Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d5fo02451j

Lactobacillus plantarum RG-034 colonic soluble capsules alleviate functional constipation in rats by promoting short-chain fatty acid generation

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Functional constipation is a chronic gastrointestinal disorder that significantly impacts quality of life and healthcare costs. Current treatments have limitations such as self-management difficulty and intestinal damage. This study evaluated the efficacy of *Lactobacillus plantarum* RG-034, a probiotic delivered *via* colonic soluble capsules, in alleviating loperamide-induced functional constipation in rats. Our findings demonstrate that 28-day RG-034 administration exhibited no significant organ toxicity. Pharmacodynamic results indicated that RG-034 improved stool frequency, characteristics, and water content and accelerated intestinal transit in constipated rats, along with enhancing colonic contraction and relaxation tension in isolated colons. Moreover, RG-034 promoted the proliferation of interstitial cells of Cajal (ICCs) and enhanced cellular calcium ion uptake by upregulating the PI3K/AKT pathway. Additionally, RG-034 positively modulated gut microbiota by increasing the abundance of *Muribaculaceae* and promoting short-chain fatty acid (SCFA) production. Co-culturing RG-034 with rat feces *in vitro* demonstrated a significant increase in SCFAs in the supernatant. Subsequent exposure of primary ICCs to this co-culture supernatant indicated that RG-034 facilitates calcium ion influx and promotes cell proliferation, an effect that was reversible with the application of PI3K inhibitors. These findings suggest that *Lactobacillus plantarum* RG-034 colonic soluble capsules enhance SCFA synthesis to promote PI3K/AKT phosphorylation, which in turn stimulates ICC proliferation and intestinal relaxation and contraction, thereby alleviating constipation symptoms.

Received 11th June 2025,
Accepted 13th March 2026

DOI: 10.1039/d5fo02451j

rsc.li/food-function

Introduction

Functional constipation (FC), a chronic disorder characterized by reduced bowel frequency, difficult defecation, and incomplete evacuation, affects approximately 14.0% of adults and 9.5% of children globally.[1–3] In the clinical management of constipation, regardless of different etiologies, osmotic laxatives (*e.g.*, polyethylene glycol, lactulose), stimulant laxatives (*e.g.*, sennosides, bisacodyl and castor oil), guanosine cyclase-C agonists (*e.g.*, linaclotide), prokinetic agents (*e.g.*, the 5-HT4 receptor agonist prucalopride), and stool softeners (*e.g.*, mineral oil) are routinely employed.[1,4,5] However, these therapies are often associated with adverse effects, including abdominal pain or cramping, nausea, vomiting, flatulence, diarrhea, urgency and fecal incontinence, which may limit their tolerability and long-term utility.[6,7]

Growing evidence highlights the critical role of gastrointestinal microbiota in intestinal function, particularly the effects of probiotics on gut motility and constipation. One study demonstrated that patients with FC exhibit reduced abundances of beneficial bacteria (*e.g.*, *Lactobacillus*, *Enterococcus*, and *Bacteroides* species) alongside increased colonization by potential pathogenic bacteria such as *Escherichia coli* and *Klebsiella* species.[8] The gut microbiota influences colonic physiology through short-chain fatty acids (SCFAs) and other metabolites derived from dietary fiber fermentation, which serve as a primary energy source for colonic epithelial cells.[9] SCFAs influence colonic motility by mediating the secretion of glucagon-like peptide-1 (GLP-1) and regulating the biosynthesis of 5-hydroxytryptamine (5-HT).[10] Additionally, the production of SCFAs leads to a decrease in intestinal pH, which shortens the colonic transit time, thereby helping to alleviate constipation.[11] Therefore, modulation of the gut microbes offers a promising approach for the treatment of FC.

Probiotics are an effective, low-risk strategy for relieving constipation by balancing gut microbiota and reducing drug dependence.[12] *Lactobacillus plantarum* (*L. plantarum*) has

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gained global attention for its probiotic properties, including antimicrobial, anti-inflammatory, and microbiota-regulating effects.[13] Specific strains like *L. plantarum* KSFY06 and KFY02 have improved fecal water content, intestinal transit, and defecation time in constipated mice.[14,15] *L. plantarum* YS2 isolated from yak yogurt attenuated activated charcoal-induced constipation and promoted gastrointestinal motility in mice,[16] while *L. plantarum* NCU116 promoted intestinal motility, increased fecal water content, and reduced colonic inflammation.[17] Consequently, *L. plantarum* may be a potential therapeutic treatment for FC.

To confer health benefits, probiotics must maintain viability in products and survive transit to the colon.[18] A daily intake of 10^8–10^9 CFU probiotic microorganisms is crucial for achieving probiotic action in the human organism.[19] However, environmental stress factors during processing and storage, such as oxygen exposure, temperature fluctuations, and humidity, often compromise the viability of the bacteria.[20] Furthermore, probiotics must exhibit acid tolerance to withstand gastric conditions and adhere to intestinal epithelial cells to sustain their activity. Encapsulation plays a crucial role in preserving probiotic functionality by protecting strains from these challenges. In this study, *L. plantarum* RG-034, isolated from healthy human feces, was lyophilized with cryoprotectants into a powdered form and encapsulated in colon-targeted, acid-resistant capsules designed to resist gastric dissolution and release probiotics specifically in the colon, enhancing delivery and colonization efficiency. However, the therapeutic potential of *L. plantarum* RG-034 capsules for constipation and their regulatory effects on gut microbiota remain unexplored.

This study investigated the effects of *L. plantarum* RG-034 colonic soluble capsules on alleviating loperamide-induced constipation symptoms in rats, as well as its role in enhancing colonic motility. Subsequent experiments using primary cultured interstitial cells of Cajal (ICCs) demonstrated that RG-034 promotes the generation of SCFAs, which in turn activates the PI3K/AKT signaling pathway, thereby enhancing ICC proliferation and calcium ion influx. The findings of this study provide a theoretical basis for considering RG-034 colonic soluble capsules as a potential therapeutic agent for functional constipation.

Materials and methods

L. plantarum RG-034 colonic soluble capsule preparation

L. plantarum RG-034 was isolated from healthy human fecal samples and inoculated into De Man, Rogosa and Sharpe (MRS) medium for anaerobic fermentation at a constant temperature of 37 °C. Following cultivation, the bacterial cells were harvested by centrifugation, and the resulting pellet was mixed with a defined formulation of lyoprotectants. The mixture was homogenized, milled, and sieved to obtain *L. plantarum* RG-034 powder.

For capsule preparation, a gelatin-based solution was used to form the primary shell, which was subsequently coated with colonic solution, air-dried, and encapsulated to produce colon-targeted capsules. A prescribed quantity of *L. plantarum* RG-034 powder, pregelatinized starch, and magnesium stearate were uniformly blended and filled into the colon-targeted capsules to produce *L. plantarum* RG-034 colonic soluble capsules. Three dosage strengths were prepared, containing viable bacterial counts of 1×10^9, 1×10^8, and 1×10^7 CFU per capsule, respectively. All capsules were stored at temperatures below 25 °C to ensure stability.

Animals

Six-week-old Sprague-Dawley (SD) rats were obtained from Hangzhou Medical College and housed at the Experimental Animal Center of Southeast University under controlled conditions (23 ± 1 °C, 45%–65% humidity, 12 h light–dark cycle). All procedures were approved by the Animal Care and Use Committees of China Pharmaceutical University (approval no. 202306008). Rats were acclimatized for one week before experiments.

