The probiotic fermented milk of *Lacticaseibacillus paracasei* JY062 and *Lactobacillus gasseri* JM1 alleviates constipation via improving gastrointestinal motility and gut microbiota

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**ABSTRACT**

Constipation is directly related to the intestinal microenvironment, in which the promotion of gastrointestinal (GI) motility and improvement of gut microbiota distribution are important for alleviating symptoms. Herein, after the intervention of probiotic fermented milk (FMMIX) containing *Lacticaseibacillus paracasei* JY062 and *Lactobacillus gasseri* JM1 for 14 d in Kunming mice with loperamide-induced constipation, the results indicated that FMMIX significantly increased the secretion of serum motilin, gastrin and 5-hydroxytryptamine, as well as decreased the secretion of peptide YY, vasoactive intestinal peptide, and nitric oxide in mice. As determined by immunohistochemical analysis, FMMIX promoted an augmentation in the quantity of Cajal interstitial cells. In addition, the mRNA and protein expression of c-kit and stem cell factor (SCF) were upregulated to facilitate intestinal motility. High-throughput sequencing and gas chromatography techniques revealed that FMMIX led to an increase in the relative abundance of beneficial bacteria (*Lactobacillus*, *Oscillospira*, *Ruminococcus*, *Coprococcus*, and *Akkermansia*), reduced the presence of harmful bacteria (*Prevotella*), and resulted in elevated levels of short-chain fatty acids (SCFA) with a superior improvement compared with unfermented milk. Untargeted metabolomics revealed significant upregulation of functional metabolites such as L-pipecolinic acid, DL-phenylalanine, and naringenin in FMMIX, presumably playing a potential role in constipation relief. Overall, our results showed that FMMIX had the potential to alleviate constipation symptoms in mice by improving the secretion of serum GI regulatory peptides and neurotransmitters, increasing the expression of c-kit and SCF proteins, and modulating the gut microbiota structure and SCFA levels, and may be associated with an increase in these functional metabolites. This suggested that FMMIX could be a promising adjunctive strategy for managing constipation symptoms and could contribute to the development of functional foods aimed at improving gut health.

**Key words:** compound probiotic fermented milk, constipation, gastrointestinal motility, gut microbiota, functional metabolites

**INTRODUCTION**

Constipation is a prevalent functional gastrointestinal (GI) disorder characterized by symptoms such as infrequent or difficult stool movements, prolonged GI transit time, hard stools, and abdominal discomfort (Barberio et al., 2021). It is a common condition worldwide, with a prevalence ranging from 14% to 30%, and it is more frequently observed in women and older adults (Li et al., 2020; Zhao et al., 2021). It can be classified into 3 main categories: normal transit constipation, slow transit constipation, and defecation disorders or rectal defecation disorders. Slow transit constipation is recognized as the primary type of morbidity, leading to the reduction of colonic transit rate and difficulties in stool passage, accounting for approximately 30% of all constipation cases (Dinning et al., 2015a). The main underlying mechanism is attributed to abnormal neurocolonic motility caused by dysfunction in the smooth muscle or innervation of the colon, leading to an impaired or faulty pattern of colon propulsion movement (Dinning et al., 2015b). This condition also predisposes individuals to a range of symptoms, including abdominal pain, bloating, loss of appetite, nausea, headaches, halitosis, restlessness, anxiety, and depression. Prolonged constipation could result in intestinal damage, the accumulation of intestinal toxins, and an increased risk of colon cancer (Liu et al., 2017). The
treatment of constipation often involves the use of prescription drugs, such as prokinetic drugs, *Helicobacter pylori* eradication drugs, proton pump inhibitors, or selective serotonin reuptake inhibitors (Feinle-Bisette et al., 2004). However, it is worth noting that the effectiveness of these drugs can vary depending on the underlying cause of the condition. Additionally, because constipation can be caused by multiple factors and may have overlapping effects, pharmacotherapy has limitations such as a low response rate and frequent relapses after discontinuation of the medication. Therefore, it is crucial to investigate potential alternative treatment strategies that do not rely on prescription drugs.

Dietary interventions, specifically the use of probiotics, are an effective and low-risk strategy for relieving constipation (Zhao et al., 2019). Emerging evidence has suggested that probiotics such as *Lactiplantibacillus plantarum* CQPC05, *Limosilactobacillus reuteri* DSM17938, and *Bifidobacterium lactis* HN019 exhibited promising effects in alleviating constipation. The main mechanisms by which these probiotics exerted their regulatory effects included modulation of neurotransmitter contents, regulation of the intestinal lumen pH level, and enhancement of functional intestinal motility frequency (Li et al., 2019; Cheng et al., 2021; Saviano et al., 2021). Indeed, fermented milk serves as an excellent carrier for probiotics, which can provide sufficient nutrients for the growth and metabolism of strains (Lourens-Hattingh and Viljoen, 2001). Probiotic fermented milk contains some active probiotic bacteria, which have been shown to confer health benefits when consumed in sufficient quantities (more than 10⁹ cfu/mL; Lourens-Hattingh and Viljoen, 2001). In addition, probiotic fermented milk contains high-quality protein, minerals, niacin, vitamin B₃, riboflavin, and other bioactive substances, contributing to the unique functional value of fermented dairy products (Peng et al., 2022). Studies have shown the potential of probiotic fermented milk to improve, alleviate, and prevent various diseases, such as enhancing immunity, improving cognitive impairment, and regulating metabolic homeostasis (Sakandar and Zhang, 2021). The beneficial effects of probiotic fermented milk on GI function have also been well-documented. For instance, Ozaki et al. (2018) discovered that probiotic fermented milk containing strain *Lactococcus lactis* ssp. cremoris FC improved intestinal motility function and fecal characteristics in healthy women. In a similar study, Anzawa et al. (2019) found that probiotic fermented milk containing *B. lactis* GCL2505 significantly influenced the population of *Bifidobacterium* in the gut microbiota, thereby regulating the intestinal environment. This pivotal role in modulating the gut microbiota has been shown to alleviate GI disorders, including constipation.

