antimicrobials are urgently needed in people, animals, and agricultural systems. The systematic examination of postbiotics to prevent and treat the spread of antimicrobial-resistant infections is of current and emerging importance to global health. Postbiotic production using probiotics that are native to mammals, alongside widely available prebiotics from foods such as rice bran, is a promising area for future application to the problem of antimicrobial resistance. The application of both non-targeted metabolomics and bioactivity-guided fractionation to well-studied probiotic strains and rice bran has filled knowledge gaps of postbiotic metabolic diversity and provides rationale for developing predictive models that assess postbiotic metabolic capacity. Increasing our understanding of postbiotic metabolite production can fuel the development of targeted microbial-based preventives and treatments against multidrug-resistant pathogens that include but are not limited to *S.* Typhimurium.

Data availability statement

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

Author contributions

NN: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. SB: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. HH: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. ER: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by the Bill and Melinda Gates Foundation, Seattle, WA (OPP1015267, 2011), the National Institutes of Health-National Cancer Institute, Bethesda, MD (NIH-NCI, 1R01CA20112-02, 2015), the National Institutes of Health-Ruth L. Kirschstein-National Research Service Program, Bethesda, MD (5T32OD012201-05), and department startup funds awarded to ER.

Acknowledgments

The authors would like to thank Amethyst V. Holder and Paige Lahaie for their assistance in performing the postbiotic cell-free supernatant assay and Charli Geer for editorial assistance. The authors extend their further acknowledgements to Dr. Brian Cranmer for assistance with the bioactivity-guided ractionation experiments, Dr. Sangeeta Rao for providing the antimicrobial resistant *S.* Typhimurium

isolate used in this study, and Dr. Joshua Daniels for establishing the antimicrobial-resistant profile of the *S.* Typhimurium isolate. The datasets used for *Salmonella* growth suppression assays, global, non-targeted metabolomics, and bioactivity-guided fractionation metabolomics datasets were included in Dr. Nora Jean Nealon's doctoral thesis, which is publicly available online through the Colorado State University Proquest electronic database (Nealon, 2019).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1362266/full#supplementary-material

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