Current Research in Food Science 9 (2024) 100875

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Lacticaseibacillus paracasei NCU-04 relieves constipation and the depressive-like behaviors induced by loperamide in mice through the microbiome-gut-brain axis

Shengjie Li [a,b,c], Yi Li [b,c], Yujie Cai [b,c], Zizhou Yan [b,d], Jing Wei [b,c], Hongyan Zhang [a,***], Fenfang Yue [b,c,**], Tingtao Chen [a,b,c,d,*]

[a] *Medical Center of Burn Plastic and Wound Repair, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, 330006, China*
[b] *National Engineering Research Center for Bioengineering Drugs and the Technologies, Jiangxi Province Key Laboratory of Bioengineering Drugs, Institute of Translational Medicine, Jiangxi Medical College, Nanchang University, Nanchang, 330031, China*
[c] *The MOE Basic Research and Innovation Center for the Targeted Therapeutics of Solid Tumors, Jiangxi Medical College, Nanchang University, Nanchang, 330031, China*
[d] *Jiangxi Province Key Laboratory of Bioengineering Drugs, School of Pharmacy, Jiangxi Medical College, Nanchang University, Nanchang, 330031, China*

ARTICLE INFO

Handling Editor: Dr. Quancai Sun

Keywords:
Lacticaseibacillus paracasei NCU-04
Constipation
5-HT
Depression-like behaviors
Microbiota-gut-brain axis

ABSTRACT

Constipation is a prevalent gastrointestinal condition that significantly affects patients' physical and mental well-being, yet current treatments often lack safety and efficacy. Emerging evidence highlights the critical role of the microbiota-gut-brain axis (MBGA) in managing constipation, paving the way for probiotics as an adjuvant treatment to improve constipation symptoms. In this study, we isolated a gut probiotic strain, *Lacticaseibacillus paracasei* NCU-04, and investigated its improvement effects on loperamide-induced constipation in mice. We demonstrated that *L. paracasei* NCU-04 exhibited excellent probiotic properties, including robust growth, strong antibacterial and antioxidant capacities, and a lack of hemolytic activity *in vitro*. The administration of *L. paracasei* NCU-04 effectively improved the defecation-related indicators such as the fecal water content, time to the first black stool defecation, and intestine transit rate, suggesting enhanced gut immobility in constipated mice. Additionally, *L. paracasei* NCU-04 significantly reduced colon inflammation induced by loperamide. Further, *L. paracasei* NCU-04 increased levels of colonic motilin, 5-hydroxytryptamine (5-HT), and c-kit, while decreased that of aquaporin 3, vasoactive intestinal peptide, and peptide YY. Notably, *L. paracasei* NCU-04 effectively upregulated the expression of 5-HT and its receptor (i.e., 5-HT4R) in the brains of constipated mice. High-throughput sequencing revealed that *L. paracasei* NCU-04 restored the diversity and composition of the gut microbiota disturbed by loperamide, and significantly increased the relative abundance of *Prevotella* and *Lactobacillus* genera in the stool, while decreased that of *Odoribacter*, *Rikenella*, and *Parabacteroides*. Importantly, *L. paracasei* NCU-04 also effectively improved the depression-like behaviors associated with constipation, possibly through 5-HT mediated MGBA. These results suggest that *L. paracasei* NCU-04 may offer a promising approach for treating constipation and its related depressive symptoms, supporting its potential as a functional food or adjuvant therapy for human health.

* Corresponding author. Medical Center of Burn Plastic and Wound Repair, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, 330006, China.
** Corresponding author. National Engineering Research Center for Bioengineering Drugs and the Technologies, Jiangxi Province Key Laboratory of Bioengineering Drugs, Institute of Translational Medicine, Jiangxi Medical College, Nanchang University, Nanchang, 330031, China.
*** Corresponding author. Medical Center of Burn Plastic and Wound Repair, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang, 330006, China.
E-mail addresses: ndyfy00672@ncu.edu.cn (H. Zhang), yff9904@163.com (F. Yue), chentingtao1984@163.com (T. Chen).

https://doi.org/10.1016/j.crfs.2024.100875
Received 21 August 2024; Received in revised form 23 September 2024; Accepted 1 October 2024
Available online 4 October 2024

1. Introduction

Constipation is a common yet often overlooked gastrointestinal (GI) condition that characterized by infrequent bowel movements, difficulty passing stools, and a sensation of incomplete evacuation. It affects a significant portion of the population. One study estimated that the global prevalence of function constipation ranges from 15.3% to 10.1% according to distinct Rome criteria with a 14.0% pooled overall prevalence, suggesting that one in six to one in ten people globally experience function constipation at any point in time (Barberio et al., 2021). In China, the prevalence of function constipation has risen over the past three decades, increasing from 5.5% between 1991 and 2000 to 10.9% between 2011 and 2020, with an overall prevalence of 8.5% (Z. Chen et al., 2022). The long-term constipation gravely impacts patients' quality of life, leading to physical discomfort, psychological distress, and disruptions in daily activities (Camilleri et al., 2017). Current treatments for constipation include lifestyle and dietary changes, biofeedback therapy, and medications such as laxatives, prokinetic agents, and prosecretory drugs (Sharma et al., 2021). However, these treatments are often criticized for their limited effectiveness and potential side effects, highlighting the need for alternative strategies to manage and prevent constipation more effectively (Sharma et al., 2021).

The pathogenesis of constipation is complex and not fully understood. Key factors such as abnormalities in the enteric and central nervous systems (ENS and CNS), imbalances in the gut microbiome, and alterations in GI dynamics are closely related to constipation (Q. Chen et al., 2021). These factors are interconnected through the microbiota-gut-brain axis (MGBA), a bidirectional communication pathway where gut microbiota influence gut and brain function through various mechanisms (Cryan & O'Mahony, 2011; Dinan and Cryan, 2017). Gut microbes can stimulate the production of various neurotransmitters to regulate the ENS and GI peristalsis. Studies demonstrated that most neurotransmitters, including motilin (MTL), peptide YY (PYY), vasoactive intestinal peptide (VIP), and 5-hydroxytryptamine (5-HT), changed in the serum and colon of constipated mice (Cheng et al., 2024; Li et al., 2023; C. Zhang et al., 2023). Among these, 5-HT plays a central role in GI motility, secretion, and sensation, suggesting that serotonergic agents could be effective in treating constipation (Cash and Chey, 2005). Additionally, the interstitial cells of Cajal (ICC) in the GI tract respond to enteric neurotransmitters to coordinate the GI motility, making ICC an effector of MGBA and a potential therapeutic target for GI motility conditions (Huizinga et al., 1997; Lyford et al., 2002).

Increasing evidence points to a strong connection between gut microbiota and constipation, indicating that microbiota-based therapies could be promising in treating constipation (Pan et al., 2022; S. Zhang et al., 2021). Given the special crosstalk between probiotics, gut microbiota, and host, the usage of probiotics as nutrient supplements or adjunctive therapy to manipulate gut microbiota and thereby treat constipation is a well-established concept in biomedicine (Cammarota et al., 2014; Ma et al., 2023). The contribution of probiotics, such as the common species of *Lactobacilli* and *Bifidobacteria*, to the relief of constipation symptoms has been documented in various study (Pan et al., 2022). For instance, *L. casei* Shirota (Matsumoto et al., 2006), *B. longum* BB536 (Kondo et al., 2013), *L. reuteri* DSM19738 (Riezzo et al., 2019), and *B. lactis* HN019 (Waller et al., 2011) have shown positive effects on constipation by modulating the intestinal microenvironment and ENS/CNS functions in clinical studies. However, the effectiveness of probiotics varies due to factors like study heterogeneity, risk of bias, and species- and strain-specific differences (Dimidi et al., 2020).

Lacticaseibacillus paracasei (formerly *Lactobacillus paracasei*) is a type of *Lactobacilli* found in the intestine, fermented foods, and dairy products. Recent review had highlighted the health-promoting properties of *L. paracasei* isolated from different sources, offering insights into its potential as a functional food or adjuvant therapy (Bengoa et al., 2021). For instance, *L. paracasei* has been shown to heighten intestinal barrier integrity and reduce gut permeability, which contributes to overall GI health (Algieri et al., 2023). It also exhibits significant immunomodulatory properties, promoting the production of anti-inflammatory cytokines (e.g., IL-10), and reducing the levels of pro-inflammatory cytokines (e.g., TNF-α and IL-6). These effects make it a promising candidate for supporting immune health and managing inflammation conditions, such as inflammatory bowel diseases (Kim et al., 2019). Furthermore, *L. paracasei* and its derivatives have also shown promise in treating constipation in both human and animal studies (C.-L. Chen et al., 2020; Riezzo et al., 2012; Valerio et al., 2010; Wang et al., 2023).

In this study, we investigated a new *L. paracasei* strain isolated from healthy infant feces, termed *L. paracasei* NCU-04, which exhibits strong probiotic properties *in vitro*. We aimed to illustrate the effects of this strain on loperamide-induced constipation in mice, focusing on its role in regulating enteric neurotransmitters, restoring the gut microbiota, and reducing colon inflammation. In particular, we demonstrated that 5-HT-mediated gut-brain axis may contribute to explaining the underlying mechanism of this strain in relieving constipation and its-related depression behaviors. This study aims to identify a new *L. paracasei* strain as a potential therapeutic option for treating constipation.

2. Materials and methods

2.1. Strains and probiotic properties testing

L. paracasei NCU-04 was isolated from the feces of breastfed healthy infants and stocked in our laboratory. It was further identified and preserved by the China General Microbiological Culture Collection Center (CGMCC) under the deposit number CGMCC No.25506.

The performance of its growth capacity was detected by the growth curve, where the OD_{600} value was measured every 2 h during the cultured period of 24 h. The sterile defibrinated goat blood was used to prepare the agar plate and test the possible hemolysis capacity of this strain (Varada et al., 2023). The performance of its acid tolerance was tested by viable counting recorded as colony-forming unit per milliliter (CFU/mL), where the activated cultures ($\sim 10^{10}$ CFU) were incubated with 1 × sterile phosphate buffer solution (PBS) with different pH values for 4 h and then the number of viable counts was measured. The scavenging capacity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (-OH), and superoxide (O_2^-) radicals, combined with the chelating capacity and total reducing power for Fe^{2+} were used to evaluate its antioxidant activities.