Previous studies have shown that the compound probiotic of *Lacticaseibacillus paracasei* JY062 (*L. paracasei* JY062) and *Lactobacillus gasseri* JM1 (*L. gasseri* JM1) possessed the functional attribute of promoting GI motility (Cheng et al., 2023). However, the effect of the compound probiotic fermented milk containing *L. paracasei* JY062 and *L. gasseri* JM1 on relieving constipation remains uncertain, partly due to the influence of matrix components present in the fermented milk. Therefore, to assess the laxative effect of the compound probiotic fermented milk containing *L. paracasei* JY062 and *L. gasseri* JM1, we conducted evaluations on the GI motility, fecal characteristics, neurotransmitter secretion, expression of c-kit and stem cell factor (SCF) signaling pathway, gut microbiota composition, and levels of short-chain fatty acids (SCFA). The functional components of fermented milk were further analyzed to further elucidate the factors that may affect constipation. The findings from this study will offer a theoretical foundation for the development of functional dairy products.

**MATERIALS AND METHODS**

**Preparation of Probiotic Fermented Milk**

The probiotic fermented milk was prepared with slight modifications to the method described previously (Yang et al., 2021). Commercially available raw milk was filtered and standardized at 20 MPa, sterilized at 90°C for 5 min, and rapidly cooled to 39.5°C. It was added 7.5% (wt/vol) sucrose and stirred to disperse well, and then inoculated the combination of *L. paracasei* JY062 (also known as *L. paracasei* TD062) and *L. gasseri* JM1 (viable count of *L. paracasei* JY062: viable count of *L. gasseri* JM = 1:2, abbreviated 1:2 later) at 1 × 10⁷ cfu/mL. The inoculated milk was fermented at 39.5°C until the pH reached 4.5, at which point fermentation was stopped. We isolated *L. paracasei* JY062 (GenBank accession number CP044361-CP044367) and *L. gasseri* JM1 (GenBank accession number CP044412-CP044414) from traditional fermented dairy products and healthy infant feces, respectively, by Northeast Agricultural University.

**Mouse Model and Experimental Design**

The male Kunming mice (n = 40, 6–7 wk) were from Vital River Laboratory Animal Technology Co., Ltd. The animal experiments in this study were approved by the Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (no. NEAU-2022-07-0215-23). The experiment was conducted with minor modifications based on a previous
protocol (Ren et al., 2017). Mice were housed in cages under controlled conditions of a room temperature of 22 ± 2°C, a humidity level of 55 ± 5%, and a 12-h light/dark cycle. In the initial week, all mice were allowed free access to food and water to facilitate acclimation to the environment. Subsequently, the mice were randomly divided into 5 groups (n = 8 in each group): normal control (NC), model (M), unfermented milk (UFM), probiotic fermented milk containing L. paracasei JY062 and L. gasseri JM1 in a 1:2 ratio (FM-MIX), and positive control fermented milk (PCFM). Except for the NC group, all groups of mice received loperamide at a dosage of 5 mg/kg of BW per day via gavage for a continuous period of 7 d. Simultaneously, the NC group received an equal volume of PBS via gavage. Following this, the mice in the UFM, FMMIX, and PCFM groups were gavaged unfermented milk, probiotic fermented milk, and positive control fermented milk (intended to regulate gut microbiota and promote intestinal digestion, containing Streptococcus thermophilus, Lactobacillus bulgaricus, Bifidobacterium lactis, and Lactobacillus paracasei) at a dosage of 10 mg/kg of BW per day for 2 consecutive weeks, and those of NC and M groups were gavaged with equal volumes of PBS, respectively. After the experiment, the mice were euthanized by intraperitoneal injection of ketamine and diazepam.

**Physiological Indicators**

The mice were fasted for 24 h and euthanized after gavage with 0.5 mL of ink for 30 min. The determination of the gastric emptying rate and small intestine propulsive rate in the mice involved the following modifications based on the previous methods (Guo et al., 2021): (1) The weights of the ink \(a_1\), the total stomach \(a_2\), and the stomach without stomach contents \(a_3\) were determined to calculate the gastric emptying rate. (2) The distance from the pylorus to the front end of the ink \(L_1\) and the distance from the pylorus to the ileocecal region \(L_2\) were determined to calculate the small intestinal propulsive rate. (3) The low-temperature freeze-drying method was used to determine the fecal moisture content. The weight of the plate \(b_1\) and the total weight of the plate and feces before freeze-drying \(b_2\) and after freeze-drying \(b_3\) were determined. The formulas were as follows:

\[
\text{gastric emptying rate (\%)} = \left(1 - \frac{a_3 - a_2}{a_1}\right) \times 100,
\]

\[
\text{intestine propulsive rate (\%)} = \frac{L_1}{L_2} \times 100, \text{ and}
\]

\[
\text{fecal moisture content (\%)} = \frac{b_2 - b_3}{b_2 - b_1} \times 100.
\]

**Histological Analysis**

The gastric antrum, small intestine, and colon of the mice were aseptically collected, and some tissues were fixed in 4% paraformaldehyde for 48 h. Subsequently, 5-mm-thick sections were obtained from the fixed tissues for hematoxylin-eosin staining, and their histological characteristics were analyzed (Wu et al., 2021).

**Determination of GI Regulatory Peptides, Neurotransmitters, and Digestive Enzymes Activity**

With the reference to the methodology of the previous study (Ren et al., 2017; Chen et al., 2019), the concentrations of motilin (MTL), gastrin (GAS), peptide YY (PYY), vasoactive intestinal peptide (VIP), and 5-hydroxytryptamine (5-HT) in serum were determined using commercially available ELISA kits (Nanjing Jiancheng Co. Ltd.). The concentration of nitric oxide (NO) in serum was determined using the nitric acid reductase biochemical kit (Beijing Solarbio Science & Technology Co. Ltd.). The activity of α-amylase, lipase, and trypsin in chyme were determined using a commercially available α-amylase activity assay kit, lipase activity assay kit, and trypsin activity assay kit (all from Beijing Solarbio Science & Technology Co. Ltd., respectively).

**Reverse Transcription and Quantitative PCR**

Determination of gene expression in mouse colon tissues was based on the previous method with minor modifications (Li et al., 2021). Briefly, the total RNA of colon tissues was extracted and the cDNA was synthesized with a Simple P total RNA extraction kit (Bioer Technology Co., Ltd.) and PrimeScript RT reagent kit (Takara Bio), respectively. The mRNA levels were determined using the QuantStudio 3 Real-Time PCR system (Applied Biosystems) with TB Green Premix Ex Taq II (Takara Bio). The relative expression of mRNA was calculated using the 2−ΔΔ(Cycle threshold) method to determine the expression of related genes (Aqp4, Aqp8, Tph1, 5-HT, SERT, VIPR1, NOS, c-kit, SCF). We used GAPDH as the internal reference gene. The primer sequences used were listed in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.24189648.v1; Cheng, 2023).