Animal treatment

To assess the safety of *L. plantarum* RG-034, rats were divided into two groups (n = 10 per group, 5 males and 5 females): a control group and an RG-034 group. The RG-034 group received daily oral gavage of *L. plantarum* RG-034 solution (4×10^{11} CFU in purified water), while the control group was administered an equivalent volume of purified water for 28 consecutive days. Throughout the experimental period, general behavior, physical appearance, and potential adverse reactions were monitored daily. Blood samples were collected for comprehensive hematological and biochemical analyses, and urine samples were subjected to routine urinalysis to evaluate the impact of *L. plantarum* RG-034 on physiological parameters following the final administration.

In the pharmacodynamic evaluation, rats were randomly assigned to two primary groups: a control group (n = 10, 5 males and 5 females) and a model group (n = 50, 25 males and 25 females). The control group received daily oral administration of purified water (5 mL kg^{-1}), while the model group was induced with constipation *via* oral loperamide suspension (3.0 mg kg^{-1}) for 3 weeks. The administration of loperamide is a widely used protocol for inducing constipation in rats.[21] Starting from day 7 of loperamide treatment, the model group was further subdivided into five subgroups (n = 10, balanced sex distribution): model subgroup (constipation-induced, no treatment), lactulose group (2.01 mg kg^{-1}, positive control) and *L. plantarum* RG-034 colonic soluble capsule (2×10^9, 2×10^8, 2×10^7 CFU, respectively) groups. Each capsule was formulated to deliver 10^9, 10^8, and 10^7 CFU per dose, administered twice daily. All treatments were delivered *via* oral gavage for 2 weeks.

ICC isolation and cultivation

Primary ICCs were isolated from rat colonic tissue following the method studied by Zhou *et al.*[22] The colon tissue was washed with D-Hank's solution (G4203, Servicebio) to remove

intestinal contents, and the mucosal and submucosal layers were carefully stripped. The tissue was then cut into 1–2 mm^3 pieces and digested with 2 mL of type II collagenase (2 mg mL^{-1}, C8150, Solarbio) and 2 mL of 0.25% trypsin (BL512A, Biosharp) at 37 °C for 50 min. The digestion was terminated by adding M199 medium (KGL1401, KeyGEN) supplemented with 10% FBS (AB-FBS-05005, ABW). The resulting cell suspension was filtered through a 75 μm mesh and centrifuged. After resuspension, the cells were cultured in M199 medium in 25 mm^2 flasks and maintained in a 5% CO_2 incubator at 37 °C. ICCs from the control, model and RG-034 (2×10^9, 2×10^8, 2×10^7 CFU) groups were cultured in M199 medium. The cells were then collected for immunofluorescence staining and western blotting.

Feces co-culture with *L. plantarum* RG-034 *in vitro*

Five grams of healthy rat feces were collected and mixed with 10 mL purified water, vortexed, autoclaved, and divided into two groups: control and *L. plantarum* RG-034 solution groups. The latter received 1.5×10^8 CFU of RG-034 and was incubated at 37 °C for 24 h. The fecal supernatant was subjected to SCFA analysis and co-incubated with ICCs after centrifugation.

ICC treatment

Rat primary ICCs were extracted and seeded in 24-well plates (1.5×10^5 cells per mL), and cultured for approximately one week until maturation. LY294002, a specific inhibitor of PI3K, was used for further verification. The cells were divided into PBS, fecal supernatant, RG-034 fecal supernatant, LY294002 and RG-034 fecal supernatant plus LY294002 groups. The RG-034 fecal supernatant group received 400 μL of RG-034 fecal supernatant, while equal volumes of the control fecal supernatant and PBS were added to the fecal supernatant group and PBS group, respectively. LY294002 was set at 10 μmol L^{-1}, which was based on previously published articles.[23] In the LY294002 and RG-034 fecal supernatant plus LY294002 groups, cells were pre-incubated with LY294002 for 12 h prior to treatment with either PBS or the RG-034 fecal supernatant. All groups were incubated in a CO_2 incubator for 6 h prior to subsequent analysis.

Blood and urine parameter analysis

On day 28 of dosing, all rats were fasted overnight with free access to water. One milliliter of whole blood was drawn from the common carotid artery into a tube containing the anticoagulant EDTA-K2. Additionally, 1.5 mL of whole blood was collected into non-anticoagulant tubes and centrifuged to isolate serum. Rat urine was collected overnight using metabolic cages. Whole blood parameters were measured using an automated hematology analyzer (ADVIA 2120, Siemens, Germany). Serum biochemical parameters were measured with an automated clinical chemistry analyzer (Dimension Xpand Plus, Siemens, Germany) using commercially available diagnostic kits. Urinalysis was conducted *via* dipstick testing and an automated urine chemistry analyzer (URIT 200B, Urit, China).

Hemolysis assay

L. plantarum RG-034 was anaerobically cultured in MRS broth at 37.0 °C for 24 h, while *Staphylococcus aureus* (positive control) was aerobically cultured in tryptic soy broth under the same conditions. After incubation, bacterial suspensions of RG-034 and *Staphylococcus aureus* were streaked onto Columbia blood agar plates and incubated at 37 °C for 24 h to assess hemolytic activity. The lack of a hemolytic ring indicated a negative result for hemolysis, while a grass-green ring suggested α-hemolysis positivity, and a clear ring was indicative of β-hemolysis positivity.[24]

Organ index

The rats were sacrificed and the hearts, livers, spleens, lungs and kidneys were obtained and weighed. The organ index was detected according to the formula: organ index = organ weight (g)/body weight (g) × 100%.

Intestinal motility detection

Following administration on days 3, 7, and 14, rats were orally administered 1 mL of 10% activated charcoal. Each rat was then transferred to a clean individual cage for assessment of stool numbers, Bristol's stool scale, stool water content, and intestinal transport time.

Stool numbers. The number of stool pellets excreted by each rat in 10 h was recorded.

Bristol's stool scale. Stool consistency was scored using the Bristol stool form scale based on cohesion and surface cracking: 1, separate hard blocks (such as nuts); 2, sausage shaped, but with blocks; 3, like sausage, but with cracks on the surface; 4, like sausage, smooth and soft; 5, soft spots, with transparent cutting edges; 6, paste, with uneven edges; 7, no solid, liquid-like water.[25]

Stool water content. All feces excreted by the rats in 10 h were collected to record wet weight, and then dried at 60 °C for 12 h. The stool water content of the feces was calculated using the following formula: water content = (wet weight − dry weight)/wet weight × 100%.[26]

Intestinal transport time. The time taken to defecate the first black stool was recorded. The time limit for recording was 12 h, with records longer than 12 h being recorded as 12 h.

Intestinal transit rate. On day 14 of administration, rats were euthanized, and the total intestinal length (from pylorus to cecum) and the distance traveled by activated charcoal were measured. Intestinal transit rate = distance traveled by activated charcoal (cm)/total intestinal length (cm) × 100%.[27]

Contraction and relaxation tone of isolated colon

The colonic contraction and relaxation detection method was slightly modified from that previously described by Wei *et al.*[28] Rats were euthanized, and a 3 cm segment of the colon was excised and placed in oxygenated Tyrode's solution. The colon was flushed and oxygenated at 37 °C. Both ends were sutured, with the upper suture connected to a tension sensor and the lower end secured to the bath. After contraction stabilized,

1 μmol L^{-1} acetylcholine was added, and changes in contraction were recorded. Following the addition of 1 μmol L^{-1} epinephrine, relaxation changes were observed and recorded.