Additionally, the antibiotics resistance of *L. paracasei* NCU-04 was detected by disk diffusion method, where 10 different antibiotics, including minomycin (MY), chloramphenicol (C), penicillin (PEN), ampicillin (AMP), tetracycline (TET), cephalosporin (CTR), ciprofloxacin (CIP), gentamicin (GEN), cotrimoxazole (SXT), and erythromycin (E) were selected for testing (Varada et al., 2023). And its antagonistic effects on pathogenic bacteria, including *Salmonella typhimurium* ATCC13311, *Shigella fowleri* ATCC12022, *Streptococcus necrosis*, *Shigella dysenteriae* 301, *Escherichia coli* O157:H7, *Streptococcus enteritidis* ATCC13076, *Listeria monocytogenes* ATCC19111, and *Staphylococcus aureus* Cowan1, were tested by oxford cup method. *L. paracasei* NCU-04 was anaerobically cultured in de Man-Rogosa-Sharpe (MRS) medium at 37 °C, and the pathogens were aerobically cultured in LB medium at 37 °C.

2.2. Probiotic suspension preparation

L. paracasei NCU-04 was activated twice in MRS medium before each dose, then cultured in 1-Liter fresh MRS liquid with a 1% inoculum for 24 h. The cultures were centrifuged at 5000 rpm for 10 min to collect the bacterial pellet, which was washed twice with sterile PBS. The pellet was then resuspended in a 5% gelatin solution (as a protective agent) and adjusted to concentrations for subsequent gavage: approximately 5×10^8 CFU/mL for the low-dose group and 5×10^9 CFU/mL for the high-dose group, respectively.

2.3. Animals and experimental design

Six-week-old, SPF-grade BALB/c mice were purchased from Hunan Slaughter Kingda Laboratory Animal Co., Ltd. (Changsha, China). All the animal experimental protocols were reviewed and approved by the Ethics Committee of Research Involving Animal of Nanchang University (Approval No. NCULAE-20221228046). Mice were housed in the standard conditions with a temperature of 23 ± 3 °C, a relative humidity of 51 ± 13%, and a 12-h light/dark cycle. Animal management adhered to the Guidelines for Care and Use of Laboratory Animals of Nanchang University.

Initially, fifty mice were acclimatized for one week and then randomly divided into five groups: the normal control group (C), model group (M), positive group treated by polyethylene glycol (Y), low-dose group (L), and high-dose group (H). During the modeling period, the M, Y, L, and H groups were administered loperamide hydrochloride (1.5 mg/kg twice daily), while the C group received sterile water. During the intervention period, the C and M groups were orally given a gelatin solution as a control, while the Y, L, and H groups were taken intragastric administration of 3.0 g/kg/d polyethylene glycol, 1×10^8 CFU/d per mouse, and 1×10^9 CFU/d per mouse of *L. paracasei* NCU-04, respectively. Each mouse received 0.2 mL per gavage, and the interventions lasted 14 consecutive days.

Subsequently, another forty mice were purchased and randomly divided into four groups: C, M, H, and inhibitor group (P). The C, M, and H groups were treated as the described above; while the P group were modeled by loperamide and then intraperitoneally injected with chlorophenylalanine (0.3 g/kg/d, 0.1 mL injection volume), a tryptophan hydroxylase inhibitor to deplete 5-HT levels (Cremata Jr and Koe, 1966).

2.4. Determination of fecal water content

At the end of the intervention, fresh fecal samples were collected from five randomly selected mice per group for water content analysis. Samples were weighed, dried, and reweighed. Water content was calculated using the formula:

$$\textit{Faecal water content}\ (\%) = \frac{\textit{the wet weight}\ (mg) - \textit{the dry weight}\ (mg)}{\textit{the wet weight}\ (mg)} \times 100\%$$

2.5. Determination of the time to the first black stool defecation

The activated carbon was mixed with the corresponding gavage suspensions at first. After collected the fresh fecal samples, five randomly selected mice in each group were intragastric administration of the activated carbon mixtures. The time between the gavage mixtures to the time when each mouse defecated its first black stool were recorded for comparative analysis.

2.6. Determination of gastrointestinal transit rate

We also randomly selected five mice in each group to determine their GI transit rate according to the protocol published previously (Wang et al., 2022). Briefly, mice were fasted overnight (only provided water) to empty the GI contents. At the next day, they were intragastric administrated with the activated carbon mixtures prepared as mentioned above and executed for 30 min. Subsequently, mice were sacrificed and their entire GI tracts from the pylorus to the anus were removed. The length of GI tract was measured as "the whole length of GI tract"; and the distance from the pylorus to the activated carbon front was measured as "the length of activated carbon movement". The GI transit rate was calculated as follows,

$$\textit{GI transit rate}\ (\%) = \frac{\textit{the length of activated carbon movement}\ (cm)}{\text{the whole length of GI tract}\ (\text{cm})} \times 100\%$$

2.7. Determination of sucrose consumption rate

Mice were first trained to consume two bottles of 1% sucrose water solution for 12 h, followed by pure water for another 12 h, and then fasted from water for 24 h to test sucrose water preference. After fasting, each mouse was exposed to one bottle of pure water and one bottle of sucrose water. The initial and final volumes of both were recorded after 12 h. The sucrose water consumption rate was calculated according to formula:

$$\textit{Sucrose comsumption rate}\ (\%) = \frac{\textit{the volume of sucrose water comsumption}\ (mL)}{\textit{the total volume of water and sucrose water comsumption}\ (mL)} \times 100\%$$

2.8. Determination of immobility time

The tail suspension test (TST) was performed to record the immobility time of mice (Steru et al., 1985). In brief, mice were acclimated for 1 h in a quiet environment, then suspended by the tail on hooks 50 cm above the ground in a device box (55 cm height × 15 cm length × 12 cm width) with a clean tray at the bottom. Activity was recorded for 6 min, and the device was cleaned before the next mouse. The immobility time was counted and statistically analyzed.

2.9. Biochemical analyses

The content of selected neurotransmitters and GI hormones, including motilin (MTL), peptide YY (PYY), and 5-hydroxytryptamine (5-HT), in the colon, serum or brain tissues were measured using ELISA assays. Protein expression levels of aquaporin-3 (AQP3), vasoactive intestinal peptide (VIP), and serotonin 4 receptor (5-HT4R) in the relevant tissues were analyzed by Western blot. Other protein factors, including c-kit, 5-HT4R, and PNDF, were detected by immunohistochemistry or immunofluorescence staining. Colon tissue histopathology was assessed using hematoxylin and eosin (HE) staining, and the transcription levels of colon inflammatory cytokines, including TNF-α, IL-6, and IL-1β, were analyzed by real-time quantitative PCR (Yue et al., 2024). ELISA kits were obtained from Shanghai Enzyme Link Biotech Co., Ltd (Shanghai, China), while HE, immunohistochemistry, and immunofluorescence staining were performed by Wuhan ServiceBio Co. (Wuhan, China).

2.10. 16S rDNA sequencing analysis

Microbial genomic DNA was extracted from fecal samples, and the V4 regions of the 16S rDNA were amplified by PCR. PCR products were purified, quantified, pooled in equimolar amounts, and sequenced on the Illumina HiSeq 2000 platform by Shanghai Personalbio Technology Co., Ltd (Shanghai, China). Bioinformatic analysis was conducted on the genescloud platform of Personalbio (https://www.genescloud.cn/home). Sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database under the identification number

PRJNA1144596.

2.11. Statistical analyses

Data were analyzed using GraphPad Prism (version 9, https://www.graphpad.com/) and ImageJ software. Statistical differences among groups were determined using one-way analysis of variance (ANOVA) combined with Tukey's multiple. Data were presented as the mean ± standard deviation (SD). *$p < 0.05$ or **$p < 0.01$ denoted a statistically significant difference for the comparison between two groups.

3. Results

3.1. Characterization of the probiotic properties of L. paracasei NCU-04 in vitro

To ensure the efficacy of *L. paracasei* NCU-04 in the GI tract, its probiotic properties were evaluated *in vitro* (Fig. 1). The growth curve showed that *L. paracasei* NCU-04 had robust growth performance as its OD_{600} could reach over 2.5 after cultured for 20 h (Fig. 1A), indicating its great advantage in industrial production. Compared to the pathogen of *S. aureus*, *L. paracasei* NCU-04 did not exhibit hemolytic activity on sterile defibrillated sheep blood, as no hemolysis ring was observed for this strain (Fig. 1B). The acid tolerance results showed that approximately ~10^8 CFU/mL viable strains survived in pH 2.0–3.0 without any additional protection (Fig. 1C). We used the total bacterial lysate to test the antioxidant activities of *L. paracasei* NCU-04. And the results exhibited that the scavenging rate of O^{2-}, -OH, and DPPH free radicals was about 67.62%, 86.86%, and 87.57%, respectively (Fig. 1D). Meanwhile, the chelating ability of Fe^{2+} was 68.50%, accompanied by a total reducing power at the A_{700} value of 0.71 (Fig. 1D). The antibiotics susceptibility test showed that *L. paracasei* NCU-04 was resistant to MY, CTR, CIP, GEN, and SXT; and in contrast, it was sensitive to C, PEN, AMP, TET, and E (Fig. 1E). Furthermore, we tested the antibacterial effects of *L. paracasei* NCU-04 on eight different pathogens. The results showed that the fermented supernatant and bacterial lysate of *L. paracasei* NCU-04 could effectively inhibit the growth of these pathogens, as indicated by the obvious inhibitory circles observed by the Oxford cup assay (Fig. 1F). Notably, the antibacterial activities of the bacterial lysate were stronger than that of the fermentation liquid, as indicated by larger inhibition zones (Fig. 1F). According to the above results, it is concluded that *L. paracasei* NCU-04 is safe and may be potentially suitable for industrial applications.