**Immunohistochemistry Analysis**

With minor modifications to the method described by Ren et al. (2017), interstitial cells of Cajal (ICC)
were identified through immunohistochemistry analysis to label the c-kit. Briefly, the colon sections were dewaxed in xylene and subjected to antigen retrieval using a citrate antigen retrieval solution. After cooling to 25°C, the sections were blocked with goat serum for 1 h and then incubated overnight at 4°C with an anti-c-kit antibody, followed by incubation with biotinylated secondary antibody at 25°C for 1 h and horseradish enzyme-labeled avidin at 37°C for 30 min. The sections were stained with diaminobenzidine and observed under a light microscope. The mean density was determined using Image-pro Plus 6.0 software (Media Cybernetics Inc.).

**Western Blot Analysis**

The protein expression of c-kit and SCF was determined by western blot analysis, with modifications made to previously described methods (He et al., 2017; Li et al., 2021). Specifically, frozen colonic tissue (100 mg) was homogenized in protein lysate (1 mL) containing a protease inhibitor cocktail (1:200). The homogenate was centrifuged at 13,000 × g for 5 min, and the supernatant was collected to determine the protein concentration using the bicinchoninic acid protein assay kit (Roche). Equal amounts of protein samples (40 µg) were separated by 10% SDS-PAGE (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST; Beyotime) at 37°C for 2 h. We used GAPDH as an internal reference. The membranes were incubated overnight at 4°C with the primary antibody (1:1,000). After washing in TBST, the secondary antibody (1:5,000) was added and incubated at 25°C for 30 min. The blots were subsequently washed with TBST and exposed to enhanced chemiluminescence-plus reagents for chemiluminescence detection. The grayscale of the target bands was analyzed using Image-pro Plus 6.0 software (Media Cybernetics Inc.).

**Gut Microbiota Sequencing and Analysis**

The cecum contents of the mice were collected to analyze the gut microbiota composition. Total DNA extraction was performed using TIANamp Stool DNA kits (Tiangen), and the V3–V4 region of the 16S rRNA gene was amplified using previously described primers (forward: ACTCTCTACGGGAGGCAGCAG, reverse: GGAATTACHVGGGTWTCTAAT; Tang et al., 2022). The DNA samples were sequenced using an Illumina MiSeq. After sequencing, bacterial diversity analysis was conducted on the 16S rRNA sequence data (https://github.com/QIIME2/).

**Determination of SCFA**

The cecum contents of the mice were homogenized and sonicated to create a suspension and centrifuged at 2,400 × g for 20 min at 4°C. The extract (0.8 mL, 25 mg/L methyl tert-butyl ether stock solution) and 50% H2SO4 (0.1 mL) were added as internal standards to the supernatant (0.8 mL). The mixture was sonicated for 10 min in an ice bath and then centrifuged at 9,600 × g for 15 min at 4°C. The supernatant was subsequently transferred to a fresh glass vial for GC-MS analysis (Wang et al., 2020a).

**Determination of Nonvolatile Components of Probiotic Fermented Milk**

The samples were prepared following Wang’s method with slight modifications (Wang et al., 2021). Briefly, milk samples (5 mL) were added to a pre-cooled solution of methanol/acetonitrile/water (2:2:1, vol/vol). The solution was mixed by vortexing and sonicated at low temperature for 30 min, and stood at −20°C for 10 min. The solution was then centrifuged at 14,000 × g for 20 min at 4°C, and the supernatant was collected for vacuum drying. The dried sample was then reconstituted by adding 100 µL of acetonitrile-water solution (acetonitrile:water = 1:1, vol/vol). The solution was centrifuged at 14,000 × g for 15 min at 4°C. The supernatant was collected and filtered through a 0.22-µm microporous membrane (Agilent) for analysis. In addition, the quality control samples were prepared by mixing equal amounts of the test samples and were determined before, during, and after the injection of the test samples in the liquid chromatography MS system. The samples were separated using an ultra-high-performance liquid chromatography system (Agilent 1290 Infinity LC). For hydrophilic interaction liquid chromatography separation, samples were analyzed using a 2.1-mm × 100-mm Acquity UPLC BEH 1.7-µm column (Waters, Ireland). The column temperature was set at 50°C, and the flow rate was 0.5 mL/min. A 2-µL injection volume was used. The mobile phase A consisted of water, 25 mM ammonium acetate, and 25 mM ammonia, and the mobile phase B was acetonitrile. The gradient elution program was as follows: 0 to 0.5 min, 95% B; 0.5 to 7 min, linear change of B from 95% to 65%; 7 to 8 min, linear change of B from 65% to 40%; 8 to 9 min, B maintained at 40%; 9 to 9.1 min, linear change of B from 40% to 95%; 9.1 to 12 min, B maintained at 95%. Throughout the entire analysis, the samples were kept in an autosampler at 4°C. The sample spectra were acquired using an AB Triple TOF 6600 mass spectrometer (AB SCIEX) in both positive ion mode and negative ion mode. The
electrospray ionization source conditions are as follows: ion source gas 1 = 60, ion source gas 2 = 60, curtain gas = 30, source temperature = 600°C, ion spray voltage floating = ±5500 V, TOF MS scan m/z range = 60–1,000 Da, product ion scan m/z range = 25–1,000 Da, TOF MS scan accumulation time = 0.20 s/spectra, product ion scan accumulation time = 0.05 s/spectra. The second-level mass spectrometry was acquired using information-dependent acquisition and the high sensitivity mode. The declustering potential was ±60 V and the collision energy was 35 ± 15 eV.

Statistical Methods

A one-way ANOVA was performed by SPSS (IBS SPSS Statistics 23) and Origin 2018 software. Graph production was performed by Excel (Microsoft Excel Office 2019) and GraphPad Prism 8.02. Experimental results were repeated 3 times and expressed as mean ± standard deviation (mean ± SD), and P < 0.05 indicated a significant difference. The raw data in .wiff format was converted to .mzXML format using ProteoWizard (http://www.proteowizard.org/). The XCMS software (https://xcmsonline.scripps.edu/) was then used for peak alignment, retention time correction, and peak area extraction. The data extracted using XCMS were subjected to metabolite structure identification, data preprocessing, and quality evaluation. Multivariate statistical analysis, such as principal component analysis and orthogonal partial least squares discriminant analysis, as well as a Student’s t-test, was employed to identify differential metabolites in the samples. The differential metabolites were further analyzed using the Kyoto Encyclopedia of Genes and Genomes database (https://www.genome.jp/kegg/pathway.html) to determine the metabolic pathways involved.

