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 (MTL), gastrin (GAS) and 5-hydroxytryptamine (5-HT) and decreased those of peptide YY (PYY), vasoactive intestinal peptide (VIP) and nitric oxide (NO) in mice. As determined by immunohistochemical analysis, FMMIX promoted an augmentation in the quantity of Cajal interstitial cells (ICC). And mRNA and protein expression of c-kit and SCF was 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 (SCFAs) with a superior improvement compared with UFM. 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. In conclusion, 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 SCFAs levels, and may be associated with an increase in the above functional components in FMMIX. 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.

Keywords: 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 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 neuromotoric 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 to a range of symptoms including abdominal pain, bloating, loss of appetite, nausea, headaches, halitosis, restlessness, anxiety, and/or depression. Prolonged constipation could result in intestinal damage, the accumulation of intestinal toxins, and even 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, H. pylori eradication drugs, proton pump inhibitors, or selective serotonin reuptake inhibitors (Feinle-Bisset 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, there are limitations to pharmacotherapy, such as a low response rate and frequent relapses after discontinuation of the medication. Therefore, it becomes crucial to investigate potential alternative treatment strategies that do not rely on prescription drugs.

Dietary interventions, specifically the use of probiotics, as an effective and low-risk strategy for relieving constipation (Zhao et al., 2019). Emerging evidence suggested that probiotics such as L. plantarum CQPC05, L. reuteri DSM17938, and B. lactis HN019 exhibited promising effects in alleviating constipation. The main mechanisms by which these probiotics exert their regulatory effects included modulation of neurotransmitter contents, regulation of intestinal luminal pH level, and enhancement of functional intestinal motility frequency (Cheng et al., 2021; Li et al., 2019; 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 10⁶ cfu/mL) (Lourens-Hattingh and Viljoen, 2001). In addition, probiotic fermented milk contains high-quality protein, minerals, niacin, vitamin B₁₂, 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). And 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 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 Bifidobacteria in 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 Lactcaseibacillus 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 impact 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 signaling pathway, gut microbiota composition, and levels of short-chain fatty acids (SCFAs). And 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. L. paracasei JY062 (GenBank accession number CP044361-CP044367) and L. gasseri JM1 (GenBank accession number CP044412-CP044414) were isolated 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 weeks) were from Vital River Laboratory Animal Technology Co., Ltd. The animal experiments in this study were allowed by Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (#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 (FMMIX), and positive control fermented milk (PCFM). Except for the NC group, all groups of mice received loperamid at a dosage of 5 mg/(kg·d) body weight via gavage for a continuous period of 7 d. Simultaneously, the NC group received an equal volume of phosphate buffer saline (PBS) via gavage. Following this, the mice of UFM, FMMIX, and PCFM groups were gavaged unfermented milk, probiotic fermented milk, and positive control fermented milk (regulate gut microbiota and promote intestinal digestion as a promotional point, containing Streptococcus thermophilus, Lactobacillus bulgaricus, Bifidobacterium lactis and Lactaseibacillus paracasei) at a dosage of 10 mg/(kg·d) body weight via gavage for 2 consecutive weeks, while those of NC and M groups were gavaged with equal volumes of PBS, respectively. After the experiment, mice were euthanized by intraperitoneal injection of ketamine and diazepam.

**Physiological Indicators**

Mice were fasted for 24 h and sacrificed after gavage with 0.5 mL of ink for 30 min. The determination of gastric emptying rate and small intestine propulsive rate in mice involved the following modifications based on the previous methods (Guo et al., 2021). (1) The weight of the ink \(a_2\), the total stomach \(a_3\), and the stomach without stomach contents \(a_4\) 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 \(b_1\), 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:

\[
\text{gastric emptying rate} \% = 1 - \frac{a_4 - a_3}{a_4} \times 100, \\
\text{intestine propulsive rate} \% = \frac{L_1}{L_2} \times 100, \\
\text{fecal moisture content} \% = \frac{b_2 - b_1}{b_2 - b_3} \times 100.
\]

**Histological Analysis**

The gastric antrum, small intestine, and colon of mice were aseptically collected, and some tissues were fixed in 4% paraformaldehyde for 48 h. Subsequently, the 5 mm-thick sections were obtained from the fixed tissues for hematoxylin-eosin (HE) staining, and 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), 5-hydroxytryptamine (5-HT) in serum were determined by commercially available ELISA kits (Nanjing Jiancheng Co., Ltd.). The concentration of nitric oxide (NO) in serum was determined by the nitric acid reductase biochemical kit (Beijing Solarbio Science & Technology Co., Ltd.). The activity of α-amylase, lipase, and trypsin in chyme were determined by commercially available α-amylase activity assay kit, lipase activity assay kit, and trypsin activity assay kit (Beijing Solarbio Science & Technology Co., Ltd.), respectively.