3.2. L. paracasei NCU-04 relieves constipation symptoms and colon inflammation

The schematic illustration of the animal experiment was shown in Fig. 2A, which has been described in detail in the methods section. Compared to the C group, the application of loperamide hydrochloride effectively caused constipation in mice, as indicated by the significantly changed defecation parameters in the M group (Fig. 2B–D), including lower fecal water content (56.74 ± 4.56% *vs.* 27.90 ± 3.32%, $p < 0.01$), the longer time to the first black stool (160.80 ± 25.90 min *vs.* 320.40 ± 34.27 min, $p < 0.01$), and the slower GI transit rate (54.33 ± 3.11% *vs.* 13.11 ± 2.92%, $p < 0.01$). However, compared to the M group, mice in

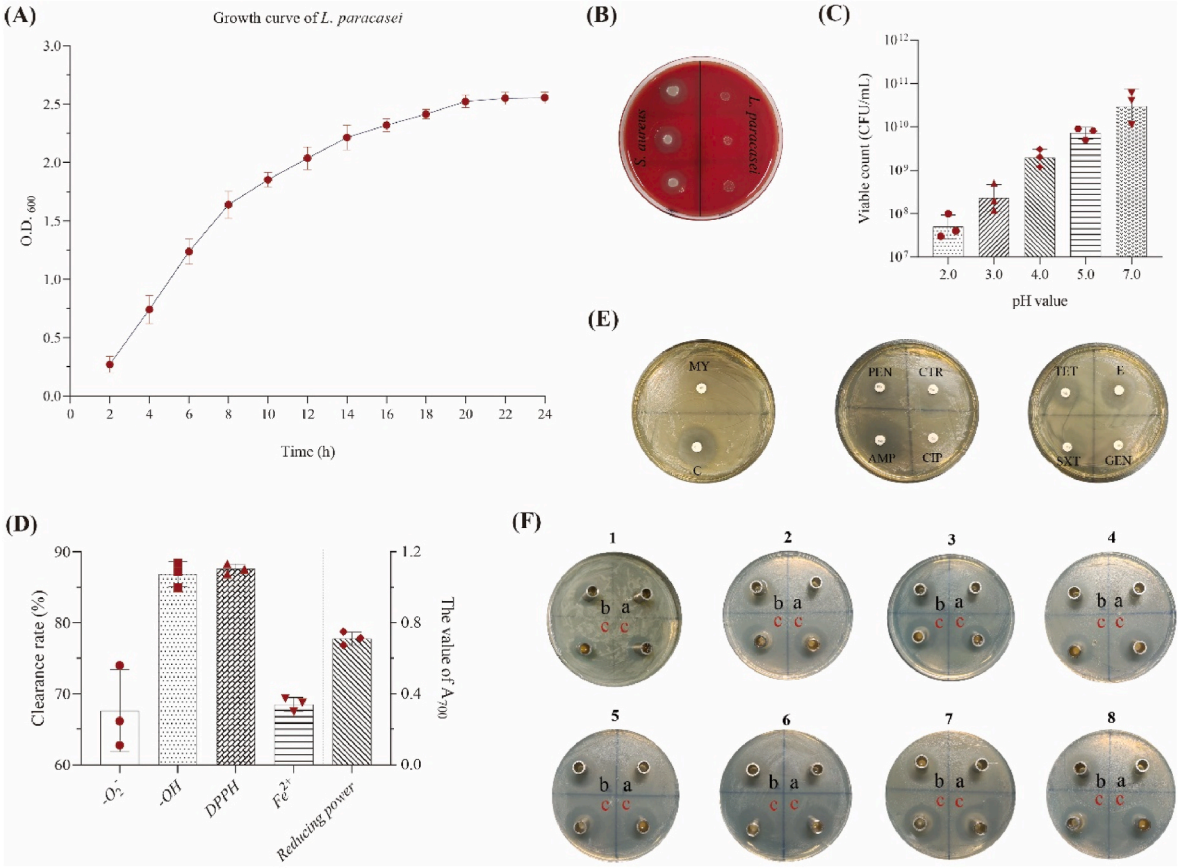

Fig. 1. Characterization of the probiotic properties of *L. paracasei* NCU-04 *in vitro*. (A) Growth curve of *L. parecasei* NCU-04. (B) Hemolysis test. (c) Acid resistance test, (D) Antioxidative analysis. (E) Antibiotics resistance test. MY, minomycin; CTR, cephalosporin; CIP, ciprofloxacin; GEN, gentamicin; SXT, cotrimoxazole; C, chloramphenicol; PEN, penicillin; AMP, ampicillin; TET, tetracycline; E, erythromycin. (F) Bacteriostatic activity test. a, MRS medium; b, the fermented liquid; c, the total bacterial lysate. 1, *S. typhimurium*; 2, *S. fowleri*; 3, *S. necrosis*; 4, *S. dysenteriae*; 5, *E. coli*; 6. *S. enteritidis*; 7, *L. monocytogenes*; 8. *S. aureus*. Data were showed as Mean ± SD, calculated from three independent assays.

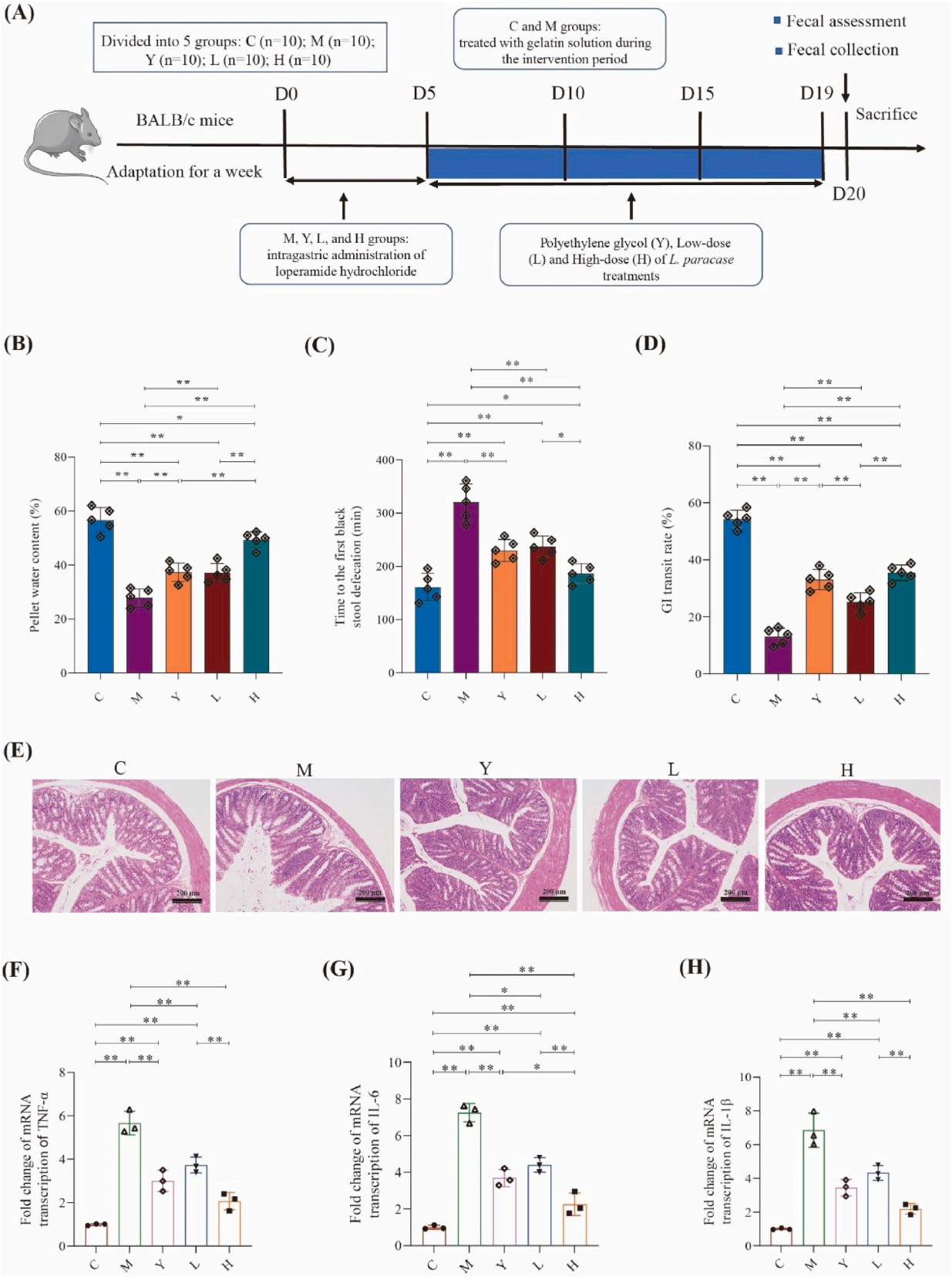

Fig. 2. Improvement effects of *L. paracasei* NCU-04 on the defecation-related parameters and colon inflammation in constipated mice. (A) The schedule of animal experiment in this study; (B) Pellet water content; (C) The time to the first black stool defecation; (D) The gastrointestinal transit rate; (E) HE staining of colon tissues; (F)–(H) The gene transcription level of TNF-α, IL-6, and IL-1β in the colon tissues. C, the normal control group; M, the modeling group; Y, the polyethylene glycol-treated group; L, the low-dose of *L. paracasei* NCU-04-treated group; H, the high-dose of *L. paracasei* NCU-04-treated group.

the Y, L, and H groups showed obviously improved defecation parameters. It's noted that the effects of low-dose of *L. paracasei* NCU-04 on faecal water content and the time to the first black stool were equal to that of polyethylene glycol ($p > 0.05$), yet its effect on the GI transit rate was significantly poor than polyethylene glycol ($p < 0.01$). Interestingly, the treatment with high-dose of *L. paracasei* NCU-04 exhibited the better pronounced improvement reaction to these parameters than that of low-dose of *L. paracasei* NCU-04, indicating a dosage dependent effects of *L. paracasei* NCU-04 on constipation.

The HE staining results showed that the muscle layer of colon was prominently thinned in M group when compared with the C group, accompanied by a clear inflammatory cell infiltration, a significant decreased goblet cells amount, and disrupted colon crypts (Fig. 2E). In contrast, the polyethylene glycol, low-dosage *L. paracasei* NCU-04, and high-dosage *L. paracasei* NCU-04 treatments alleviated the pathological changes in the colon of constipated mice to varying degrees (Fig. 2E). Moreover, compared to the C group, the transcription levels of inflammatory factors, including TNF-α, IL-6, and IL-1β in the colon of constipated mice were significantly upregulated ($p < 0.01$) (Fig. 2F–H). However, the treatments of polyethylene glycol and *L. paracasei* NCU-04 remarkedly downregulated their transcriptions in the order of high-dosage *L. paracasei* NCU-04, polyethylene glycol, to low-dosage *L. paracasei* NCU-04 (Fig. 2F–H). Collectively, these results indicated that *L. paracasei* NCU-04 attenuated loperamide-induced difficult defecation and colon inflammation in constipated mice.