RESULTS

Physiological Indicators and Histological Analysis in Constipated Mice

As shown in Figure 1A, 1B, and 1C, the gastric emptying rate, intestine propulsive rate, and fecal moisture content were significantly reduced in the M group compared with the NC group, indicating a significant modeling effect in mice with constipation induced by loperamide (P < 0.05). Compared with the M group, the UFM, FMMIX, and PCFM groups showed a significant increase in gastric emptying rate, intestine propulsive rate, and fecal moisture content (P < 0.05). We observed no significant difference between FMMIX and PCFM (P > 0.05), but the improvement was most obvious in FMMIX. We also found that fecal moisture content in the FMMIX group reached a similar level to that of the NC group, without a significant difference between them (P > 0.05). Thus, it was shown that FMMIX improved the delayed gastric emptying rate, delayed intestine propulsive rate, and fecal dryness caused by constipation.

The hematoxylin-eosin staining results (Figure 1D) showed that the histological structure of gastric antrum in the M group was disorganized, and some closely arranged tubular glands with disorganized glandular arrangement were still visible in the gastric sinus (black arrows). The small intestinal villi were loosely arranged and shortened in length. Both the gastric antrum and small intestine showed epithelial cell erosion and detachment (red arrows) and inflammatory cell infiltration (blue arrows). The crypt structure of the colonic mucosal layer in the M group partially disappeared, along with focal infiltration of inflammatory cells (blue arrows). Some goblet cells were also present (green arrows). After the intervention, UFM, FMMIX, and PCFM all alleviated the degree of tissue injury in the gastric antrum, small intestine, and colon. Notably, we observed visible cell necrosis in the small intestine of the UFM group (yellow arrows), but all others were alleviated to some extent. In addition, the degree of tissue injury was significantly diminished in FMMIX and PCFM (P < 0.05), which was superior to that of UFM.

GI Regulatory Peptide, Neurotransmitter, and Digestive Enzyme Activity

Excitatory GI regulatory peptides (MTL and GAS) and inhibitory GI regulatory peptides (PYY and VIP) play an essential role in the regulation of GI functional disorders. The levels of excitatory GI regulatory peptides MTL and GAS were significantly lower in the M group compared with the NC group (P < 0.05; Figure 2A and 2B). After the dietary intervention, the levels of MTL and GAS were significantly increased in the UFM, FMMIX, and PCFM groups (P < 0.05), especially in the FMMIX group, which had higher levels than the UFM and PCFM groups. Meanwhile, the levels of inhibitory GI regulatory peptides PYY and VIP were significantly increased in the M group (P < 0.05; Figure 2C and 2D). After the FMMIX intervention, the levels of both PYY and VIP were significantly reduced (P < 0.05), without significant difference compared with the PCFM group (P > 0.05). We also observed that the level of VIP in the FMMIX group reached a similar level to that of the NC group. Interestingly, we found lower levels of PYY in the UFM group than that in the FMMIX group, but no significant difference (P > 0.05). Simultaneously, the level of excitatory neurotransmitter 5-HT was significantly reduced, and the level of inhibi-
tory neurotransmitter NO was significantly increased in the M group ($P < 0.05$). These levels were reversed after the dietary intervention, with the most significant effect being observed in the FMMIX group (Figure 2E and 2F). The activity of digestive enzymes ($\alpha$-amylase, lipase, and trypsin) was directly related to GI digestive function. Specifically, digestive enzyme activities were significantly decreased in the M group, whereas those in the FMMIX group were significantly increased, with $\alpha$-amylase activity being significantly higher than that in the PCFM group ($P < 0.05$) (Figure 2G, 2H, and 2I). Unexpectedly, lipase activity increased the most after the UFM intervention. Therefore, the UFM, FMMIX, and PCFM interventions all showed improvement in the aforementioned GI regulatory peptides, neurotransmitters, and digestive enzyme activities in constipated mice to a certain extent, but overall the improvement was particularly pronounced after the FMMIX intervention.

Figure 1. Alleviating effect of probiotic fermented milk on constipated mice and changes in gastric emptying rate (A), small intestinal propulsive rate (B), and fecal moisture content in mice (C). Each bar represents mean $\pm$ SD, $n = 4$. Different letters (a–d) indicate significant differences in values ($P < 0.05$). The upper and lower edges represent the interquartile range of the entire dataset. Midlines represent the median of the entire dataset. Whiskers represent the lower 25% and upper 25% of the range of data values. Panel (D) shows hematoxylin-eosin staining results for the gastric antrum, small intestine, and colon sections (original magnification $\times$200). Disorganized glandular arrangement is indicated by black arrows, epithelial cell erosion and detachment by red arrows, inflammatory cell infiltration by blue arrows, goblet cells by green arrows, and cell necrosis by yellow arrows. NC = normal control, M = model, UFM = unfermented milk, FMMIX = probiotic fermented milk, PCFM = positive control fermented milk.
Expression of Related Genes

The expression of related genes was determined in this study to elucidate the intervention mechanism of FMMIX in constipated mice. Compared with the NC group, the expression levels of Aqp4 and Aqp8 were significantly increased in the M group (P < 0.05), but those levels were decreased after dietary intervention, especially in the FMMIX group, which reached a similar level to that of the NC group (Figure 3A and 3B). In addition, the gene expression levels of Tph1 and 5-HT₄R were significantly reduced in the M group, whereas that of SERT was significantly increased (P < 0.05), but their expressions were all improved after the dietary intervention. Specifically, the effects of the 2 fermented milks were significantly stronger than that of unfermented milk (P < 0.05), among which the effect of FMMIX in Tph1 and 5-HT₄R expression was superior.
to that of PCFM, whereas the improvement in SERT expression was more pronounced in the PCFM group, but we did not find a significant difference between them ($P > 0.05$; Figure 3C, 3D, and 3E). Concurrently, we found that VIPR1 and NOS expression levels were decreased after the dietary intervention, especially in the FMMIX group (Figure 3F and 3G). It is worth noting that the expression levels of c-kit and SCF were reduced in the M group and increased in the diet intervention groups. The c-kit and SCF levels in the FMMIX group were significantly higher than those in the UFM and PCFM groups ($P < 0.05$), without significant differences from the NC group ($P > 0.05$; Figure 3H and 3I). The above results further validated the modulatory effects of FMMIX on GI regulatory peptides and neurotransmitters by gene expression, indicating that it may improve GI motility and suggesting a need to initiate further studies.