**Reverse Transcription (RT) and Quantitative PCR (qPCR)**

Determination of gene expression in mouse colon tissues was based on the previous method with minor modifications (Li et al., 2021). Briefly, total RNA of colon tissues was extracted and cDNA was synthesized by Simple P total RNA extraction kit (Bioer Technology Co., Ltd.) and PrimeScript RT reagent kit (TaKaRa Bio), respectively. The mRNA levels were determined by 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^ΔΔCt method to determine the expression of related genes (Aqp4, Aqp8, Tph1, 5-HT₄R, SERT, VIPR1, NOS, c-kit, SCF). GAPDH was used as the internal reference gene. The primer sequences used were listed in Supplementary Table 1 (https://doi.org/10.6084/m9.figshare.24189648.v1).
**Gut Microbiota Sequencing and Analysis**

Total DNA extraction was performed using TIANamp Stool DNA kits (Tiangen), and the V3-V4 region of 16S rRNA gene was amplified using previously described primers (F: ACTTCTACGGGAGGCAGCAG, R: GGACTACHVGGGTWTCTAAT) (Tang et al., 2022). DNA samples were sequenced by Illumina MiSeq. After sequencing, bacterial diversity analysis was conducted on the 16S rRNA sequence data (https://github.com/QIIME2/).

**Western blot (WB) Analysis**

The protein expression of c-kit and SCF was determined by WB 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 PVDF membranes (Millipore). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) at 37°C for 2 h. GAPDH was used as an internal reference. Membranes were incubated overnight at 4°C with the primary antibody (1:1000). After washing in TBST, the secondary antibody (1:5000) was added and incubated at 25°C for 30 min. The blots were subsequently washed with TBST and exposed to enhanced chemiluminescence reagents (ECL) for chemiluminescence detection. The grayscale of the target bands was analyzed using Image-pro Plus 6.0 software (Media Cybernetics, Inc.).

**Immunohistochemistry Analysis**

Based on the method described by Ren et al. (2017) with minor modifications, interstitial cells of Cajal (ICC) were identified by 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. And then 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 (DAB) and observed under a light microscope. The Mean Density (IOD/AREA) was determined using Image-pro Plus 6.0 software (Media Cybernetics, Inc.).

**Determination of SCFAs**

The cecum contents of the mice were homogenized and sonicated to create a suspension and centrifuged at 5000 rpm for 20 min. 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 10,000 rpm for 15 min. The supernatant was subsequently transferred to a fresh glass vial for GC-MS analysis (Wang et al., 2020a).

**Determination of non-volatile 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. Then, it was centrifuged at 14,000 g for 20 min and the supernatant was collected for vacuum drying. After that, the dried sample was 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. The supernatant was collected and filtered through a 0.22 µm microporous membrane (Agilent) for analysis. In addition, the quality control (QC) samples were prepared by mixing equal amounts of the test samples and should be determined before, during, and after the injection of the test samples in the LC-MS system.

The samples were separated using an Ultra-High-Performance Liquid Chromatography (UHPLC) system (Agilent 1290 Infinity LC). For HILIC separation, samples were analyzed using a 2.1 mm × 100 mm AC-QUIY UPLC BEH 1.7 µm column (waters, Ireland). The column temperature was set at 53°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, while the mobile phase B was acetonitrile. The gradient elution program was as follows: 0–0.5 min, 95% B; 0.5–7 min, linear change of B from 95% to 65%; 7–8 min, linear change of B from 65% to 40%; 8–9 min, B maintained at 40%; 9–9.1 min, linear change of B from 40% to 95%; 9.1–12 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 in both positive ion mode (ESI+) and negative ion mode (ESI). The ESI source conditions are as follows: ion source gas1: 60, ion source gas2: 60, curtain gas: 30, source temperature: 600°C, ion spray voltage floating: ± 5500 V; TOF MS scan m/z range: 60–1000 Da, product ion scan m/z range: 25–1000 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 (IDA) and the high sensitivity mode. Dech毛孔tering potential was ± 60 V and collision energy was 35 ± 15 eV.