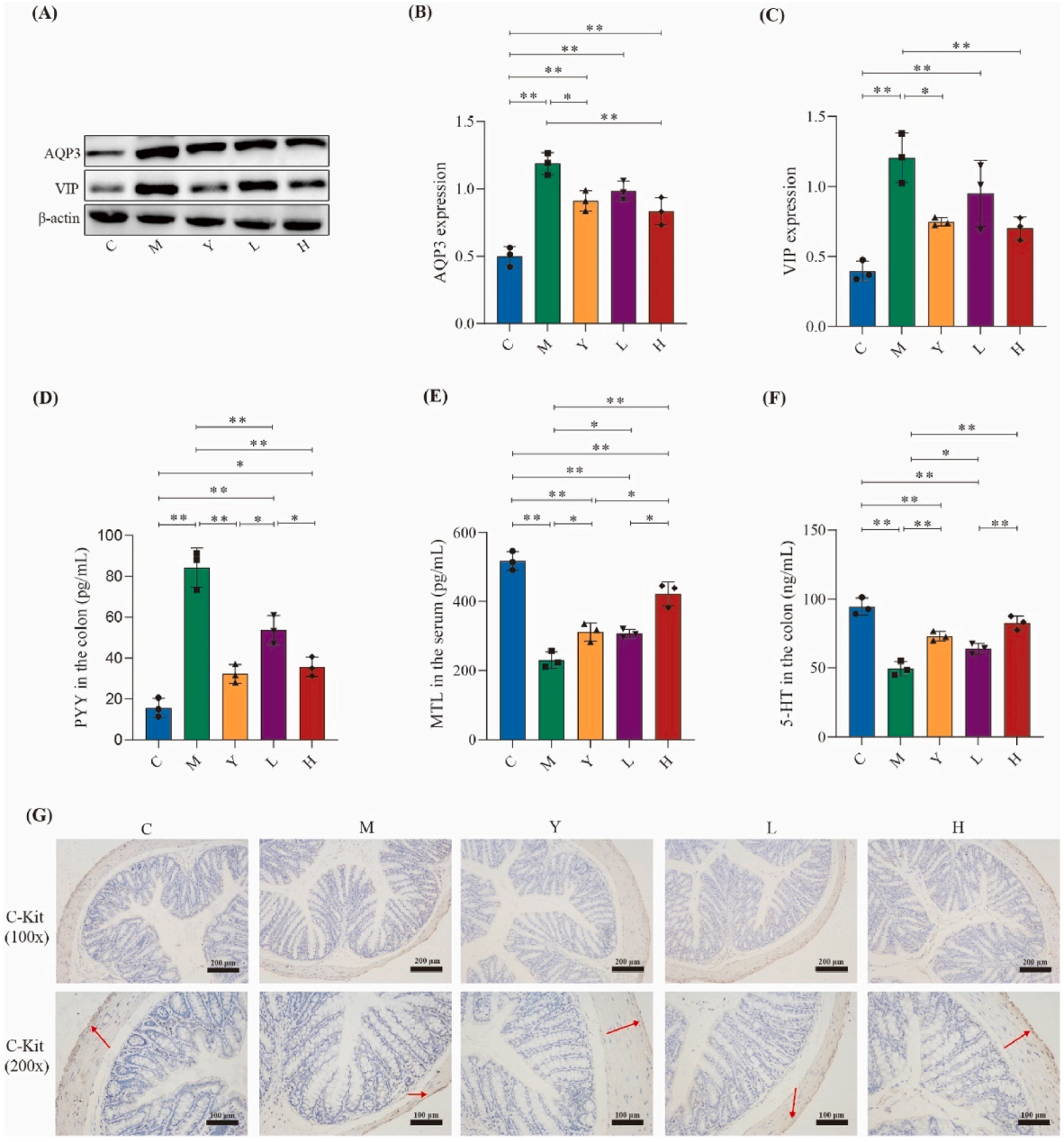

Fig. 3. Effects of L. *paracasei* NCU-04 on the expression of defecation-related factors in the colon of constipated mice. (A)–(C) Western blot and quantification analysis of the expression of AQP3 and VIP proteins in the colon of constipated mice. (D)–(F) Detection of the gut neurotransmitters in constipated mice, including motilin (MTL), peptide YY (PYY), and 5-hydroxytryptamine (5-HT). (G) Immunohistochemical staining of c-kit in the colon tissues of constipated mice. C, the normal control group; M, the modeling group; Y, the polyethylene glycol-treated group; L, the low-dosage *L. paracasei* NCU-04-treated group; H, the high-dosage *L. paracasei* NCU-04-treated group.

3.3. *L. paracasei* NCU-04 regulates the levels of intestinal neurotransmitters and proteins involved in the gut mobility

It has been reported that the upregulation of colonic AQP3, a key aquaporin responsible for the transmembrane transport of water molecules, can contribute to varying degrees of constipation (Kon et al., 2015). As evidenced in Fig. 3A and B, the expression levels of AQP3 in the colon of M group was dramatically higher than that of C group ($p < 0.01$). The treatment of polyethylene glycol and high-dosage *L. paracasei* NCU-04 significantly downregulated the expression of colonic AQP3 when compared to the M group ($p < 0.05$). It's noted that there was no statistically significant difference in AQP3 expression among the three treatment groups, even though high-dosage *L. paracasei* NCU-04 exhibited the best efficacy in decreasing the expression of AQP3 ($p > 0.05$). Additionally, compared to the C group, these three treatments could not restore the AQP3 expression to the normal levels ($p < 0.01$), indicating there might be other signaling pathways involved in the constipation improving of *L. paracasei* NCU-04.

Several representative intestinal neurotransmitters, including the inhibitory ones (VIP and PYY) and excitatory ones (MTL and 5-HT), were selected to investigate the effects of *L. paracasei* NCU-04 on constipation. Compared to the C group, the expression of VIP (Fig. 3A and C) and the level of PYY (Fig. 3D) were significantly increased in the M group ($p < 0.01$); in contrast, the levels of circulation MTL (Fig. 3E) and colonic 5-HT (Fig. 3F) obviously decreased ($p < 0.01$). After the treatments with polyethylene glycol and *L. paracasei* NCU-04, the changes in the levels of these neurotransmitters were significantly reversed in the colon of constipated mice, with the best efficacy in the H group. Additionally, we observed that the expressions of VIP and AQP3 in the L group were lower than that of M group, but there was no statistical difference by western-blot detection ($p > 0.05$).

The receptor tyrosine protein kinase kit (c-kit), a biomarker of ICC, plays a crucial role in gut motility (Huizinga et al., 1997). The results of immunohistochemical staining showed that the expression of colonic c-kit in the muscularis externa of the M group was obviously reduced when compared with that of the C group, indicating poor distribution and population of ICC caused by loperamide (Fig. 3G). However, the expression of c-kit effectively enhanced in the three treatment groups, particularly in the H group showing the strongest expression of c-kit, suggesting that high-dosage *L. paracasei* NCU-04 may improve intestinal patency and peristalsis through regulating the population of ICC.

3.4. *L. paracasei* NCU-04 regulates 5-HT and its receptor 4 (5-HT4R) expression in the brain of constipated mice

There is evidence that 5-HT not only regulates the development of the ENS, directly influencing gut motility, but also affects the CNS, thereby indirectly impacting intestinal function (Israelyan et al., 2019). Therefore, the reduced release of 5-HT from CNS might lead to defects in brain development and constipation. As illustrated in Fig. 4, the production of brain 5-HT was significantly lower in the M group than that of the C group ($p < 0.01$) (Fig. 4A), accompanied by the remarkedly decreased expression of 5-HT receptor 4 (5-HT4R) (Fig. 4B and C), a member of the family of serotonin receptors that stimulate cAMP production in response to 5-HT. The treatment of polyethylene glycol and *L. paracasei* NCU-04 dramatically increased the 5-HT yields and 5-HT4R expression when compared to the M group, except for there was no statistically different between the Y and L groups. In particular, the treatment of high-dosage *L. paracasei* NCU-04 showed the best ability to reverse the changes of brain 5-HT and its receptor 4. These results suggested that the administration of *L. paracasei* NCU-04 improved constipation might through regulating the production of brain 5-HT and 5-HT4R expression, indicating a 5-HT-mediated comorbid relationship between brain disorders and constipation.

3.5. *L. paracasei* NCU-04 modulates the gut microbiota in constipated mice

We performed 16S rRNA high-throughput sequencing to analyze the effects of *L. paracasei* NCU-04 on the gut microbiota of constipated mice. As shown in Fig. 5A–C, the Shannon, Simpson, and Pielou_e indices of the M group were significantly reduced compared to the C group ($p < 0.01$), suggesting a lower microbial α-diversity in the loperamide-induced constipation mice. The treatment of polyethylene glycol did not show significant changes in these indices when compared to the M group ($p > 0.05$). While the treatment of low-dosage *L. paracasei* NCU-04 could significantly increase the Simpson and Pielou_e indices ($p < 0.05$), yet showed no statistic difference on Shannon index. It's noted that the treatment of high-dosage *L. paracasei* NCU-04 more effectively restored the α-diversity of gut microbiota to the normal level when compared to the Y and L groups, suggesting a dose-dependent effect of *L. paracasei* NCU-04 on the community diversity of gut microbiota in constipated mice. PcoA analysis based on Bray-Curtis distance matrices showed that the microbial communities of mice in the M group showed a separation with that of the C group, as indicated by the long distance of each sample in the M group to that of C group (Fig. 5D). Meanwhile, fecal samples from the M, Y, and L groups were almost clustered together, except one