Hematoxylin and eosin (H&E) staining

The heart, liver, spleen, lungs, kidneys, testes, ovaries and colon were excised and fixed in 4% paraformaldehyde. Tissues were processed according to standard protocols, including sampling, paraffin embedding, sectioning, and H&E staining, followed by coverslipping. Morphological features of each organ were examined under a light microscope. Colon histopathological changes were evaluated using the scoring system established by Chiu *et al*.[29] Pathologic scoring criteria were as follows: 0, normal mucosal villi; 1, development of sub-epithelial Gruenhagen's gap at the apex of the villus, often with capillary congestion; 2, elevation of the epithelial layer from the lamina propria and dilatation of the sub-epithelial gap; 3, massive intestinal mucosal epithelium elevated, with villi inverted to the sides, and some of the apical portions of the villi detached; 4, detached villi with lamina propria and dilated capillaries exposed, and increased cellularity of lamina propria can be noted; 5, digestion and disintegration of lamina propria accompanied by hemorrhage and ulceration.

Quantitative real-time PCR (qPCR)

***L. plantarum* concentration.** *L. plantarum* standard reference (6×10^{10} CFU) was dissolved in 2.4 mL sterile water, and DNA was extracted using the genomic DNA extraction kit (DP304, Tiangen). The DNA was diluted 500-fold and serially diluted 10-fold to create eight concentrations (ranging from 2×10^{-3} to 2×10^{-10}), which served as templates for qPCR amplification. The DNA from each dilution was amplified according to the qPCR reaction system and conditions. A standard curve was constructed by plotting the Ct values on the vertical axis and the logarithmic concentrations on the horizontal axis. DNA from the samples was extracted using the same kit and amplified under identical qPCR conditions: an initial cycle at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and a final cycle at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Target analyte concentrations were calculated using Ct values and the standard curve. The primer sequences used were: *L. plantarum* (forward: 5′-AGCTTGAAAGATGGCTTCGG-3′, reverse: 5′-GGTCGGCTACGTATCATTGC-3′).

C-kit expression. Total RNA was extracted from rat colon tissues using TRIzol reagent (R401-01, Vazyme). RNA concentration and purity were measured with an ultramicrospectrophotometer (Nano-100, Allsheng), and RNA was diluted to equal concentrations with DEPC water. Reverse transcription was performed using the HiScript® III RT SuperMix kit (R323, Vazyme). The cDNA was then subjected to the quantitative reverse-transcription polymerase chain reaction using the ChamQ SYBR qPCR Maser Mix (R331, Vazyme) and real-time PCR systems (QuantStudio 3, Thermo Fisher Scientific). The qPCR conditions were the same as those described in section "*L. plantarum* concentration". Target mRNA levels were normalized to β-actin. The primer sequences were as follows: C-kit (forward: 5-CTGTTGCAGCCAGTTCCCTTA-3, reverse: 5-AGTGCCATTGCTGTCCATTG-3); β-actin (forward: 5-GAAGATCAAGATCATTGCTCCT-3, reverse: 5-TACTCCTGCTTGCTGATCCA-3). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

16S rRNA high-throughput sequencing

Genomic DNA was extracted from rat feces as described previously by Shi *et al*., with a few modifications.[30] The rats were euthanized after the last administration, and about 8 cm of the colon was removed, and the contents were scooped out with a sterile scalpel for the determination of intestinal flora. The sampling process was done at 4 °C and immediately stored at −80 °C. DNA of the intestinal contents was extracted and quantified using Nanodrop and the quality of DNA extraction was checked by 1.2% agarose gel electrophoresis. The V4–V5 region of the bacterial 16S rRNA gene was amplified by the polymerase chain reaction, and the amplified product was recovered by magnetic bead purification. Subsequently, the PCR amplification and recovery products were subjected to fluorescence quantification using the Quant-iT PicoGreen dsDNA assay kit. According to the results of fluorescence quantification, each sample was mixed in an appropriate proportion according to the sequencing amount required for each sample. Illumina's TruSeq Nano DNA LT Library prep kit was used to prepare sequencing libraries for high-throughput sequencing. Bioinformatics analysis was conducted on the platform provided by Suzhou PANOMIX Biomedical Tech Co., Ltd.

SCFA assay

SCFA concentration in colonic contents. Colonic contents were collected, weighed, and thoroughly mixed with water and glass beads. Subsequently, 200 μL of supernatant was collected by centrifugation (12 000*g*, 10 min, 4 °C). To the supernatant, 100 μL of 15% phosphoric acid, 20 μL of 375 μg mL^{-1} internal standard (4-methylpentanoic acid), and 280 μL of diethyl ether were added, followed by mixing for 1 min. The supernatant was again collected by centrifugation (12 000*g*, 10 min, 4 °C) and analyzed by gas chromatography-mass spectrometry (GC-MS) with an Agilent HP-INNOWAX capillary column (30 m × 0.25 mm ID × 0.25 μm). GC-MS conditions included a split injection (1 μL, 10 : 1 split ratio), an injector temperature at 250 °C, an ion source at 300 °C, and a transfer line at 250 °C. The temperature program started at 90 °C, increased to 120 °C at 10 °C min^{-1}, and then to 150 °C at 5 °C min^{-1}, and finally to 250 °C at 25 °C min^{-1}, holding for 2 min. Helium was used as the carrier gas (1.0 mL min^{-1}). Analysis was conducted on a Thermo ISQ 7000 mass spectrometer (Thermo Fisher Scientific, USA) with electron impact ionization at 70 eV in selected ion monitoring mode.

SCFA concentration in the fecal supernatant. SCFAs in the fecal supernatant were detected using a gas chromatography (GC) system (PerkinElmer Clarus 680, PerkinElmer, USA) as previously reported, with a few modifications.[31] A standard curve was prepared using 2-ethylbutyric acid as the internal

standard (22.33 mg mL^{-1}), and *n*-butyric acid was used as the standard (23.74 mg mL^{-1}). Methanol was added to the supernatant and the supernatant was collected after centrifugation. A 200 μL sample was mixed with 4 μL of the internal standard solution for butyric acid detection. GC analysis was conducted using an HP-5 MS column (30 m × 0.25 mm × 0.25 μm) and a hydrogen flame ionization detector (FID), with a carrier gas split ratio of 100 : 1. The temperature program was as follows: 90 °C for 1 min, increased to 150 °C at 7.5 °C min^{-1}, held for 1 min, and then increased to 180 °C at 4.5 °C min^{-1} and held for 2 min.

Calcium ion concentration

The ICCs were incubated with 2 μmol L^{-1} Fluo-4 AM (S1060, Beyotime) for 30 min, and then washed three times with PBS for 5 min each time. The fluorescence intensity of the cells was then observed using a fluorescence microscope (MF52-N, MSHOT, China).