**Statistical Methods**

One-way ANOVA (ANOVA) was performed by SPSS and Origin software. Graph production was performed by Excel 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 was then used for peak alignment, retention time correction, and peak area extraction. The data extracted by XCMS was subjected to metabolite structure identification, data preprocessing, and quality evaluation. Multivariate statistical analysis, such as principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), 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 KEGG database (https://www.genome.jp/kegg/pathway.html) to determine the involved metabolic pathways.

**RESULTS**

**Physiological Indicators and Histological Analysis in Constipated Mice**

As shown in Figure 1A-C, 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, UFM, FMMIX, and PCFM groups were significantly increased in the above 3 indicators with significant improvement (P < 0.05), among which there was 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 could reach the level of the NC group without significant differences 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.

From the HE staining results (Figure 1D), it could be seen 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 sinuses (black arrows). The small intestinal villi were loosely arranged and shortened in length. Both gastric antrum and small intestine showed epithelial cell erosion and detachment (red arrows) and inflammatory cell infiltration (blue arrows). The crypt structure of colonic mucosal layer in the M group partially disappeared, along with focal infiltration of inflammatory cells (blue arrows), and there were some goblet cells (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, there was 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 Peptides, Neurotransmitters, and Digestive Enzymes 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 B). 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 D). 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), and the level of VIP in the FMMIX group could reach that of the NC group. Interestingly, we found lower levels of PYY in the UFM group than that in the FMMIX group, but there was no significant difference (P > 0.05). Simultaneously, the level of excitatory neurotransmitter 5-HT was significantly reduced and the level of inhibitory neurotransmitter NO was significantly increased in the M group.
(\(P < 0.05\)), and their levels were reversed after the dietary intervention, with the most significant effect in the FMMIX group (Figure 2E and F). 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 while those in the FMMIX group were significantly increased, with \(\alpha\)-amylase activity significantly higher than that in the PCFM group (\(P < 0.05\)) (Figure 2G-I). 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 certain content, but overall the improvement was particularly pronounced after the FMMIX intervention.

**Expression of Related Genes**

The expression of related genes was determined in this study to elucidate the intervention mechanism of FMMIM in constipated mice. Compared with the NC group, the expression levels of \(Aqp4\) and \(Aqp8\) were significantly increased in the M group (\(P < 0.05\)), while those were decreased after dietary intervention,

![Graphs showing changes in gastric emptying rate, small intestinal propulsive rate, and fecal moisture content](image)

**Figure 1.** Alleviating effect of probiotic fermented milk on constipated mice, and changes of gastric emptying rate (A), small intestinal propulsive rate (B), and fecal moisture content in mice (C). Each bar represents mean ± standard deviation (SD), n = 4. And hematoxylin-eosin (HE) staining results (D) for gastric antrum, small intestine, and colon sections (200 × ).
especially in the FMMIX group that could reach the level of the NC group (Figure 3A and B). In addition, the gene expression levels of $Thp1$ and $5-HT_4R$ 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 2 fermented milk were significantly better than that of unfermented milk ($P < 0.05$), among which the effect of FMMIX in $Thp1$ and $5-HT_4R$ expression was superior to that of PCFM, while the improvement in $SERT$ expression was more pronounced in the PCFM group, but there was no significant difference between them ($P > 0.05$) (Figure 3C-E). Concurrently, we found that VIPR1 and NOS expression levels were decreased after the dietary intervention, especially in the FMMIX group (Figure 3F and G). It was worth noting that the gene $c$-kit and SCF expression levels 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

**Figure 2.** The contents of gastrointestinal (GI) regulatory peptides, neurotransmitters, and digestive enzymes activity in constipated mice, including the content of GI regulatory peptide motilin (MTL) (A), gastrin (GAS) (B), peptide YY (PYY) (C) and vasoactive intestinal peptide (VIP) (D), the content of neurotransmitter 5-hydroxytryptamine (5-HT) (E) and nitric oxide (NO) (F), and the activity of $\alpha$-amylase (G), lipase (H) and trypsin(I). Each bar represents mean ± standard deviation (SD), n = 4.
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 need to initiate further studies.