Figure 3. Regulatory effect of probiotic fermented milk on 9 related genes in constipated mice: Aqp4 (A), Aqp8 (B), Tph1 (C), 5-HT$_4$R (D), SERT (E), VIPR1 (F), NOS (G), c-kit (H), and SCF (I). Different letters (a–d) indicate significant differences in values ($P < 0.05$). Each bar represents mean ± SD, n = 4. NC = normal control, M = model, UFM = unfermented milk, FMMIX = probiotic fermented milk, PCFM = positive control fermented milk.
**ICC Distribution and Protein Expression of c-kit and SCF**

Improving GI motility can be effective in alleviating constipation, and ICC is important pacemaker cells of the intestine, closely related to intestinal motility (Jeon et al., 2019). The distribution of ICC and the semi-quantitative number of ICC were observed by labeling the tyrosine kinase receptor c-kit on the ICC membrane with a c-kit antibody. The brownish-yellow color in the results of immunohistochemistry (Figure 4A) indicated positive markers, and the darker color indicated a higher degree of positivity. Mice in the M group had lighter colonic staining, whereas constipated mice had darker colonic staining after FMMIX intervention, indicating an increase in the extent of c-kit labeling and an increase in the number of ICC in the colon of mice. Simultaneously, the mean density of the M group was significantly lower than that of the NC group, which was significantly higher after the dietary intervention (P < 0.05), especially in the FMMIX group (Figure 4B). This was consistent with the results of staining and labeling. Therefore, intervention with FMMIX could increase the number of ICC, elucidating its effect on promoting intestinal motility.

To further explore the mechanism affecting the number of ICC, we determined the protein expression of c-kit and SCF. As shown by the results in Figure 4C and 4D, the expression of c-kit and SCF proteins was significantly decreased (P < 0.05) in the M group (0.11 ± 0.024 and 0.15 ± 0.033) compared with the NC group (0.91 ± 0.022 and 0.84 ± 0.039), with decreased rates of 87.4% and 82.5%, respectively. Compared with the M group, the expression of c-kit and SCF proteins was significantly increased after the dietary interventions (P < 0.05), with the most prominent levels in the FM-MIX group (0.56 ± 0.038 and 0.59 ± 0.051), as shown by the growth rates of 390.0% and 305.9%, respectively. Simultaneously, we found that the protein levels of c-kit and SCF in the FMMIX group were significantly higher than those in the PCFM group (P < 0.05). However, the expression of c-kit protein in the UFM group was not significantly different from that in the M group (P > 0.05), which was consistent with the results of immunohistochemistry.

**Gut Microbiota**

Gut microbiota plays an important role in maintaining intestinal homeostasis and regulating GI function (Zhou et al., 2022). We analyzed the gut microbiota of constipated mice and explored the effect of dietary intervention on the gut microbiota of constipated mice. Alpha diversity analysis characterized the richness using Chao1 and observed species indices and the diversity using Shannon and Faith's phylogenetic diversity indices. The results showed that the indices were decreased in the M group and increased in the FMMIX group, indicating that the richness and diversity of gut microbiota were improved in FMMIX-intervened mice (Figure 5A–5D). Although we did not find a significant difference between the groups, the results were similar to those of Zhang et al. (2022). Beta diversity analysis by principal coordinates analysis and nonmetric multidimensional scaling showed that the dietary intervention group was separated from the M group to a different extent, indicating that the gut microbiota of mice was regulated after the dietary intervention (Figure 5E and 5F).

At the phylum level of gut microbiota composition (Figure 5G), *Firmicutes* and *Bacteroidetes* were predominant in all groups of mice, with both accounting for more than 90% of the total. The abundance of *Firmicutes* was decreased and that of *Bacteroidetes* was increased in the M group, but these trends were reversed after the dietary intervention. The most noticeable improvement observed in the FMMIX group was that both *Firmicutes* and *Bacteroidetes* in the FMMIX group (62.72% and 30.71%, respectively) were close to those in the NC group (63.73% and 30.74%, respectively). At the family level of gut microbiota composition (Figure 5H), the NC group was dominated by *Lachnospiraceae* and *Ruminococcaceae*, and the M group had a higher abundance of *S24-7* and *Prevotellaceae* than other groups. Compared with the M group, the abundance of *Lachnospiraceae*, *Ruminococcaceae*, and *Rikenellaceae* was increased and the abundance of *S24-7* and *Prevotellaceae* was decreased after dietary intervention in constipated mice. We also found that the decrease in the abundance of *S24-7* and *Prevotellaceae* was most prominent in the PCFM group, and the increase in *Lachnospiraceae*, *Ruminococcaceae*, and *Rikenellaceae* was most prominent in the FMMIX group, indicating that the FMMIX intervention increased the colonization of beneficial bacteria in the intestine of mice. At the genus level of gut microbiota composition (Figure 5I), it is noteworthy that the abundance of *Lactobacillus* was 0.61%, 0.23%, 0.34%, 1.39%, and 0.77% in the NC, M, UFM, FM-MIX, and PCFM groups, respectively, indicating that the abundance of *Lactobacillus* was increased and higher in the FMMIX group than that in the NC group. In addition, the abundance of *Oscillospira*, *Ruminococcus*, and *Coproccocus* was increased and that of *Prevotella* was decreased in the FMMIX group compared with the M group. These changes effectively improved the composition of gut microbiota in constipated mice, reduced the abundance of harmful bacteria, and promoted the colonization of beneficial bacteria.
As shown by the heat map of species composition on relative abundance at the top 50 in genus level (Figure 6A), we found a clear aggregation between different samples within the M group, indicating a high degree of similarity in the community structure of their gut microbiota, with the dominant genera mainly concentrated in *Anaerostipes*, *Acinetobacter*, *Pseudomonas*, and *Prevotella*, with *Shigella* or *Staphylococcus* appearing in the intestines of individual mice. *Blautia*, *Weissella*, and *Pelomonas* were increased in individual mice of the UFM group; *Lactobacillus*, *Streptococcus*, and *Rikenella* were increased in individual mice of the PCFM group; *Lactobacillus*, *Coprococcus*, *Bifidobacterium*, *Akkermansia*, *Faecalibacterium*, and *Oscillospira* were increased in individual mice of FMMIX group. Meanwhile, the abundance of *Shigella* and *Staphylococcus* was reduced in the NC and dietary intervention groups. Overall, the NC group was far away from the M group, and the gut microbiota of mice after the dietary intervention clustered with the NC group, which also further supported the results of beta diversity analysis. Linear discriminant analysis effect size analysis (Figure 6B, 6C, and 6D) showed that in the comparison of M, UFM, PCFM, and FMMIX groups, the FMMIX group was predominantly enriched in *Akkermansia*, *Adlercreutzia*, *Megamonas*, and *Oscillospira*; the M group was mainly enriched in *Dehalobacterium* and *Mogibacteriaceae*; the UFM group was mainly enriched in *Desulfovibrionales* and *Bilophila*; and the PCFM group was mainly enriched in *Streptococcus*, *Dehalobacterium*, and