Immunofluorescence staining

Colon tissue was sectioned into 4 μm-thick slices embedded with paraffin, which were then deparaffined and rehydrated. Subsequently, the sections were boiled in a pH 8.0 Tris–EDTA antigen repair solution (G1206, Servicebio) for 30 min to induce antigen retrieval. The primary ICCs were seeded in glass bottom cell culture dishes (801002, Nest), and the cells were incubated with Edu (KTA2030, Abbkine) for 5 h. Then, the cells were fixed with 4% paraformaldehyde. The sections or cells were blocked with 8% goat serum (C0265, Beyotime) for 2 h and then incubated at 4 °C overnight with antibodies: anti-C-kit (1 : 100, WL00125, Wanleibio) and anti-ki67 (1 : 100, TW0001, Abmart), followed by incubation for 2 h with a secondary antibody: Cy3-conjugated secondary antibody (1 : 400, AS007, ABclonal) for 1 h at 25 °C in the dark. Cells underwent a Click-iT reaction according to the reagent kit, and then the sections and cells were mounted with mounting medium with DAPI (P0131, Beyotime).

Western blotting

Tissues and cells were lysed using radio immunoprecipitation assay (RIPA) buffer (P0013B, Beyotime) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (ST506, Beyotime). The lysate was then centrifuged at 12 000*g* for 10 min at 4 °C to collect the total protein in the supernatant. Protein concentration was determined using a BCA kit (FD2001, Fude Biological Technology). The protein was diluted to an equal concentration with PBS and boiled with 5× loading buffer (B1012, Applygen) for 10 min. Proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (IPVH00010, Millipore). After blocking with 5% degrease milk for 2 h, the membranes were incubated overnight at 4 °C with the following primary antibody: anti-PI3K (1 : 2000, A4992, Abclonal), anti-AKT (1 : 4000, ab179463, Abcam), anti-p-PI3K (1 : 1000, AP0854, Abclonal), anti-p-AKT (1 : 4000, ab81283, Abcam) and anti-GAPDH (1 : 4000, AF0721, Affinity). After washing with TBST (G0004, Servicebio), the membrane was incubated for 2 h with a horseradish peroxidase (HRP)-conjugated secondary antibody: goat anti-rabbit IgG (1 : 5000, AS014, ABclonal). Membranes were treated with enhanced chemiluminescence (ECL) reagents (K1231, APExBIO) and visualized with a Tanon Chemi Dog 5200T.

Statistical analysis

Data analysis and processing were performed using ImageJ and SPSS 26.0. For *in vivo* safety assessment, intergroup differences were compared using an independent samples *t*-test. Homogeneity of variance was assessed by Levene's test, and two-tailed tests were applied for statistical significance. For other experiments, one-way ANOVA followed by a *post hoc* LSD test (homogeneous variance) or the Games–Howell test (heterogeneous variance) was introduced for comparisons between groups. A p-value <0.05 was considered statistically significant.

Results

L. plantarum RG-034 has no evident toxicity on the main organs of rats

There were no abnormalities observed in food intake, water consumption, behavioral activity, or mental state among the rats throughout 28 days of continuous oral gavage administration with *L. plantarum* RG-034 (4×10^{11} CFU). No significant differences were observed in body weight (Fig. 1A) and organ indices as well (Fig. 1B) between the *L. plantarum* RG-034 (4×10^{11} CFU) group and the control group. *L. plantarum* primarily colonized the colon (approximately 3.8×10^4 CFU per g), with no bacteria detected in the blood or major organs such as the heart, liver, spleen, lungs, and kidneys. H&E staining revealed intact histological structures in the aforementioned major organs of the RG-034-treated rats, showing no abnormalities compared to the control group. Moreover, testicular and ovarian development were normal, indicating an absence of reproductive toxicity (Fig. 1C). In blood agar assays, *Staphylococcus aureus* exhibited β-hemolysis, whereas *L. plantarum* RG-034 formed no hemolytic rings (Fig. 1D).

Hematological assessments revealed that all parameters remained stable in the treated group compared to the control group, with only minor fluctuations in the mean corpuscular hemoglobin concentration (MCHC), while the mean erythrocyte hemoglobin level (MCH) showed no significant difference (Table 1). Biochemical analysis indicated transient decreases in creatinine, blood urea nitrogen, and triglycerides ($P < 0.05$), while other indicators reflecting hepatic and renal function remained within normal ranges (Table 2). Further urinalysis results indicated that all parameters remained within normal ranges, with negative urine protein detection (Table 3). Therefore, considering the normal results from urinalysis as well as liver and kidney tissue histopathological examinations, it is suggested that these abnormal parameters may lack significant clinical relevance. In summary, the comprehensive evaluation of

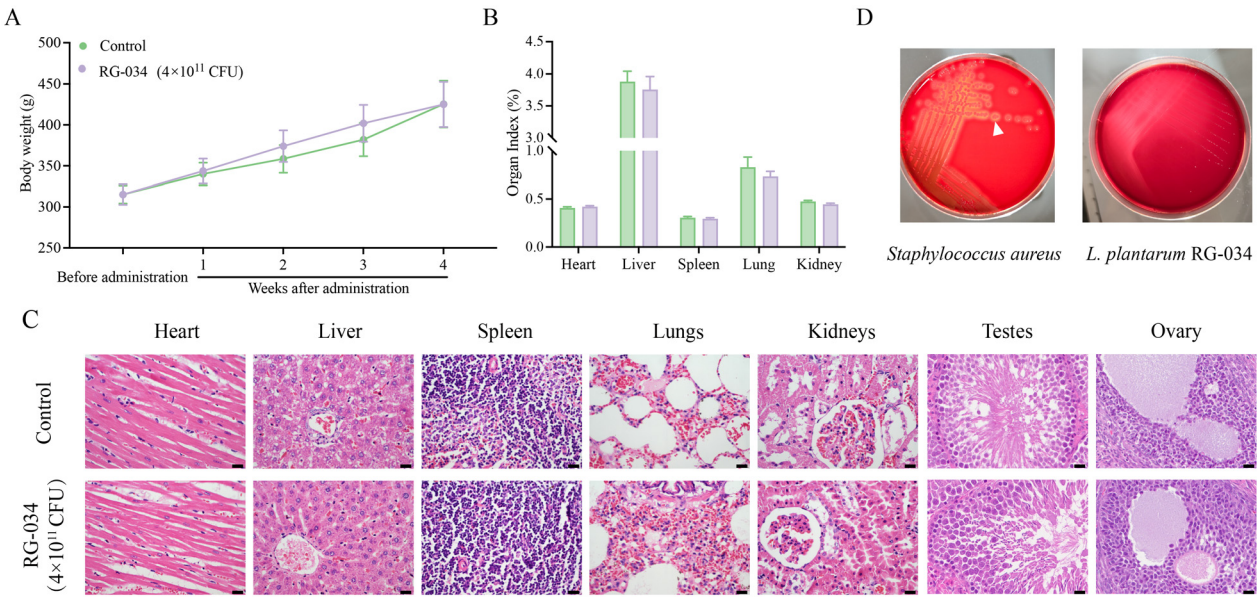

Fig. 1 Toxicity of *L. plantarum* RG-034 after the 28-day oral gavage (mean ± SEM). (A) The effect of RG-034 on the body weight of the rats, n = 10. (B) The effect of RG-034 on the organ index of the rats, n = 10. (C) The effect of RG-034 on the pathology of vital organs in the rats, n = 3, scale bar = 20 μm. (D) Hemolytic activity of RG-034 on blood agar plates, n = 3.

hematological, biochemical, urinary, and histopathological data supports the safety profile of RG-034 at the tested dose.