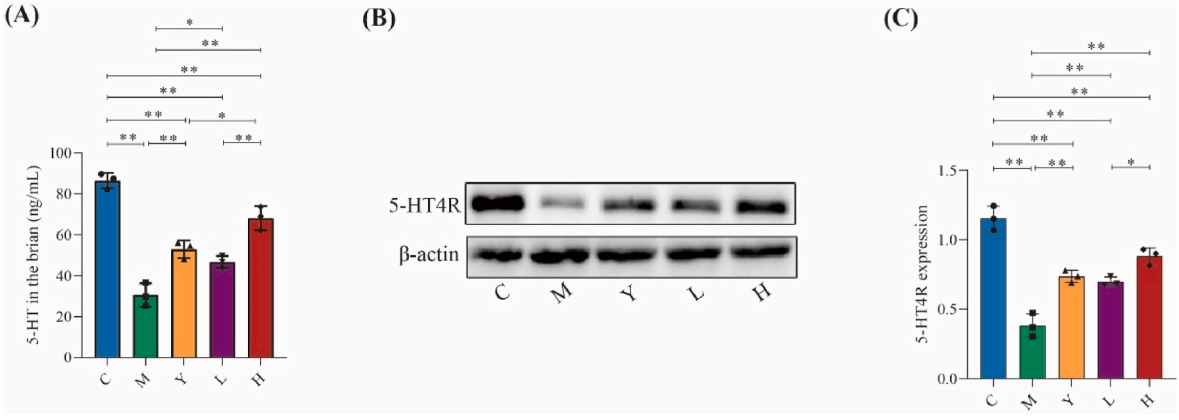

Fig. 4. Effects of L. *paracasei* NCU-04 on the 5-HT level and its related receptor in the brain of constipated mice. (A) Detection of the content of 5-HT in the brain of constipated mice. (B) Western blot and quantification analysis (C) of the expression of 5-HT4R in the brain of constipated mice. C, the normal control group; M, the modeling group; Y, the polyethylene glycol-treated group; L, the low-dose of *L. paracasei* NCU-04-treated group; H, the high-dose of *L. paracasei* NCU-04-treated group.

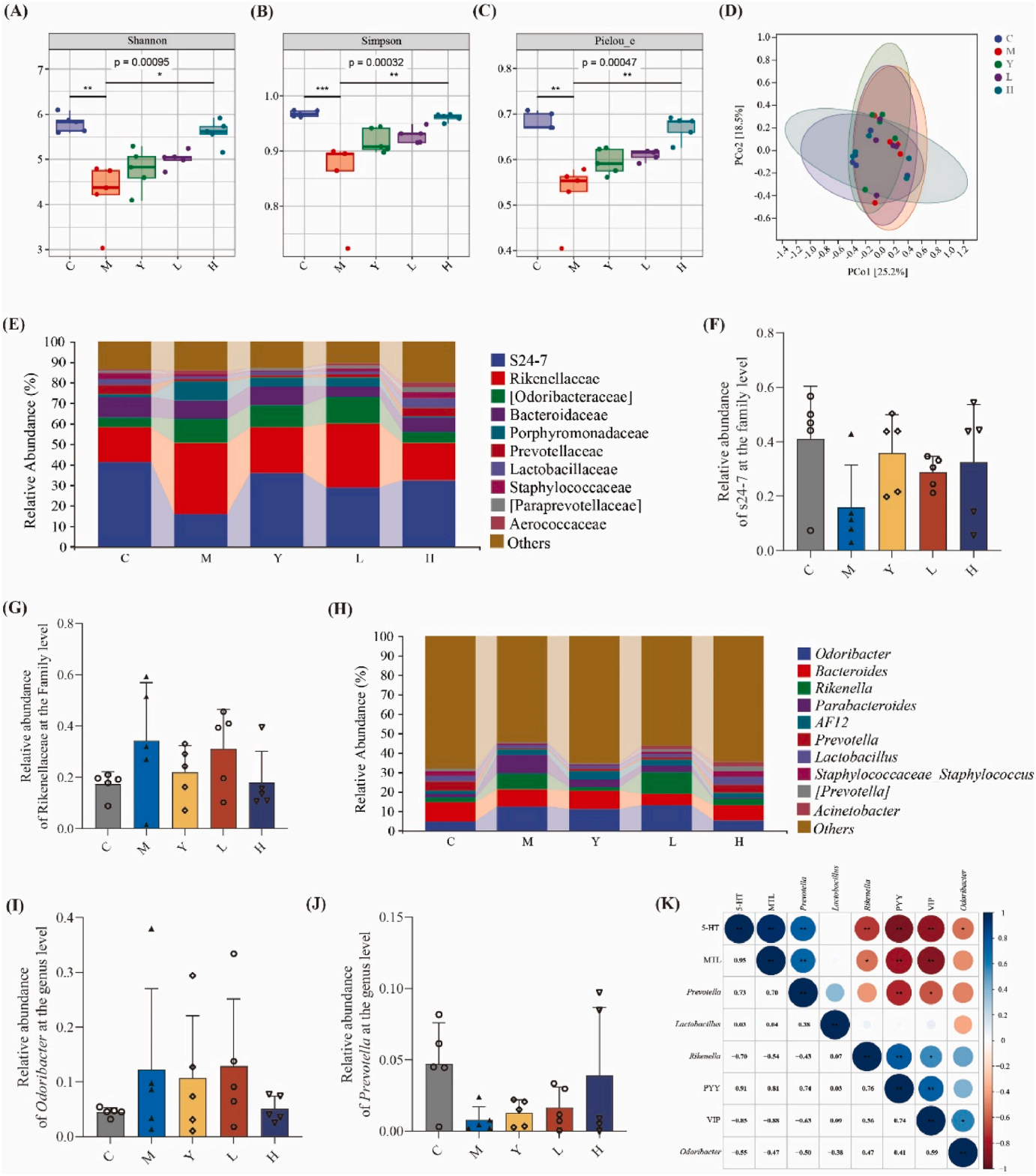

Fig. 5. Effects of L. *paracasei* NCU-04 on the gut microbiota of the constipated mice. (A)–(C) The Shannon, Simpson, and Pielou_e indices of α-diversity. (D) The PcoA analysis of β-diversity based on Bray-Curtis distance matrices. (E) The gut microbial distribution at the family level. (F)–(G) Relative abundances of the family s24-7 and Rikenellaceae. (H) The gut microbial distribution at the genus level. (I)–(J) Relative abundance of the genera *Odoribacter* and *Prevetlla*. (K) The Spearman correlation analysis of the changed gut microbes and neurotransmitters. C, the normal control group; M, the modeling group; Y, the polyethylene glycol-treated group; L, the low-dose of *L. paracasei* NCU-04-treated group; H, the high-dose of *L. paracasei* NCU-04-treated group.

sample from the L group was clustered with the C and H groups, suggesting a high degree of similarity in their microbiome β-diversity (Fig. 5D). In contrast, fecal samples in the H group were close to that of the C group, indicating that the treatment with high-dosage *L. paracasei* NCU-04 could restore the microbial community composition of constipated mice to the normal level (Fig. 5D).

At the family level of gut microbiota composition (Fig. 5E), several bacterial families, including s24-7, *Bacteroidacese*, *Prevotellaceae*, and *Lactobacillaceae* were relatively enriched in the C group; while *Rikenellaceae* and *Porphyromonadaceae* were enriched in the M group. The abundance of s24-7 and *Rikenellaceae*, two representative bacterial families, was increased and decreased after the treatments in constipated mice, respectively (Fig. 5F and G). In particular, the decrease in the abundance of *Rikenellaceae* and *Porphyromonadaceae* and the increase in the abundance of *Prevotellaceae* and *Lactobacillaceae* were most prominent in the H group, accompanied by a most similarity microbial profile between the two groups. At the genus level, the abundance of *Odoribacter*, *Rikenella*, and *Parabacteroides* was increased and that of *Prevotella* and *Lactobacillus* was decreased in the M group compared with the C group (Fig. 5H). It is noteworthy that the relative abundance of *Odoribacter* was obviously reduced and that of *Prevotella* was higher in the H group than that of the M group (Fig. 5I and J). And the changing trend of other genera in the H group, including *Rikenella*, *Prabaceroides*, and *Lactobacillus* was similar with that of either *Odoribacter* or *Prevotella*, showing a close microbial composition between H and C groups (Fig. 5H). Interestingly, the correlation analysis showed that 5-HT and MTL were significantly positively correlated with *Provetella*, yet negatively with *Rikenella* and *Odoribacter* (Fig. 5K). In contrast, PYY and VIP were negatively correlated with *Prevotella*, but positively with *Rikenella* and *Odoribacter* (Fig. 5K). However, no obvious correlation was shown between *Lactobacillus* genus and these four regulated neurotransmitters. These results suggested that the gut microbiota might be involved in regulating the synthesis and secretion of gut neurotransmitters or peptides, contributing to intestinal motility and normal defecation. Collectively, these results suggested that high-dosage *L. paracasei* NCU-04 effectively restored the disturbed gut microbiota in constipated mice, characterized by the improved microbial diversity and composition of gut microbiota.

3.6. *L. paracasei* NCU-04 alleviates depression-like behaviors in constipated mice

A large-scale cohort study has reported a higher risk of depression among individuals with constipation, highlighting the importance of addressing depressive symptoms in these individuals (Yun et al., 2024). Narek Israelyan et al. have proposed that neuronal production of 5-HT mediates the link between constipation and depression (Israelyan et al., 2019). Therefore, we designed an additional animal experiment to investigate the improvement effects of *L. paracasei* NCU-04 on the depressive-like behaviors in constipated mice (Fig. 6A). Anhedonia, the inability to experience pleasure from typically enjoyable activities, is a key symptom of depression (Arioz et al., 2019). The sucrose preference test was commonly used to assess the degree of anhedonia in animal models of depression. Similarly, the tail suspension test (TST) is a standard method for evaluating despair-like behavior, another hallmark of depression.

As showed in Fig. 6B and C, the loperamide-induced constipated mice showed a lower preference for sucrose solution and a longer immobility time compared to the normal control mice ($p < 0.01$), and these impairments could be effectively improved by the treatment of *L. paracasei* NCU-04 ($p < 0.01$). However, the preference for sucrose water was dramatically decreased and the immobility time was significantly increased in the P group when compared with the H group ($p < 0.01$). Additionally, the levels of colonic and brain 5-HT were also significantly reduced in the P group (Fig. 6D and E), accompanied by the downregulation of 5-HT4R around the hippocampus in the brain (Fig. 6F), suggesting that the intraperitoneal injection of chlorophenylalanine resulted in the defects of 5-HT in constipated mice.