![Figure 4.](image-url)
Erysipelotrichaceae_Clostridium. Overall, these were consistent with the above description. In all, the balance of the intestinal microenvironment was disrupted in the M group mice, which were prone to colonization by harmful bacteria, and the beneficial bacteria were increased after the dietary intervention to increase probiotic colonization, thereby regulating gut microbiota and improving the intestinal microenvironment to alleviate constipation.

SCFA Levers and Correlation Analysis

Studies have shown that SCFA, metabolites of gut microbiota, could regulate intestinal function (Kaji et al., 2015; Wang et al., 2020b). We explored the levels of SCFA in the cecum contents of each group of mice and determined whether and to what extent they affected constipation. As shown in Figure 7A, 7B, 7C, 7D, 7E, and 7F, the levels of SCFA (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) were significantly decreased in the M group compared with the NC group ($P < 0.05$). However, the levels of SCFA were reversed in the UFM, FMMIX, and PCFM groups after the dietary intervention, among which the levels of acetic acid, propionic acid, isobutyric acid and valeric acid in the FMMIX group were not significantly different from those in the PCFM group ($P > 0.05$). It was also notable that the levels of butyric acid and isovaleric acid in the FMMIX group were significantly elevated compared with those in the PCFM group.
group ($P < 0.05$). Thus, the levels of SCFA in constipated mice were improved by FMMIX intervention, which was superior to those of PCFM intervention.

Further correlation analysis of gut microbiota composition and physiological indexes (Figure 7G) showed that the levels of 5-HT, SCFA, c-kit, and SCF protein were positively correlated with the abundance of \textit{Lactobacillus} and negatively correlated with \textit{Prevotella}. Among them, isobutyric acid was significantly and positively correlated with \textit{Lactobacillus}, whereas acetic acid and isobutyric acid were significantly and negatively correlated with \textit{Prevotella} ($P < 0.05$). Meanwhile, the preceding study found that the abundance of \textit{Lactobacillus} increased and the abundance of \textit{Prevotella} decreased in the gut microbiota of mice after FMMIX intervention, which could regulate the intestinal microenvironment by improving gut microbiota and thus alleviate constipation.

**Metabolic Profiles Analysis in the Probiotic Fermented Milk**

Based on the functionality of FMMIX, we further revealed the functional components of this fermented milk. FMMIX and UFM were distinguished in a principal component analysis of both positive and negative ion modes (Figure 8A and 8B). Screening of significantly different metabolites with strict orthogonal partial least squares discriminant analysis VIP $> 1$, $P$ value $< 0.05$ and fold change ($FC$) $> 2$ or $FC < 0.5$ criteria (Supplemental Table S2; https://doi.org/10.6084/m9.figshare.24189648.v1; Cheng, 2023) and clustering heat map analysis (Figure 8C) resulted in 2 clusters, a and b, suggesting that the upregulated metabolites fermented by \textit{L. paracasei} JY062 and \textit{L. gasseri} JM1 were significantly greater than the downregulated metabolites. Compared with UFM, the
contents of phenyllactic acid, pyridoxal (vitamin B₆), DL-lactate, naringenin, isoliquiritigenin, L-pipecolinic acid, and DL-phenylalanine were significantly increased, and hippuric acid was significantly decreased in FMMIX (P < 0.05; Figure 8D). In the enrichment analysis of metabolic pathways (Figure 8E), the main enriched pathways were ATP-binding cassette transporters, pyrimidine metabolism, amino sugar and nucleotide sugar metabolism, galactose metabolism, the phosphotransferase system, arginine and proline metabolism, and glyoxylate and dicarboxylate metabolism.

**DISCUSSION**

The etiology of constipation is currently understood to be heterogeneous and multifactorial, with dietary interventions being widely explored as a means to alleviate symptoms (Bharucha and Lacy, 2020; Li et al., 2022). In this study, we aimed to investigate the mechanisms by which FMMIX (fermented by the compound of *L. paracasei* JY062 and *L. gasseri* JM1) alleviated constipation, in terms of GI regulatory peptides, neurotransmitters, GI motility, and gut microbiota.
Figure 8. The metabolic profiles and differential metabolites in the probiotic fermented milk (FMMIX) and unfermented milk (UFM), including principal component analysis (PCA) in positive (A) and negative (B) ion mode, heat map of nonvolatile metabolic profiles (C), differential metabolites between FMMIX and UFM batches (D), and the enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved by significantly different metabolites when comparing FMMIX with UFM (E). n = 3. [1] and [2] represent principal component 1 and 2, respectively. In panel D, the upper and lower edges represent the interquartile range of the entire dataset. Midlines represent the median of the entire dataset. Whiskers represent the lower 25% and upper 25% of the range of data values.
We first demonstrated the alleviating effect of probiotic fermented milk containing *L. paracasei* JY062 and *L. gasseri* JM1 on constipated mice.

Loperamide acts on intestinal smooth muscle, which inhibits the contraction of intestinal smooth muscle, slows down intestinal motility, and prolongs the residence time of food in the intestine (Merritt et al., 1982). It has been extensively employed in the establishment of constipated mice models and consistently demonstrated its efficacy in numerous research studies (Zhang et al., 2021; He et al., 2022). In this mouse experiment, the use of 5 mg/(kg·d) body weight of loperamide by gavage for 7 d has been studied (Eor et al., 2019). Concurrently, the gastric emptying rate, small intestine propulsive rate, and fecal moisture content of the M group were found to be significantly lower than those of the unmodeled mice, indicating the successful establishment of a mouse model of constipation. Following intervention with UFM, FMMIX, and PCFM at the dose of 10 mg/(kg·d) body weight (Li et al., 2021), we observed an improvement in the aforementioned indexes in mice, suggesting that dietary intervention in this study has a beneficial effect on alleviating constipation.