L. plantarum RG-034 colonic soluble capsules attenuate functional constipation in rats

Treatment with *L. plantarum* RG-034 significantly increased its concentration in the colon, indicating successful colonization, whereas no colonization was observed in the colons of rats not treated with RG-034 (Fig. 2A). On the third day of administration, the number of stools significantly increased in the RG-034 (2×10^9 and 2×10^7 CFU) groups compared with the model group. By day 7, all treatment groups exhibited elevated Bristol stool scores, with the RG-034 (2×10^9 CFU) group showing a notable increase in stool number. By day 14, all treatment groups demonstrated improvements in Bristol stool scores, with the RG-034 (2×10^9 CFU) group showing the most pronounced increase in water content (Fig. 2B–D). H&E staining revealed that the model group exhibited damaged colon structure, dilated submucosal space, injured intestinal villi, and reduced muscle thickness, consistent with the literature. The RG-034 (2×10^9 CFU) group showed restored muscle thickness and submucosal space morphology, indicating reduced colon lesions and lower pathological scores (Fig. 2E and F).

In addition to the fecal parameters, intestinal motility was assessed. Constipated rats prior to dosing failed to pass stool within 12 h. Rats in the RG-034 (2×10^9 CFU) group exhibited earlier first black stool defecation times on days 3, 7, and 14 post-administration compared to the model group (Fig. 3A). All treatment groups exhibited significantly higher intestinal transit rates than the model group (Fig. 3B). Isolated colon segments were analyzed for contraction and relaxation tension under acetylcholine and adrenaline treatments. The model group showed significantly reduced colon contraction and relaxation tension compared to the control group. The RG-034 (2×10^9 CFU) and RG-034 (2×10^8 CFU) groups demonstrated increased contraction tension, while RG-034 (2×10^9 CFU) also exhibited enhanced relaxation tension (Fig. 3C–E).

L. plantarum RG-034 colonic soluble capsules promote proliferation of ICCs in the colons of functional constipation rats

ICCs, known as intestinal "pacemaker cells", generate electrical signals that initiate and coordinate digestive muscle contractions, playing a vital role in intestinal motility and digestive function. The expression of C-kit mRNA, which encodes a marker protein of ICCs, was significantly higher in the RG-034 (2×10^9 CFU) group than in the model group (Fig. 4A). Additionally, RG-034 increased ICC proliferation, as evidenced by higher $Ki67^+$/$C\text{-}kit^+$ cell counts in the RG-034 (2×10^9 CFU) group (Fig. 4B and C). Moreover, research has shown that ICCs express molecules and proteins related to the PI3K/AKT signaling pathway which can promote cell proliferation by phosphorylating and regulating cell cycle regulatory proteins.[32] Western blot analysis revealed that PI3K/AKT signaling was activated in the colons of the RG-034 treatment group (Fig. 4D–F). These findings suggest that RG-034 potentially enhances gastrointestinal motility by promoting ICC proliferation through upregulating PI3K/AKT phosphorylation.

L. plantarum RG-034 colonic soluble capsules facilitate cell proliferation and Ca^{2+} uptake of primary ICCs

To investigate the effect of RG-034 on ICC proliferation *in vitro*, rats were gavaged with RG-034 colonic soluble capsules for two weeks, followed by extraction of primary ICCs. Given that Ca^{2+} influx is a key mechanism for slow-wave initiation in ICCs,

Table 1 The effect of RG-034 on the hematological parameters of the rats

Parameters	Control	RG-034
WBC (10^9 L^{-1})	4.58 ± 0.62	3.49 ± 0.73
RBC (10^{12} L^{-1})	7.53 ± 0.26	6.83 ± 0.34
HGB (g dL^{-1})	13.28 ± 0.49	12.15 ± 0.63
HCT (%)	40.31 ± 1.55	37.5 ± 2.02
MCV (fL)	53.54 ± 0.32	54.84 ± 0.54
MCH (pg)	17.66 ± 0.12	17.8 ± 0.2
MCHC (g dL^{-1})	32.94 ± 0.11	32.46 ± 0.2*
PLT (10^9 L^{-1})	1013.6 ± 45.18	936 ± 66.67
NEU (%)	18.3 ± 5.18	18.11 ± 4.22
LYM (%)	73.85 ± 5.38	73.67 ± 4.59
MONO (%)	2.31 ± 0.4	2.23 ± 0.33
EOS (%)	3.56 ± 0.41	3.28 ± 0.23
BASO (%)	0.3 ± 0.04	0.31 ± 0.04
#NEUT (10^9 L^{-1})	0.88 ± 0.31	0.85 ± 0.47
#LYMP (10^9 L^{-1})	3.35 ± 0.53	2.34 ± 0.29
#MONO (10^9 L^{-1})	0.11 ± 0.02	0.07 ± 0.01
#EOS (10^9 L^{-1})	0.17 ± 0.04	0.12 ± 0.03
#BASO (10^9 L^{-1})	0.01 ± 0	0.01 ± 0
CHCM (g dL^{-1})	34.95 ± 0.22	34.97 ± 0.29
RDW (%)	11.5 ± 0.13	11.87 ± 0.22
HDW (g dL^{-1})	2.69 ± 0.06	2.58 ± 0.02
MPV (fL)	11.95 ± 0.21	11.75 ± 0.33
LUC (%)	1.68 ± 0.41	2.4 ± 0.51

Data are presented as mean ± SEM (n = 10). *P < 0.05 *vs.* the control. WBC: white blood cell count, expressed in 10^9 cells per liter (10^9 L^{-1}); RBC: red blood cell count, expressed in 10^{12} cells per liter (10^{12} L^{-1}); HGB: hemoglobin, expressed in grams per deciliter (g dL^{-1}); HCT: hematocrit, expressed as a percentage (%); MCV: mean corpuscular volume, expressed in femtoliters (fL); MCH: mean corpuscular hemoglobin, expressed in picograms (pg); MCHC: mean corpuscular hemoglobin concentration, expressed in grams per deciliter (g dL^{-1}); PLT: platelet count, expressed in 10^9 cells per liter (10^9 L^{-1}); NEU%: percentage of neutrophils; LYM%: percentage of lymphocytes; MONO%: percentage of monocytes; EOS%: percentage of eosinophils; BASO%: percentage of basophils; #NEUT: neutrophils expressed in 10^9 cells per liter (10^9 L^{-1}); #LYMP: lymphocytes expressed in 10^9 cells per liter (10^9 L^{-1}); #MONO: monocytes expressed in 10^9 cells per liter (10^9 L^{-1}); #EOS: eosinophils expressed in 10^9 cells per liter (10^9 L^{-1}); #BASO: basophils expressed in 10^9 cells per liter (10^9 L^{-1}); CHCM: cellular hemoglobin concentration mean, expressed in grams per deciliter (g dL^{-1}); RDW: percentage of red cell distribution width; HDW: hemoglobin distribution width, expressed in grams per deciliter (g dL^{-1}); MPV: mean platelet volume, expressed in femtoliters (fL); LUC: percentage of large unstained cells.