Brain-derived neurotrophic factor (BDNF) is a well-studied neurotrophin known to promote the development and maintenance of serotonergic neurons, thereby influencing 5-HT biosynthesis and availability (Correia et al., 2023). Also, BNDF has been reported to accelerate gut motility in slow-transit constipation (F. Chen et al., 2014). Immunofluorescence staining revealed that BNDF expression in the hippocampus of constipated mice was reduced compared to the normal control mice. However, the treatment of *L. paracasei* NCU-04 upregulated its expression to varying degrees (Fig. 6G). Despite this, defects in the production of hippocampal 5-HT led to the repression of BNDF, counteracting the effects of *L. paracasei* NCU-04 on BNDF expression. These results indicated that the depletion of 5-HT successfully counteracted the therapeutic efficacy of *L. paracasei* NCU-04 on depression-like behaviors in constipated mice, supporting the important role of 5-HT in mediating constipation and depression.

4. Discussion

Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts (Hill et al., 2014), are increasingly being studied for their potential to promote health. Here, we isolated a probiotic strain of *Lacticaseibacillus paracasei*, termed *L. paracasei* NCU-04, from healthy infant feces. This strain exhibited excellent probiotic properties *in vitro*, such as rapid growth ability, strong antibacterial and antioxidant capacity, and no hemolytic ability (Fig. 1), implying its potential as a new gut probiotic. Consequently, we investigated the therapeutic effects of *L. paracasei* NCU-04 on constipated mice induced by loperamide.

Loperamide-induced animal models are well-established for constipation research they can mimic the physiological mechanisms of human constipation. Studies based on these models consistently reproduced the symptoms of constipation such as reduced fecal water content, slowed intestinal motility, colon lesions and inflammation, and even depression-related behaviors (Chai et al., 2021; C.-L. Chen et al., 2020; Li et al., 2023; Wang et al., 2022; Wang et al., 2023; Wu et al., 2023; C. Zhang et al., 2023), which aligned with our findings in this study (Figs. 2 and 6). Interestingly, these symptoms could be ameliorated by probiotics and probiotics-enriched products, although their effectiveness remains varied due to species- and strains-specific (Dimidi et al., 2020). Here, we demonstrated that the administration of *L. paracasei* NCU-04 effectively improved the general manifestations of constipation and depression-like behaviors in mice, providing additional evidence for the use of probiotics in treating constipation.

Probiotics alleviate constipation through multiple mechanisms (Dimidi et al., 2017). AQP3, a water channel primarily expressed in the colon, plays a crucial role in regulating fecal water content (Kon et al., 2015). Some studies have suggested that constipation rodents experienced increased water absorption in their colons and reduced intestinal fluid secretion due to the upregulated AQP3 expression (Kon et al., 2015; Wu et al., 2023; X. Zhang et al., 2021). This upregulation led to intestinal smooth muscle spasms, enhanced water absorption, altered frequency of intestinal peristalsis, and ultimately, constipation. Similarly, in our study, we also observed an upregulation of AQP3 expression in constipated mice, which was repressed by *L. paracasei* NCU-04 treatment, resulting in increased fecal water content (Fig. 3). ICC, which specifically express c-kit, are specialized pacemaker cells in the colon that coordinate smooth muscle contractions and regulate GI motility (Komuro, 2006). In constipation mice, the reduced colonic c-kit expression and ICC population indicates the poor intestinal patency and peristalsis, leading to long-time and difficulty in defecation, which could be improved by probiotics (Cheng et al., 2024; Li et al., 2023). Consistent with these findings, our study demonstrated significantly reduced colonic c-kit expression in constipation mice, while *L. paracasei* NCU-04 treatment increased its expression, suggesting restoration of gut motility

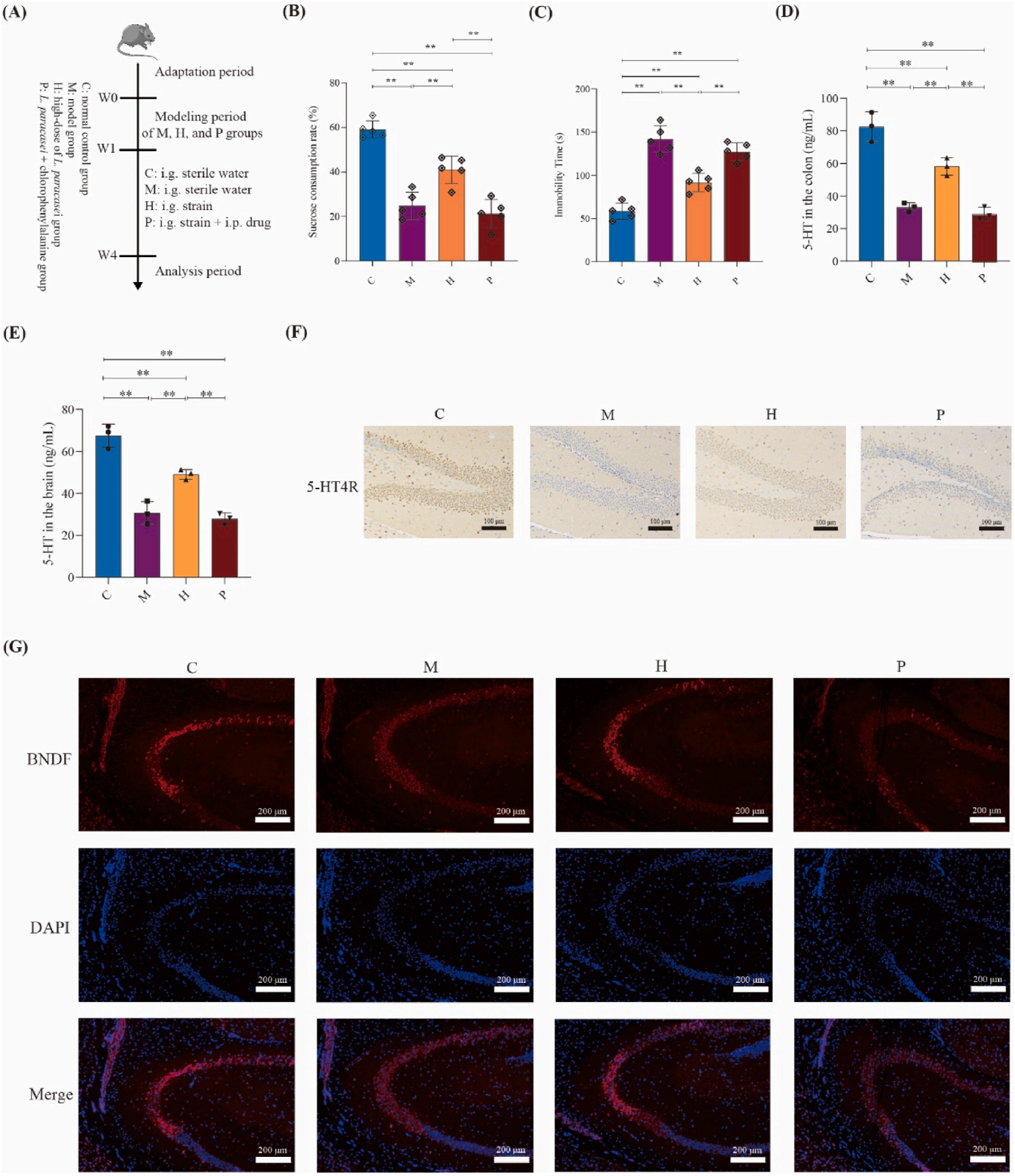

Fig. 6. Effects of L. *paracasei* NCU-04 on the depressive-related behaviors in constipated mice. (A) Animal experimental design. (B) Sucrose preference test. (C) Tail suspension test. (D)–(E) Detection of the content of 5-HT in the colon and brain of constipated mice. (F) Immunohistochemical staining of 5-HT4R in the brain of constipated mice. (G) Immunofluorescence staining of BNDF in the brain of constipated mice. C, the normal control group; M, the modeling group; H, the high-dose of *L. paracasei* NCU-04-treated group; P, the combination of high dosage of *L. paracasei* NCU-04 and the inhibitor of tryptophan hydroxylase-treated group.

and bowel movements (Fig. 3).

Various enteric neurotransmitters and gut peptides, such as VIP, PYY, MTL, and 5-HT, play crucial roles in regulating gut motility. VIP is primarily located in the cells of the GI tract, where it performs several essential roles in maintaining digestive health and ensuring the proper functioning of the GI system (Iwasaki et al., 2019). For instance, it slows the contraction of the sphincter and smooth muscles, aiding in the regulation of GI motility and preventing spasms. PYY, a peptide consisting of 36 amino acids, is an important intestinal hormone released by enteroendocrine L cells located primarily in the ileum and colon, where it inhibits gastric emptying and intestinal transit time (Ballantyne, 2006). Both VIP and PYY, regarded as inhibitory enteric neurotransmitters, are elevated in the cases of constipation (Cheng et al., 2024; C. Zhang et al., 2023). Conversely, excitatory neurotransmitters like MTL and 5-HT are decreased in response to constipation (Li et al., 2023; C. Zhang et al., 2023). MTL enhances GI motility to accelerate defecation, while 5-HT regulates the actions of ENS and CNS by activating its receptors such as 5-HT4R to improve constipation. Our findings showed similar changes in these neurotransmitters, which could be reversed by *L. paracasei* NCU-04 treatment (Fig. 3), suggesting improved gut motility in constipated mice. In particular, the expression of 5-HT and its receptor 5-HT4R in the brain were also downregulated in constipation mice, while *L. paracasei* NCU-04 treatment increased their expressions to varying degrees (Fig. 4), indicating the activation of 5-HT signal pathway in the brain. We speculated that the crosstalk between ENS and CNS might mediate this activation, as the ENS communicates bidirectionally with CNS through intrinsic and extrinsic innervations (Fleming et al., 2020).