**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, while constipated mice had deeper colonic staining after FMMIX intervention, indicating a 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 D, the expression of c-kit and SCF proteins were significantly decreased \((P < 0.05)\) in the M group \((0.11 \pm 0.024 \text{ and } 0.15 \pm 0.033)\) compared with the NC group \((0.91 \pm 0.022 \text{ and } 0.84 \pm 0.039)\), with decreased rates of 87.4% and 82.5%, respectively. Compared with the M group, the expression of c-kit and SCF proteins were significantly increased after the dietary interventions \((P < 0.05)\), with the most prominent levels in the FMMIX group \((0.56 \pm 0.038 \text{ and } 0.59 \pm 0.051)\), 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

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**Figure 4.** Cajal interstitial cells (ICC) distribution in colon \((200 \times )\) (A), the mean density \((\text{IOD/AREA})\) in the colon of constipated mice (B), blot images of c-kit and SCF (C) and protein expression ratios of c-kit to GAPDH and SCF to GAPDH in constipated mice (D). Each bar represents mean ± standard deviation (SD), \(n = 3\).
intervention on the gut microbiota of constipated mice. Alpha diversity analysis was characterized the richness by Chao1 and Observed species indices and the diversity by Shannon and Faith's PD 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-D). Although there was no significant difference between the groups, the results were similar to those of Zhang et al. (2022). Beta diversity analysis by Principal coordinates analysis (PCoA) and Nonmetric Multidimensional Scaling (NMDS) 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 F).

At the phylum level of gut microbiota composition (Figure 5G), Firmicutes and Bacteriodetes 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 Bacteriodetes was increased in the M group, while these trends were reversed after the dietary intervention. The most noticeable improvement was observed in the FMMIX group, where the abundance of both Firmicutes and Bacteriodetes in the FMMIX group (62.72% and 30.71%) tended to those in the NC group (63.73% and 30.74%). 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, FMMIX, 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 Coprococcus was increased and that of Prevotella was decreased in the FMMIX group compared with the M group, which 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), there was 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, and 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 (LefSe) analysis (Figure 6B-D) 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 Desulfovibrionaceae and Bilophila; and the PCFM group was mainly enriched in Streptococcus, Dehalobacterium and Erysipelotrichaceae_Clostridium. Overall, these were consistent with the above description. In conclusion, the balance of the intestinal microenvironment was disrupted in the M group mice, which were prone to colonize 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.

**SCFAs Levers and Correlation Analysis**

Studies have shown that SCFAs, metabolites of gut microbiota, could regulate intestinal function (Kaji et al., 2015; Wang et al., 2020b). We explored the levels of SCFAs in the cecum contents of each group of mice and determined whether and to what extent they affected constipation. As shown in Figure 7A-F, the levels of SCFAs (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 SCFAs 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 ($P > 0.05$), and it was notable that the levels of butyric acid and isovaleric acid in the FMMIX group were significantly elevated compared with those in the PCFM group ($P < 0.05$). Thus, the levels of SCFAs 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, SCFAs, c-kit and SCF protein were positively correlated with the abundance of Lactobacillus and negatively correlated with Prevotella. Among them, isobutyric acid was significantly and positively correlated with Lactobacillus, while acetic acid and isobutyric acid were significantly and negatively correlated with Prevotella ($P < 0.05$). Meanwhile, the above study found that the abundance of Lactobacillus increased and the abundance of Prevotella decreased in 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 PCA of both positive and negative ion modes (Figure 8A...
and B). Screening of significantly different metabolites with strict OPLS-DA VIP > 1, P value < 0.05 and fold change (FC) > 2 or FC < 0.5 criteria (Supplementary Table 2 https://doi.org/10.6084/m9.figshare.24189648.v1) and clustering heat map analysis (Figure 8C) resulted in 2 clusters, a and b, suggesting that the upregulated metabolites fermented by *L. paracasei* JY062 and *L. gasseri* JM1 were significantly more than the downregulated metabolites. Compared with UFM, the contents of phenyllactic acid, pyridoxal (Vitamin B6), D-lactate, naringenin, isoliquiritigenin, L-pipecolinic acid, DL-Phenylalanine were significantly increased and hippuric acid significantly decreased in FMMIX (*P* < 0.05) (Figure 8D). In the enrichment analysis of metabolic pathways (Figure 8E), the main enriched pathways were ABC transporters, pyrimidine metabolism, amino sugar and nucleotide sugar metabolism, galactose metabolism, phosphotransferase system (PTS), Arginine and proline metabolism, 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. 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 (He et al., 2022; Zhang et al., 2021). 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 modeled mice (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, FM-MIX, 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.