Table 2 The effect of RG-034 on the serum biochemical analysis of the rats

Parameters	Control	RG-034
ALP (U L^{-1})	137.1 ± 9.36	108.6 ± 9.88
ALB (g L^{-1})	12.17 ± 0.42	12.66 ± 0.36
GGT (U L^{-1})	6.18 ± 0.77	6.07 ± 0.6
CHOL (mm L^{-1})	1.47 ± 0.12	1.69 ± 0.08
CREA (μm L^{-1})	46.04 ± 3.87	30.96 ± 3.56*
TBI (μm L^{-1})	2.37 ± 0.13	2.48 ± 0.19
TGL (mm L^{-1})	0.63 ± 0.11	0.22 ± 0.03**
GLUC (mm L^{-1})	12.65 ± 1.09	9.9 ± 0.83
AST (U L^{-1})	78.2 ± 7.18	79.6 ± 6.1
ALT (U L^{-1})	42.6 ± 3.71	41.8 ± 6.31
BUN (mm L^{-1})	8.16 ± 0.3	5.93 ± 0.35**
CKI (U L^{-1})	1318.6 ± 281.79	1052.2 ± 344.99
TP (g L^{-1})	56.28 ± 0.96	58.48 ± 1.26
Na (mm L^{-1})	164.38 ± 8.13	148.03 ± 7.44
K (mm L^{-1})	5.42 ± 0.42	4.38 ± 0.35
Cl (mm L^{-1})	127.19 ± 7.24	114.32 ± 6.52

Data are presented as mean ± SEM (n = 10). **P < 0.01 and *P < 0.05 *vs.* the control. ALP: alkaline phosphatase; ALB: albumin; GGT: gamma-glutamyl transferase; CHOL: cholesterol; CREA: creatinine; TBI: total bilirubin; TGL: triglycerides; GLUC: glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; BUN: blood urea nitrogen; CKI: creatine kinase; TP: total protein; Na: sodium; K: potassium; Cl: chloride.

Table 3 The effect of RG-034 on the urinalysis findings of the rats

Parameters	RG-034	
UWBC, *n* (%)	Negative	10 (100%)
UKet, *n* (%)	Negative	8 (80%)
	1+	2 (20%)
UpH	7.20 ± 0.27	
UNit, *n* (%)	Negative	10 (100%)
Uriglu, *n* (%)	Negative	10 (100%)
USG	1.02 ± 0.001	
UBld, *n* (%)	Negative	10 (100%)
UProt, *n* (%)	Negative	10 (100%)
UBil, *n* (%)	Negative	10 (100%)
URO, *n* (%)	Normal	10 (100%)
UVC, *n* (%)	Negative	9 (90%)
	2+	1 (10%)

Data are presented as mean ± SEM (n = 10). UWBC: urine white blood cell; UKet: urine ketone; UpH: urine pH; UNit: urine nitrite; Uriglu: urine glucose; USG: urine specific gravity; UBld: urine blood; UProt: urine protein; UBil: urine bilirubin; URO: urobilinogen; UVC: urine vitamin C.

intracellular calcium ion concentrations were measured using the Fluo-4 probe. The results showed a significantly lower intracellular calcium ion concentration in the model group compared to the controls, which was restored by RG-034 treatment (Fig. 5A and B). Immunofluorescence assessment of ICC proliferation revealed reduced Edu^+/C-kit^+ cells in the model group, which increased significantly after RG-034 administration (Fig. 5C and D). Western blot analysis confirmed that RG-034 activated the PI3K/AKT pathway in primary ICCs, as evidenced by elevated phosphorylation levels (Fig. 5E–G).

L. plantarum RG-034 colonic soluble capsules regulate gut microbiota and upregulate SCFAs in intestinal contents of functional constipation rats

According to the 16S gut microbiota sequencing results, the top 20 in terms of genus ranking were *Muribaculaceae*, *Ligilactobacillus*, *Alloprevotella*, *Blautia*, *Lactobacillus*, and others. The model group exhibited reduced levels of *Muribaculaceae*, *Alloprevotella*, and *Lactobacillus* post-modeling, while RG-034 treatment increased SCFA-producing bacteria such as *Muribaculaceae*, *Ligilactobacillus*, and *Alloprevotella* compared to the model group (Fig. 6A). The species accumulation curve indicated sufficient sample size, as the curve plateaued with increasing samples (Fig. 6B). The Simpson index was significantly lower in the model group, reflecting reduced diversity post-constipation (Fig. 6C). The diversity indices (Simpson, Shannon, Pielou-e, Faith_pd) in the RG-034 treated groups were slightly lower than those in the model group with

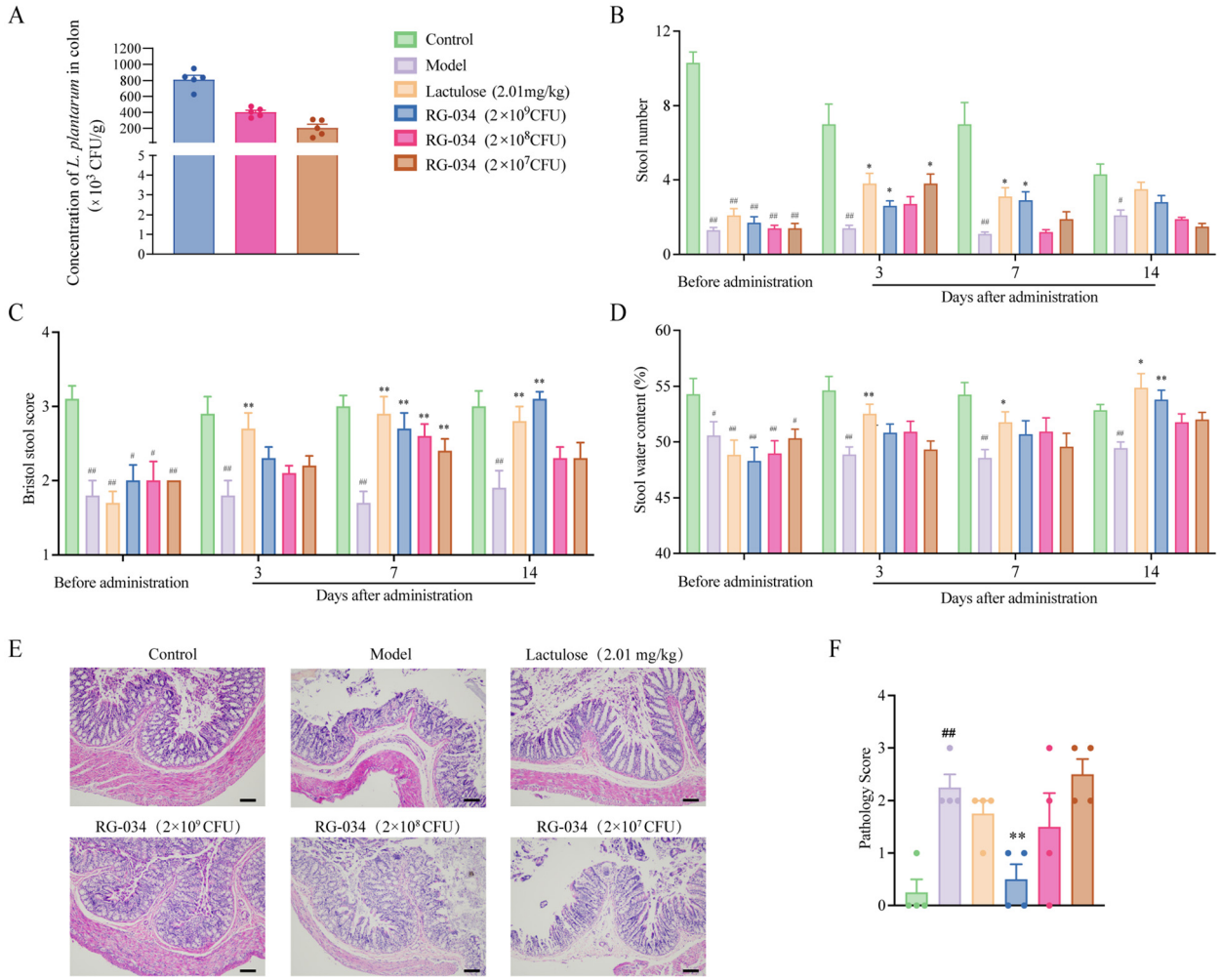

Fig. 2 RG-034 is enriched in the colon and improves functional constipation in the rats (mean ± SEM). (A) The *L. plantarum* colonization concentration in the rat colons, $n = 5$. (B) The effect of RG-034 on the stool number in 10 h, $n = 10$. (C) The effect of RG-034 on the Bristol stool score, $n = 10$. (D) The effect of RG-034 on the stool water content, $n = 10$. (E) The effect of RG-034 on the colonic pathological changes, $n = 4$, scale bar = 100 μm. (F) Statistical analysis of the pathological score, $n = 4$. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* the control; $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* the model.