Studies suggested a close relationship between constipation and the dysbiosis of gut microbiota (Dimidi et al., 2017; Pan et al., 2022; S. Zhang et al., 2021). Beyond commonly known taxa like *Lactobacilli* and *Bifidobacteria*, alterations in other gut microbiota taxa also contribute to constipation. For example, the *Bacteroidales_s24-7* family, a predominant member in the gut microbiota, showed reduced relative abundance in constipated mice (Huang et al., 2022; X. Ren et al., 2017). And the increased abundance of *Rikenellaceae* was reportedly associated with the severity of depression/anxiety in mice (Yang et al., 2022). *Prevotellaceae* had been demonstrated to be the only bacterial family significantly lowed in constipated patients and the *Prevotella* genus greatly contributed to this changing trend (Yu et al., 2023; Zhu et al., 2014); however, Cheng et al. reported an opposite results of altered *Prevotellaceae* family and *Prevotella* genus in constipated mice (Cheng et al., 2024). Our results align with the patient data, showing decreased *Prevotellaceae* and *Prevotella* in constipated mice. The genus *Odoribacter* was reportedly enriched in constipated mice and was associated with cognitive impairment in Parkinson's disease mice (T. Ren et al., 2020; Wang et al., 2023). Interestingly, these changed microbial compositions could be restored by probiotics treatment, including *L. paracasei* NCU-04 in this study (Fig. 5).

Emotional disorders such as anxiety and depression frequently co-occur with constipation, yet their shared causes were rarely examined (Yun et al., 2024). The neurotransmitter 5-HT is crucial for the development and long-term functioning of both CNS and ENS, influencing GI motility and emotion. A study base on the 5-HT synthesis-deficient transgenic mice demonstrated that the reduced release of 5-HT in the CNS led to defects in ENS and gut motility, while feeding 5-hydroxytryptophan slow-release chow restored the content of 5-HT in CNS and reduced depression-related behaviors, indicating that the production of 5-HT by neurons connects constipation with mood disorders (Israelyan et al., 2019). Additionally, the alterations in the gut microbiota, such as the increased *Odoribacter* and decreased *Lactobacillus*, inhibited the 5-HT synthesis in the colon in constipated mice by promoting the expression of intestinal 5-HT transporter and damaging the intestinal integrity (Cao et al., 2017; Wang et al., 2023). In this study, we found that *L. paracasei* NCU-04 significantly improved depression-like behaviors in constipated mice, potentially mediated by the neuron 5-HT production (Fig. 6). A gut indigenous *Ruminococcus gnavus* also reportedly alleviated constipation and stress-related behaviors in constipated mice by increasing colonic 5-HT levels (Li et al., 2023). Other probiotics, such as *Bifidobacteria* and *Lactobacilli*, have been shown to relief the symptoms of depression and constipation by regulation the 5-HT-mediated signal pathway (Tian et al., 2019; Wang et al., 2023).

In conclusion, we identified a new *L. paracasei*, NCU-04, with excellent probiotic properties and effectiveness in improving constipation and depression-like behaviors in loperamide-induced constipated mice. This effect could be achieved through the regulation of neurotransmitters, promotion of GI motility and lubrication, and maintenance of gut microbiota homeostasis (Fig. 7). In particular, we demonstrated the 5-HT may greatly involve in mediating the therapeutic efficacy of *L. paracasei* NCU-04 (Fig. 7). Collectively, *L. paracasei* NCU-04 can be regarded as a food supplementation or adjuvant strategy to alleviate constipation in the future. However, there are still several scientific questions in this study that warrant further exploration. For instance, it is important to determine whether the therapeutic effects of *L. paracasei* NCU-04 are dependent on the gut microbiota, which could be clarified using a germ-free mouse model. Although the study highlighted the role of 5-HT in mediating the effects of this strain, a more comprehensive and in-depth analysis of the molecular mechanisms by which the strain influences 5-HT signaling in vivo would provide deeper insights. Additionally, this study relied solely on a loperamide-induced constipation model in mice, potentially limiting the generalizability of the findings to clinical settings.

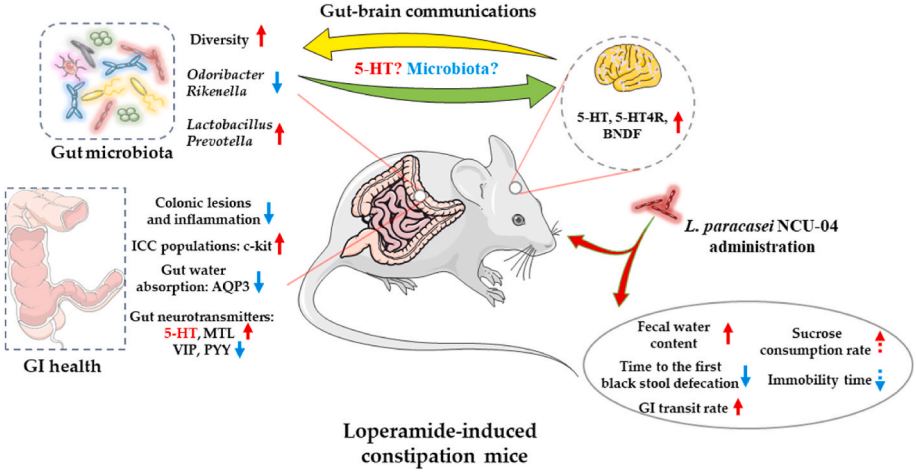

Fig. 7. Graphical abstract of this study.

CRediT authorship contribution statement

Shengjie Li: Funding acquisition, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Yi Li:** Methodology, Data curation, Investigation. **Yujie Cai:** Methodology, Data curation, Investigation. **Zizhou Yan:** Methodology, Data curation, Investigation. **Jing Wei:** Methodology, Project administration. **Hongyan Zhang:** Conceptualization, Supervision, Project administration. **Fenfang Yue:** Methodology, Software, Data curation, Investigation, Project administration, Writing – original draft, All authors approved the publication of this manuscript. **Tingtao Chen:** Conceptualization, Funding acquistion, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest in the manuscript of "***Lacticaseibacillus paracasei* NCU-04 relieves constipation and the depressive-like behaviors induced by loperamide in mice through the microbiome-gut-brain axis**".

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Jiangxi Natural Science Foundation of China (20224BAB206078 to Shengjie Li), National Natural Science Foundation of China (82460297 and 82060638 to Tingtao Chen, 82260389 to Shengjie Li), and the "Chunhui project" of Cooperative Research Foundation of Ministry of Education, China (202200648 to Shengjie Li).

References

Algieri, F., Tanaskovic, N., Rincon, C.C., Notario, E., Braga, D., Pesole, G., Rescigno, M., 2023. *Lactobacillus paracasei* CNCM I-5220-derived postbiotic protects from the leaky-gut. Front. Microbiol. 14, 1157164.

Arioz, B.I., Tastan, B., Tarakcioglu, E., Tufekci, K.U., Olcum, M., Ersoy, N., Genc, S., 2019. Melatonin attenuates LPS-induced acute depressive-like behaviors and microglial NLRP3 inflammasome activation through the SIRT1/Nrf2 pathway. Front. Immunol. 10, 1511.

Ballantyne, G.H., 2006. Peptide YY (1-36) and peptide YY (3-36): Part I. Distribution, release and actions. Obes. Surg. 16 (5), 651–658.

Barberio, B., Judge, C., Savarino, E.V., Ford, A.C., 2021. Global prevalence of functional constipation according to the Rome criteria: a systematic review and meta-analysis. Lancet Gastroenterol. Hepatol. 6 (8), 638–648.

Bengoa, A.A., Dardis, C., Garrote, G.L., Abraham, A.G., 2021. Health-promoting properties of *Lacticaseibacillus paracasei*: a focus on kefir isolates and exopolysaccharide-producing strains. Foods 10 (10), 2239.

Camilleri, M., Ford, A.C., Mawe, G.M., Dinning, P.G., Rao, S.S., Chey, W.D., Chang, L., 2017. Chronic constipation. Nat. Rev. Dis. Prim. 3 (1), 1–19.

Cammarota, G., Ianiro, G., Bibbò, S., Gasbarrini, A., 2014. Gut microbiota modulation: probiotics, antibiotics or fecal microbiota transplantation? Intern. Emerg. Med. 9, 365–373.

Cao, H., Liu, X., An, Y., Zhou, G., Liu, Y., Xu, M., Jiang, K., 2017. Dysbiosis contributes to chronic constipation development via regulation of serotonin transporter in the intestine. Sci. Rep. 7 (1), 10322.

Cash, B., Chey, W., 2005. The role of serotonergic agents in the treatment of patients with primary chronic constipation. Aliment Pharmacol. Therapeut. 22 (11-12), 1047–1060.

Chai, M., Wang, L., Li, X., Zhao, J., Zhang, H., Wang, G., Chen, W., 2021. Different *Bifidobacterium bifidum* strains change the intestinal flora composition of mice via different mechanisms to alleviate loperamide-induced constipation. Food Funct. 12 (13), 6058–6069.

Chen, C.-L., Chao, S.-H., Pan, T.-M., 2020. *Lactobacillus paracasei subsp. paracasei* NTU 101 lyophilized powder improves loperamide-induced constipation in rats. Heliyon 6 (4), 05684.

Chen, F., Yu, Y., Wang, P., Dong, Y., Wang, T., Zuo, X., Li, Y., 2014. Brain-derived neurotrophic factor accelerates gut motility in slow-transit constipation. Acta Physiol. 212 (3), 226–238.

Chen, Q., Li, N., Jiang, J., 2021. Relationship between chronic constipation and brain-gut microflora axis. Zhonghua wei Chang wai ke za zhi= Chinese Journal of Gastrointestinal Surgery 24 (12), 1048–1053.

Chen, Z., Peng, Y., Shi, Q., Chen, Y., Cao, L., Jia, J., Zhang, J., 2022. Prevalence and risk factors of functional constipation according to the Rome criteria in China: a systematic review and meta-analysis. Front. Med. 9, 815156.

Cheng, S., Li, B., Ding, Y., Hou, B., Hung, W., He, J., Man, C., 2024. The probiotic fermented milk of *Lacticaseibacillus paracasei* JY062 and *Lactobacillus gasseri* JM1 alleviates constipation via improving gastrointestinal motility and gut microbiota. J. Dairy Sci. 107 (4), 1857–1876.

Correia, A.S., Cardoso, A., Vale, N., 2023. BDNF unveiled: exploring its role in major depression disorder serotonergic imbalance and associated stress conditions. Pharmaceutics 15 (8), 2081.

Cremata Jr, V.Y., Koe, B.K., 1966. Clinical-pharmacological evaluation of p-chlorophenylalanine: a new serotonin-depleting agent. Clin. Pharmacol. Therapeut. 7 (6), 768–776.