Figure 7. The effect of probiotic fermented milk on short-chain fatty acids (SCFAs) in constipated mice, including acetic acid (A), propionic acid (B), butyric acid (C), isobutyric acid (D), valeric acid (E) and isovaleric acid (F). And the correlation between gut microbiota and physiological indexes (G). Each bar represents mean ± standard deviation (SD), n = 4.
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 isoliquiritigenin, and some studies have shown that naringenin and isoliquiritigenin possessed antioxidant and anti-inflammatory properties and could alleviate intestinal tissue damage (Yahya et al., 2022; Zhong et al., 2021). 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, while decreasing the levels of PYY, VIP, and...
NO. Particularly, 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 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 (\( Tph1 \)) and serotonin transporter (\( SERT \)) are closely related to the homeostasis of 5-HT secretion, while \( Tph1 \) and \( 5-HT, R \) are important receptors associated with intestinal motility and intestinal fluid secretion. We found that 5-HT secretion was promoted and its homeostasis was maintained through upregulation of \( Tph1 \) and \( 5-HT, R \) 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 (\( VIPR1 \)) to suppress gastric acid secretion and intestinal motility (Iwasaki et al., 2019). NO is considered as an inhibitory neurotransmitter responsible for smooth muscle relaxation, and its synthesis 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 \( VIPR1 \) 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. ICC forms a network of cells around the plexus between the circumferential and longitudinal muscular layers throughout the intestine, which can generate rhythmic slow waves in the smooth muscle cells that regulate the frequency of intestinal contractions (Zhao et al., 2021). Depletion and dysfunction of ICC had 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, it was 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. (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 propulsion rate (Hollister et al., 2020). Tang et al. (2022) and Wang et al. (2017) discovered that probiotics and oligosaccharides improved the ratio of Firmicutes to Bacteroidetes to promote GI motility, consistent with the present study. 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, SCFAs, \( c-kit \) and SCF protein levels in correlation analyses, in addition to the previous finding that \( Lactobacillus \) may relieve constipation by modulating neurotransmitters, intestinal motility, and SCFAs 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 utilize glucuronic acid (Gophna et al., 2017; Ji et al., 2020). FMMIX contained many sugars such as glucose, providing a rich source of carbon for the growth of \( Oscillospira \). Both \( Ruminococcus \) and \( Coprococcus \) could
metabolically promote the production of SCFAs, with *Coprococcus* being the butyrate-producing bacteria and *Ruminococcus* decomposing complex sugars, including cellulose, to produce fermentation products such as acetic acid (Rajilic-Stojanovic and de Vos, 2014). *Akkermansia*, a beneficial microorganism colonizing the human gut, has been shown to degrade mucin into a variety of metabolites such as SCFAs, 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, SCFAs 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 (Zhuang et al., 2019). In this study, the content of SCFAs in the cecum contents of mice was increased after dietary intervention, which was beneficial to improve intestinal motility in constipated mice. Additionally, SCFAs 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 SCFAs in the intestinal tract. Also, 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, but whether SCFAs on the expression of *Aqp4* and *Aqp8* is 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 SCFAs levels, thereby improving the constipation symptoms in mice.

*L. paracasei* JY062 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* JY062 and *L. gasseri* JM1 and may promote the probiotic function of probiotics (Baruah et al., 2022). Oligosaccharides such as maltodextrins and oligogalactans are good 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 of 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, improving protein digestion and calcium/phosphorus absorption. Phenylactic acid, a natural biological preservative, was upregulated in FMMIX compared with UFM and could inhibit some pathogenic bacteria (Dieuleveux et al., 1998). Besides, it was found that milk contained hippuric acid, which was significantly reduced in FMMIX, reducing its health risks (Guler et al., 2018). Moreover, the intervention of pipeliconic acid in mice could increase 5-HT and Ach levels and reduce the symptoms of constipation (Ou et al., 2019). L-pipeliconic acid was significantly increased in FMMIX, which may play a potential function in regulating intestinal motility in mice. Also, naringenin is 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. Of noteworthy that DL-phenylalanine in FMMIX was significantly increased, while 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 it was 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-pipeliconic acid, DL-phenylalanine and naringenin in FMMIX to relieve constipation, in addition to the pre-studied role of *L. paracasei* JY062 and *L. gasseri* JM1.

**CONCLUSION**

In conclusion, FMMIX containing *L. paracasei* JY062 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 SCFAs-producing beneficial bacteria, and promoted the content of SCFAs to alleviate the symptoms commonly associated with constipation. Also, the presence of functional components in FMMIX that favor constipation relief (L-pipeolicin acid, DL-phenylalanine and naringenin, among others) remained a potential factor for a role. Overall, FMMIX showed potential as an alternative therapeutic strategy for constipation relief.

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