no significance, potentially due to RG-034 colonization inhibiting other bacteria (Fig. 6C). PCoA analysis showed distinct microbial community structures between the model and control groups, with RG-034 and lactulose treatments significantly altering the microbiota composition compared to the model group (Fig. 6D). KEGG pathway analysis showed that compared with the model group, the pathways related to the RG-034 (2×10^9 CFU) group were enriched in carbohydrate metabolism, xenobiotics biodegradation and metabolism, and amino acid metabolism (Fig. 6E), showing RG-034's ability to modulate gut metabolites in rats.

SCFAs, produced by gut microbiota through dietary fiber fermentation, play a crucial role in intestinal health. We speculate that it is SCFAs that promote the proliferation of ICCs, so we detected the content of SCFAs in the intestinal contents of rats. The SCFAs in the model group were all lower than those in the control group, with significantly lower concentrations of butyric acid and valeric acid. Compared with the model group, all three RG-034 (2×10^9, 2×10^8 and 2×10^7 CFU) groups demonstrated a significant increase in butyric acid concentration, and the RG-034 (2×10^9 CFU) group showed a significant increase in valeric acid concentration (Fig. 7A–G). KEGG pathway analysis revealed that butyric acid metabolites in the RG-034 (2×10^9 CFU) group were significantly altered, linked to protein and carbohydrate digestion and absorption (Fig. 7H). These findings suggest that RG-034 enhances SCFA production, particularly butyric and valeric acids, in rat intestinal contents.

L. plantarum RG-034 promotes ICC proliferation and Ca^{2+} influx by upregulating SCFAs through the PI3K/AKT pathway

The current constraints of *in vitro* experiments pose a significant challenge in functional constipation research, mainly due to difficulties in creating an *in vitro* functional constipation model and delivering probiotics to cells. In this study, we developed an *ex vivo* fecal co-culture system in which RG-034

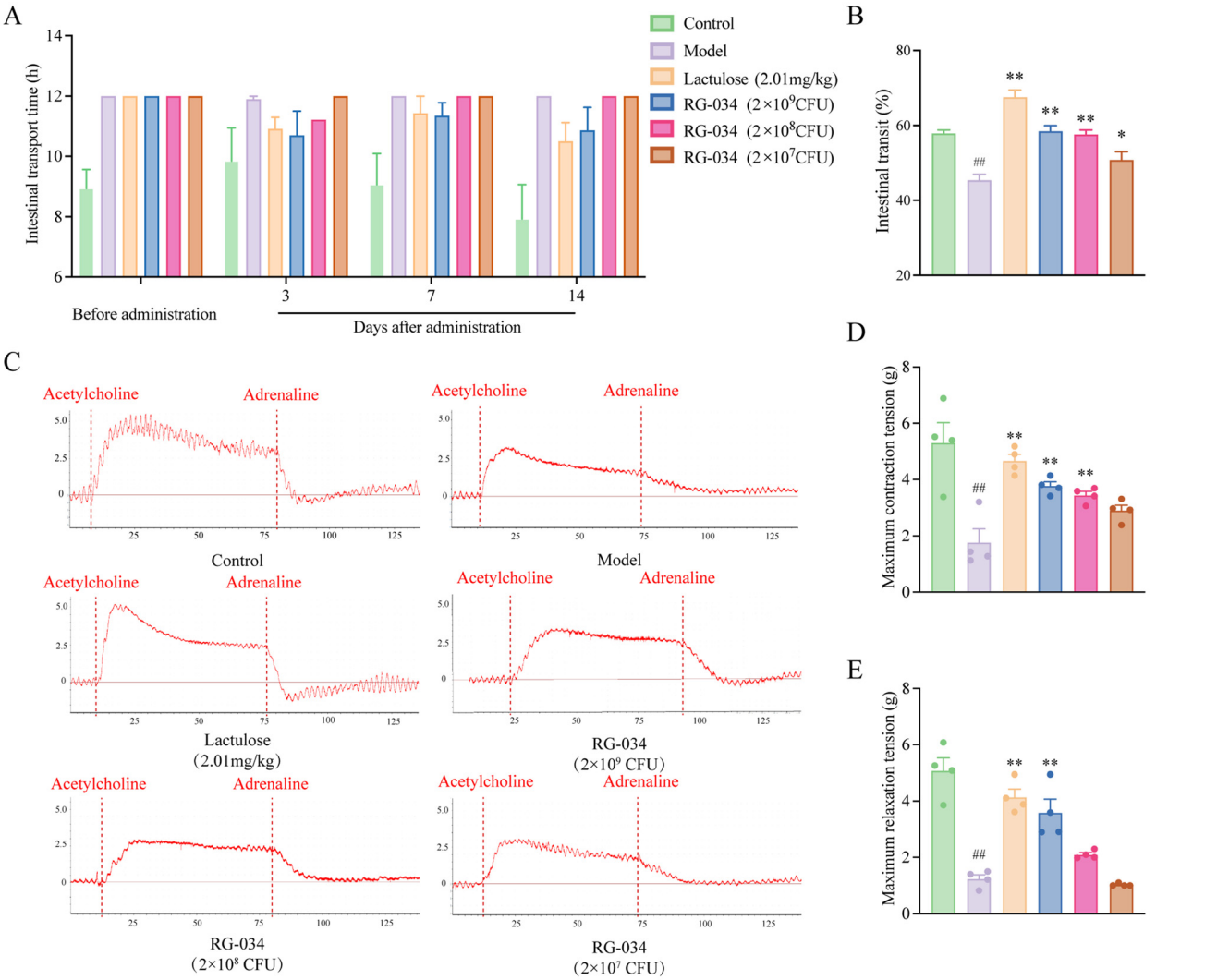

Fig. 3 RG-034 improves intestinal motility in rats with functional constipation (mean ± SEM). (A) The effect of RG-034 on the intestinal transport time, n = 10. (B) The effect of RG-034 on the intestinal transit rate, n = 10. (C) The effect of RG-034 on the contraction and relaxation tension of the isolated colons, n = 4. (D) Statistical analysis of contraction tension, n = 4. (E) Statistical analysis of relaxation tension, n = 4. $^{\#\#}P < 0.01$ *vs.* the control; $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* the model.

was incubated with rat fecal matter to simulate its natural intestinal microenvironment. Subsequent SCFA analysis of the culture supernatant revealed elevated levels of acetic acid, propionic acid, butyric acid, isobutyric acid and isovaleric acid following RG-034 co-culture (Fig. 8A). Subsequently, we treated primary ICCs with PBS, the fecal supernatant, and the RG-034 fecal supernatant. The results showed that compared with the fecal supernatant group, the Ca^{2+} uptake ability of ICCs in the RG-034 fecal supernatant group significantly increased (Fig. 8B and C), and the RG-034 fecal supernatant notably enhanced ICC proliferation *in vitro* (Fig. 8D and E).