Cryan, J.F., O'Mahony, S.M., 2011. The microbiome-gut-brain axis: from bowel to behavior. Neuro Gastroenterol. Motil. 23 (3), 187–192.

Dimidi, E., Christodoulides, S., Scott, S.M., Whelan, K., 2017. Mechanisms of action of probiotics and the gastrointestinal microbiota on gut motility and constipation. Adv. Nutr. 8 (3), 484–494.

Dimidi, E., Scott, S.M., Whelan, K., 2020. Probiotics and constipation: mechanisms of action, evidence for effectiveness and utilisation by patients and healthcare professionals. Proc. Nutr. Soc. 79 (1), 147–157.

Dinan, T.G., Cryan, J.F., 2017. The microbiome-gut-brain axis in health and disease. Gastroenterol. Clin. 46 (1), 77–89.

Fleming, M.A., Ehsan, L., Moore, S.R., Levin, D.E., 2020. The enteric nervous system and its emerging role as a therapeutic target. Gastroenterol. Res. Pract. 2020 (1), 8024171.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Salminen, S., 2014. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514.

Huang, J., Lin, B., Zhang, Y., Xie, Z., Zheng, Y., Wang, Q., Xiao, H., 2022. Bamboo shavings derived O-acetylated xylan alleviates loperamide-induced constipation in mice. Carbohydr. Polym. 276, 118761.

Huizinga, J.D., Thuneberg, L., Vanderwinden, J.-M., Rumessen, J.J., 1997. Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal motor disorders. Trends Pharmacol. Sci. 18 (10), 393–403.

Israelyan, N., Del Colle, A., Li, Z., Park, Y., Xing, A., Jacobsen, J.P., Saurman, V., 2019. Effects of serotonin and slow-release 5-hydroxytryptophan on gastrointestinal motility in a mouse model of depression. Gastroenterology 157 (2), 507–521. e504.

Iwasaki, M., Akiba, Y., Kaunitz, J.D., 2019. Recent advances in vasoactive intestinal peptide physiology and pathophysiology: focus on the gastrointestinal system. F1000Research 8.

Kim, W.-K., Jang, Y.J., Seo, B., Han, D.H., Park, S., Ko, G., 2019. Administration of *Lactobacillus paracasei* strains improves immunomodulation and changes the composition of gut microbiota leading to improvement of colitis in mice. J. Funct. Foods 52, 565–575.

Komuro, T., 2006. Structure and organization of interstitial cells of Cajal in the gastrointestinal tract. J. Physiol. 576 (3), 653–658.

Kon, R., Ikarashi, N., Hayakawa, A., Haga, Y., Fueki, A., Kusunoki, Y., Sugiyama, K., 2015. Morphine-induced constipation develops with increased aquaporin-3 expression in the colon via increased serotonin secretion. Toxicol. Sci. 145 (2), 337–347.

Kondo, J., Xiao, J.-Z., Shirahata, A., Baba, M., Abe, A., Ogawa, K., Shimoda, T., 2013. Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding. World J. Gastroenterol.: WJG 19 (14), 2162.

Li, R., Xu, S., Li, B., Zhang, B., Chen, W., Dai, D., Liu, Z., 2023. Gut indigenous *Ruminococcus gnavus* alleviates constipation and stress-related behaviors in mice with loperamide-induced constipation. Food Funct. 14 (12), 5702–5715.

Lyford, G., He, C., Soffer, E., Hull, T., Strong, S., Senagore, A., Farrugia, G., 2002. Pan-colonic decrease in interstitial cells of Cajal in patients with slow transit constipation. Gut 51 (4), 496–501.

Ma, T., Shen, X., Shi, X., Sakandar, H.A., Quan, K., Li, Y., Sun, Z., 2023. Targeting gut microbiota and metabolism as the major probiotic mechanism-An evidence-based review. Trends Food Sci. Technol. 138, 178–198.

Matsumoto, K., Takada, T., Shimizu, K., Kado, Y., Kawakami, K., Makino, I., Kajimoto, O., 2006. The effects of a probiotic milk product containing *Lactobacillus casei* strain Shirota on the defecation frequency and the intestinal microflora of sub-optimal health state volunteers: a randomized placebo-controlled cross-over study. Biosci. Microflora 25 (2), 39–48.

Pan, R., Wang, L., Xu, X., Chen, Y., Wang, H., Wang, G., Chen, W., 2022. Crosstalk between the gut microbiome and colonic motility in chronic constipation: potential mechanisms and microbiota modulation. Nutrients 14 (18), 3704.

Ren, T., Gao, Y., Qiu, Y., Jiang, S., Zhang, Q., Zhang, J., Nie, K., 2020. Gut microbiota altered in mild cognitive impairment compared with normal cognition in sporadic Parkinson's disease. Front. Neurol. 11, 137.

Ren, X., Liu, L., Gamallat, Y., Zhang, B., Xin, Y., 2017. Enteromorpha and polysaccharides from enteromorpha ameliorate loperamide-induced constipation in mice. Biomed. Pharmacother. 96, 1075–1081.

Riezzo, G., Chimienti, G., Orlando, A., D'Attoma, B., Clemente, C., Russo, F., 2019. Effects of long-term administration of *Lactobacillus reuteri* DSM-17938 on circulating levels of 5-HT and BDNF in adults with functional constipation. Benef. Microbes 10 (2), 137–147.

Riezzo, G., Orlando, A., D'attoma, B., Guerra, V., Valerio, F., Lavermicocca, P., Russo, F., 2012. Randomised clinical trial: efficacy of *Lactobacillus paracasei*-enriched artichokes in the treatment of patients with functional constipation–a double-blind, controlled, crossover study. Aliment Pharmacol. Therapeut. 35 (4), 441–450.

Sharma, A., Rao, S.S., Kearns, K., Orleck, K.D., Waldman, S.A., 2021. Diagnosis, management and patient perspectives of the spectrum of constipation disorders. Aliment Pharmacol. Therapeut. 53 (12), 1250–1267.
Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85, 367–370.
Tian, P., Wang, G., Zhao, J., Zhang, H., Chen, W., 2019. *Bifidobacterium* with the role of 5-hydroxytryptophan synthesis regulation alleviates the symptom of depression and related microbiota dysbiosis. J. Nutr. Biochem. 66, 43–51.
Valerio, F., Russo, F., de Candia, S., Riezzo, G., Orlando, A., Lonigro, S.L., Lavermicocca, P., 2010. Effects of probiotic *Lactobacillus paracasei*-enriched artichokes on constipated patients: a pilot study. J. Clin. Gastroenterol. 44, S49–S53.
Varada, V.V., Panneerselvam, D., Pushpadass, H.A., Mallapa, R.H., Ram, C., Kumar, S., 2023. In vitro safety assessment of electrohydrodynamically encapsulated Lactiplantibacillus plantarum CRD7 and Lacticaseibacillus rhamnosus CRD11 for probiotics use. Curr. Res. Food Sci. 6, 100507.
Waller, P.A., Gopal, P.K., Leyer, G.J., Ouwehand, A.C., Reifer, C., Stewart, M.E., Miller, L. E., 2011. Dose-response effect of *Bifidobacterium lactis* HN019 on whole gut transit time and functional gastrointestinal symptoms in adults. Scand. J. Gastroenterol. 46 (9), 1057–1064.
Wang, L., Chai, M., Wang, J., Qiangqing, Y., Wang, G., Zhang, H., Chen, W., 2022. *Bifidobacterium longum* relieves constipation by regulating the intestinal barrier of mice. Food Funct. 13 (9), 5037–5049.
Wang, L., Yang, S., Mei, C., Tang, N., Wang, J., Yu, Q., Chen, W., 2023. *Lactobacillus paracasei* relieves constipation by acting on the acetic acid-5-ht-intestinal motility pathway. Foods 12 (22), 4176.
Wu, Y., Li, Q., Cao, J., Fan, F., Gan, L., Wu, R., Zhang, Z., 2023. Aged black tea alleviates constipation in mice by modulating intestinal neurotransmitters and decreasing AQP3 and AQP9 expression. Food Nutr. Res. 67, 9513.
Yang, J., Zhang, Z., Xie, Z., Bai, L., Xiong, P., Chen, F., Zhou, Y., 2022. Metformin modulates microbiota-derived inosine and ameliorates methamphetamine-induced anxiety and depression-like withdrawal symptoms in mice. Biomed. Pharmacother. 149, 112837.
Yu, T., Ding, Y., Qian, D., Lin, L., Tang, Y., 2023. Characteristics of fecal microbiota in different constipation subtypes and association with colon physiology, lifestyle factors, and psychological status. Therapeut. Adva. Gastroenterol. 16, 17562848231154101.
Yue, F., Zeng, X., Wang, Y., Fang, Y., Yue, M., Zhao, X., Chen, T., 2024. *Bifidobacterium longum* SX-1326 ameliorates gastrointestinal toxicity after irinotecan chemotherapy via modulating the P53 signaling pathway and brain-gut axis. BMC Microbiol. 24 (1), 8.
Yun, Q., Wang, S., Chen, S., Luo, H., Li, B., Yip, P., Tang, J., 2024. Constipation preceding depression: a population-based cohort study. EClinicalMedicine 67.
Zhang, C., Wang, L., Liu, X., Wang, G., Guo, X., Liu, X., Chen, W., 2023. The different ways multi-strain probiotics with different ratios of *bifidobacterium* and *lactobacillus* relieve constipation induced by loperamide in mice. Nutrients 15 (19), 4230.
Zhang, S., Wang, R., Li, D., Zhao, L., Zhu, L., 2021. Role of gut microbiota in functional constipation. Gastroenterol. Rep. 9 (5), 392–401.
Zhang, X., Zheng, J., Jiang, N., Sun, G., Bao, X., Kong, M., Liu, H., 2021. Modulation of gut microbiota and intestinal metabolites by lactulose improves loperamide-induced constipation in mice. Eur. J. Pharmaceut. Sci. 158, 105676.
Zhu, L., Liu, W., Alkhouri, R., Baker, R.D., Bard, J.E., Quigley, E.M., Baker, S.S., 2014. Structural changes in the gut microbiome of constipated patients. Physiol. Genom. 46 (18), 679–686.