The extent of GI tissue damage directly or indirectly affects GI function. In this study, glandular arrangement disorder in the gastric antrum tissue, epithelial cell detachment in some mucosal layers of the small intestinal tissue, and focal infiltration of inflammatory cells in the colonic tissue were alleviated after interventions with UFM, FMMIX, and PCFM in mice. Cell necrosis was still observed in the small intestine of mice in the UFM group, which was relieved with FMMIX intervention. It also showed that the alleviation of tissue damage in mice was more significant with FMMIX intervention compared with UFM intervention. Additionally, our findings indicated that milk co-fermented with *L. paracasei* JY062 and *L. gasseri* JM1 increased the presence of functional substances such as naringenin and isoliquiritinigenin, and some studies have shown that naringenin and isoliquiritinigenin possessed antioxidant and anti-inflammatory properties and could alleviate intestinal tissue damage (Zhong et al., 2021; Yahya et al., 2022). Simultaneously, previous studies have demonstrated that *L. paracasei* JY062 and *L. gasseri* JM1 exhibited anti-inflammatory properties and enhanced the integrity of the mucosal barrier in the GI tract (Sun et al., 2020; Zhao et al., 2022a). Therefore, it was plausible that FMMIX could potentially ameliorate constipation symptoms by facilitating the repair of intestinal damage in constipated mice.

The enteric nervous system (ENS) is a highly autonomous GI neural network of neurons and neuroglia that regulates fluid exchange and blood flow over the mucosal surface, as well as the secretion of intestinal hormones and neurotransmitters. The secretion could act on the corresponding receptors to be accepted by the colon and transferred to the adjacent smooth muscle cells to control the peristalsis of the intestine and facilitate defecation (Sasselli et al., 2012). The regulation of intestinal motility was primarily carried out through the ENS. In the case of constipated mice, the intervention of FMMIX has been shown to modulate the levels of GI regulatory peptides and neurotransmitters. Specifically, FMMIX could increase the levels of serum MTL, GAS, and 5-HT, along with decreasing the levels of PYY, VIP, and NO. In particular, MTL has been found to enhance the myoelectric complex component of GI motility migration, which was closely associated with gastric emptying rate (Ohno et al., 2010). The gastric emptying rate was significantly increased by FMMIX intervention in this study, with consistent changes being observed. The peptide hormones MTL and GAS could stimulate the secretion of gastric acid and digestive enzymes, thereby promoting intestinal motility (Fourmy et al., 2011). The activity of digestive enzymes (α-amylase, lipase, and trypsin) in mouse chyme increased after dietary intervention. Notably, the highest lipase activity in mouse chyme of the UFM group was probably related to the fact that UFM was unfermented by *L. paracasei* JY062 and *L. gasseri* JM1, in which the milk fat content was higher. In addition, 5-HT is an excitatory neurotransmitter that plays a crucial role in the motility and secretory responses of the ENS, stimulating local enteric nerve reflexes to initiate secretory and propulsive motility and acting on vagal afferent nerves to regulate contractile activity (Spiller, 2007). Both tryptophan hydroxylase 1 and serotonin transporter are closely related to the homeostasis of 5-HT secretion, whereas 5-HT₃R is an important receptor associated with intestinal motility and intestinal fluid secretion. We found that 5-HT secretion was promoted and its homeostasis maintained through upregulation of *Tph1* and 5-HT₃R gene expression, as well as the downregulation of *SERT* gene expression after FMMIX intervention. In contrast, VIP is an inhibitory GI hormone that interacts with the vasoactive intestinal peptide receptor 1 to suppress gastric acid secretion and intestinal motility (Iwasaki et al., 2019). The synthesis of NO, which is considered as an inhibitory neurotransmitter responsible for smooth muscle relaxation, is mediated by the key enzyme NOS (Idrizaj et al., 2021). Interestingly, it has been shown that VIP can also stimulate NO production (Mourad et al., 2003). After the dietary intervention, we observed that the gene expression of both *VIPRI* and *NOS* was downregulated, consistent with the changes in VIP and NO levels. Moreover, our findings indicated that the
FMMIX intervention was more effective in improving constipation compared with UFM, potentially due to its modulatory effects on GI regulatory peptides and neurotransmitters in mice.

Intestinal motility, which directly affects the occurrence of constipation, involves the coordinated contraction and relaxation of the intestinal muscular layer. Throughout the intestine, ICC form a network of cells around the plexus between the circumferential and longitudinal muscular layers, which can generate rhythmic slow waves in the smooth muscle cells that regulate the frequency of intestinal contractions (Zhao et al., 2021). The depletion and dysfunction of ICC have been demonstrated to lead to a specific loss of excitatory intestinal neurotransmission, resulting in severe colonic motility disorders (Klein et al., 2013). In this study, we found that the intervention of FMMIX resulted in an increase in the number of colonic ICC, as well as an upregulation of the mRNA and protein expression levels of c-kit and its ligand SCF. This suggested that FMMIX intervention could improve the c-kit/SCF signaling pathway, which was critical for ICC differentiation and maintenance of their phenotype (Zhao et al., 2022b). The improvement in the c-kit/SCF signaling pathway and the increase in ICC numbers were consistent with the observed increase in intestinal motility, as indicated by the small intestinal propulsion rate. These findings also aligned with previous research conducted by Li et al. (2021). Overall, these results indicated that FMMIX intervention could improve intestinal motility and alleviate constipation symptoms by regulating ICC and the c-kit/SCF signaling pathway.

The symbiotic relationship between gut microbiota and the host organism is vital for maintaining intestinal health. It has been shown that patients suffering from intestinal diseases have an increased number of conditionally pathogenic bacteria in the intestinal tract, with significant changes in adverse trends (Khalif et al., 2005). The abundance of *Prevotella* in the gut microbiota of mice in the M group was increased, and this trend was reversed after dietary intervention. Simultaneously, we found an increase in the abundance of *Firmicutes* and a decrease in that of *Bacteroidetes* in the FMMIX group’s mice, with the ratio of *Firmicutes* to *Bacteroidetes* increasing. Moreover, it has been demonstrated that the ratio was positively correlated with intestinal propulsive rate (Hollister et al., 2020). Consistent with the present study, Wang et al. (2017) and Tang et al. (2022) discovered that probiotics and oligosaccharides improved the ratio of *Firmicutes* to *Bacteroidetes* to promote GI motility. In addition to this, the abundance of *Lactobacillus, Oscillospira, Ruminococcus, Coprococcus*, and *Akkermansia* was increased after FMMIX intervention in mice. Notably, *Lactobacillus* was positively correlated with 5-HT, SCFA, c-kit, and SCF protein levels in correlation analyses, in addition to the previous finding that *Lactobacillus* may relieve constipation by modulating neurotransmitters, intestinal motility, and SCFA levels (Cheng et al., 2023). *Oscillospira* is an anaerobic genus of clostridia whose growth requires a specific carbon source, particularly glucose, which significantly promotes its growth, and some of these species have been found to have the ability to use glucuronic acid (Gophna et al., 2017; Ji et al., 2020).

Because FMMIX contains many sugars, such as glucose, it provides a rich source of carbon for the growth of *Oscillospira*. Both *Ruminococcus* and *Coprococcus* could metabolically promote the production of SCFA, with *Coprococcus* being the butyrate-producing bacteria and *Ruminococcus* decomposing complex sugars, including cellulose, to produce fermentation products such as acetic acid (Rajilić-Stojanović and de Vos, 2014). *Akkermansia*, a beneficial microorganism colonizing the human gut, has been shown to degrade mucus into a variety of metabolites such as SCFA, which could regulate intestinal motility and alleviate diet-induced inflammation (Belzer et al., 2017; Plovier et al., 2017). The overall modulatory effect of FMMIX intervention on gut microbiota in mice was similar to the change in genus abundance after the intervention of herbal solid drink and lactulose in constipated rats (Deng et al., 2021), further suggesting the effect of FMMIX in relieving constipation by modulating gut microbiota.

Concurrently, SCFA are important metabolites of gut microbiota, which could stimulate intestinal mucosal receptors and vagus nerves, with direct action on colonic smooth muscle to promote intestinal motility (Zhuan et al., 2019). In this study, the content of SCFA in the cecum contents of mice was increased after dietary intervention, which was beneficial to improve intestinal motility in constipated mice. Additionally, SCFA are absorbed by colonic epithelial cells and can also be used as energy stores to maintain the physiological function and normal morphology of intestinal cells by reducing the osmotic pressure of intestinal cells, thus reducing the absorption of water in the feces of intestinal lumen by intestinal cells due to high osmotic pressure (Soret et al., 2010). We found that the fecal moisture content of mice increased significantly after dietary intervention, which was presumed to be closely related to the increase of SCFA in the intestinal tract. In addition, the mRNA expression levels of aquaporins (*Aqp4* and *Aqp8*) were downregulated in the FMMIX group, which could reduce the absorption of water from the intestinal lumen and contribute to the increase of fecal moisture content in mice. However, whether SCFA and the expression of *Aqp4* and *Aqp8* are directly related to each other still needs further study. The overall analysis
revealed that FMMIX intervention reshaped the gut microbiota distribution in constipated mice and correlated with GI regulatory peptides, neurotransmitters, intestinal motility, and SCFA levels, thereby improving the constipation symptoms in mice.

We found that *L. paracasei* JM062 and *L. gasseri* JM1 were delivered to the gastrointestinal tract when mice ingested FMMIX, and previous studies have demonstrated that this probiotic combination could promote gastrointestinal motility (Cheng et al., 2023). In addition, matrix components in the FMMIX, such as free amino acids, free fatty acids, and polysaccharides, may play an important role in the intestinal colonization of microorganisms after the co-fermentation of *L. paracasei* JM062 and *L. gasseri* JM1 and may promote the probiotic function of probiotics (Baruah et al., 2022). Oligosaccharides such as maltodextrins and oligogalactans are effective prebiotics and have been shown to improve intestinal transit rate and promote intestinal motility in mice (Jang et al., 2020; Kim et al., 2020). Meanwhile, the increased content of functional metabolites in FMMIX could regulate intestinal dysfunction. We found a significant increase in DL-lactate in FMMIX, which was conducive to prolonging the shelf life of fermented milk, giving the product a sour and refreshing taste, as well as promoting gastrointestinal motility and improving protein digestion and the absorption of calcium and phosphorus. Phenyllactic acid, a natural biological preservative, was upregulated in FMMIX compared with UFM and could inhibit some pathogenic bacteria (Dieuleveux et al., 1998). In addition, we found that milk contained hippuric acid, which was significantly reduced in FMMIX, thereby reducing its health risks (Güler et al., 2018). Moreover, the intervention of phepcolic acid in mice could increase 5-HT and Ach levels and reduce the symptoms of constipation (Ou et al., 2019). We found that L-pipecolic acid was significantly increased in FMMIX, which may play a potential function in regulating intestinal motility in mice. Naringenin is also a flavonoid that could chelate heavy metals and improve intestinal motility through metal flavonoid chelation (Salami et al., 2023). It was well known that vitamin B6 acts as a cofactor for many biochemical reactions and has been shown to have anti-inflammatory properties (Hossain et al., 2022), and vitamin B6 in FMMIX was mainly involved in amino acid biosynthesis and catabolism. It is noteworthy that DL-phenylalanine in FMMIX was significantly increased, and more studies have found that phenylalanine intervention promoted the alleviation of constipation and stimulated serum GAS secretion in mice (Feng et al., 2010; Yang et al., 2022), so we further hypothesized that the improvement of constipation by FMMIX and the increase in the content of DL-phenylalanine may be directly related. Conclusively, the constipation-alleviating effect of FMMIX in this study was hypothesized to be exerted by functional components such as L-pipecolic acid, DL-phenylalanine, and naringenin, in addition to the pre-studied role of *L. paracasei* JM062 and *L. gasseri* JM1.

CONCLUSIONS

Overall, FMMIX containing *L. paracasei* JM062 and *L. gasseri* JM1 has shown promising results in relieving loperamide-induced constipation in mice. Specifically, FMMIX improved the secretion of GI regulatory peptides and neurotransmitters and promoted GI motility by increasing the number of colonic ICC and upregulating the expression of c-kit and SCF. It also regulated the distribution of gut microbiota in constipated mice, leading to an increase in the abundance of SCFA-producing beneficial bacteria, and promoted the content of SCFA to alleviate the symptoms commonly associated with constipation. In addition, FMMIX contains functional ingredients (L-pipecolic acid, DL-phenylalanine, naringenin, and so on) that contribute to constipation relief. Overall, FMMIX showed potential as an alternative therapeutic strategy for constipation relief.

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