To investigate the mechanistic basis, we pre-treated ICCs with the PI3K inhibitor LY294002 for 12 h. LY294002 pretreatment significantly inhibited ICC proliferation and Ca^{2+} uptake, and the RG-034 supernatant failed to rescue this inhibitory effect (Fig. 8B–E). These findings suggest that SCFAs in the fecal supernatant co-cultured with RG-034 may alleviate constipation by promoting ICC proliferation and calcium ion influx through activation of the PI3K/AKT pathway.

Discussion

This study finds that *L. plantarum* RG-034 colonic soluble capsules alleviate the symptoms of functional constipation and restore intestinal motility without inducing abnormal toxic responses. The potential mechanism underlying its therapeutic effects involves RG-034 improving the gut microbiota composition and promoting SCFA production, which enhance ICC proliferation and calcium ion influx by activating the PI3K/AKT pathway.

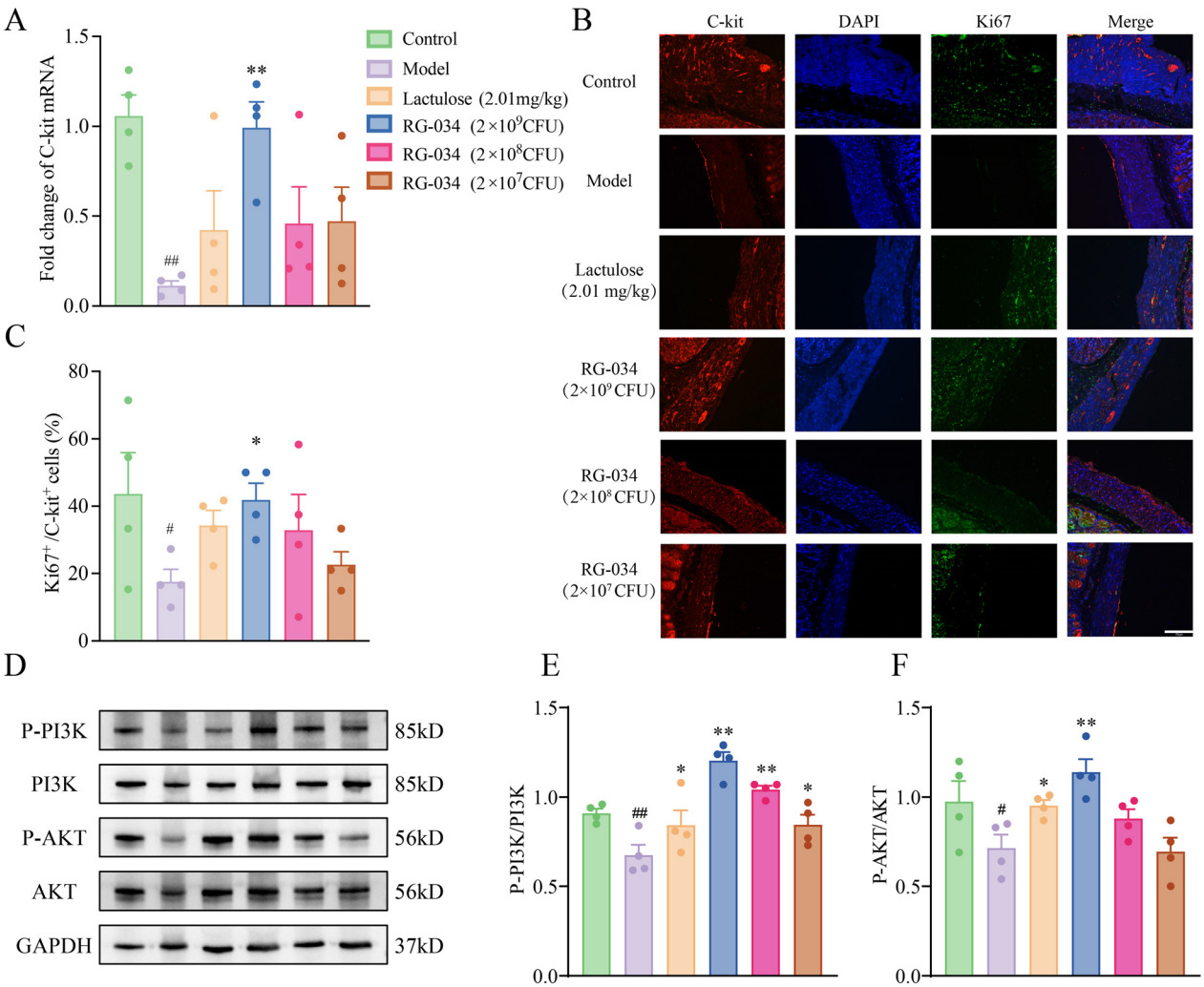

Fig. 4 RG-034 promotes the proliferation of ICCs in the intestines of rats with functional constipation (mean ± SEM, n = 4). (A) The effect of RG-034 on the expression of C-kit mRNA. (B) The effect of RG-034 on the proliferation ability of colon ICCs in the constipated rats, scale bar = 50 μm. (C) Statistical analysis of immunofluorescence. (D) The effect of RG-034 on the phosphorylation levels of PI3K and AKT in the colon. (E) Statistical analysis of the PI3K phosphorylation level. (F) Statistical analysis of the AKT phosphorylation level. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* the control; $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* the model.

The *L. plantarum* RG-034 strain was newly isolated from fecal samples of healthy individuals and has been deposited at the China General Microbiological Culture Collection Center (CGMCC, no. 25963). Unlike conventional *L. plantarum* strains, RG-034 exhibits superior lactic acid and hydrogen peroxide production capabilities, along with significant antimicrobial activity. These characteristics enable the strain to effectively modulate gut microbiota, suppress the growth of harmful bacteria, and maintain intestinal homeostasis. This functional bacterial strain is backed by intellectual property protection, with a Chinese invention patent (CN 117866797 A) publicly disclosed in April 2024. Furthermore, this study employed acid-resistant, colon-targeted capsules to achieve precise delivery of RG-034, significantly improving the formulation's stability, acid resistance, and colonic release accuracy. Investigating the role and mechanisms of *L. plantarum* RG-034 colonic soluble capsules in specific diseases not only provides novel microbial intervention strategies and theoretical foundations for treating these conditions but also lays a solid foundation for developing stable novel targeted probiotic formulations.

Following four consecutive weeks of oral gavage administration of *L. plantarum* RG-034 (4×10^{11} CFU), no significant changes were observed in the rat body weight, organ index, or histopathology of major organs. Furthermore, *L. plantarum* RG-034 primarily colonized the colon in this study without translocating to the bloodstream or major organs and posed no hemolytic risk. These findings align with previous literature reports, where administration of a fruit-derived strain for 28 days similarly induced no alterations in rat physiological parameters, organ histopathology, or detectable bacterial translocation to organs.[33] Hematological evaluation revealed that following RG-034 administration, all parameters remained

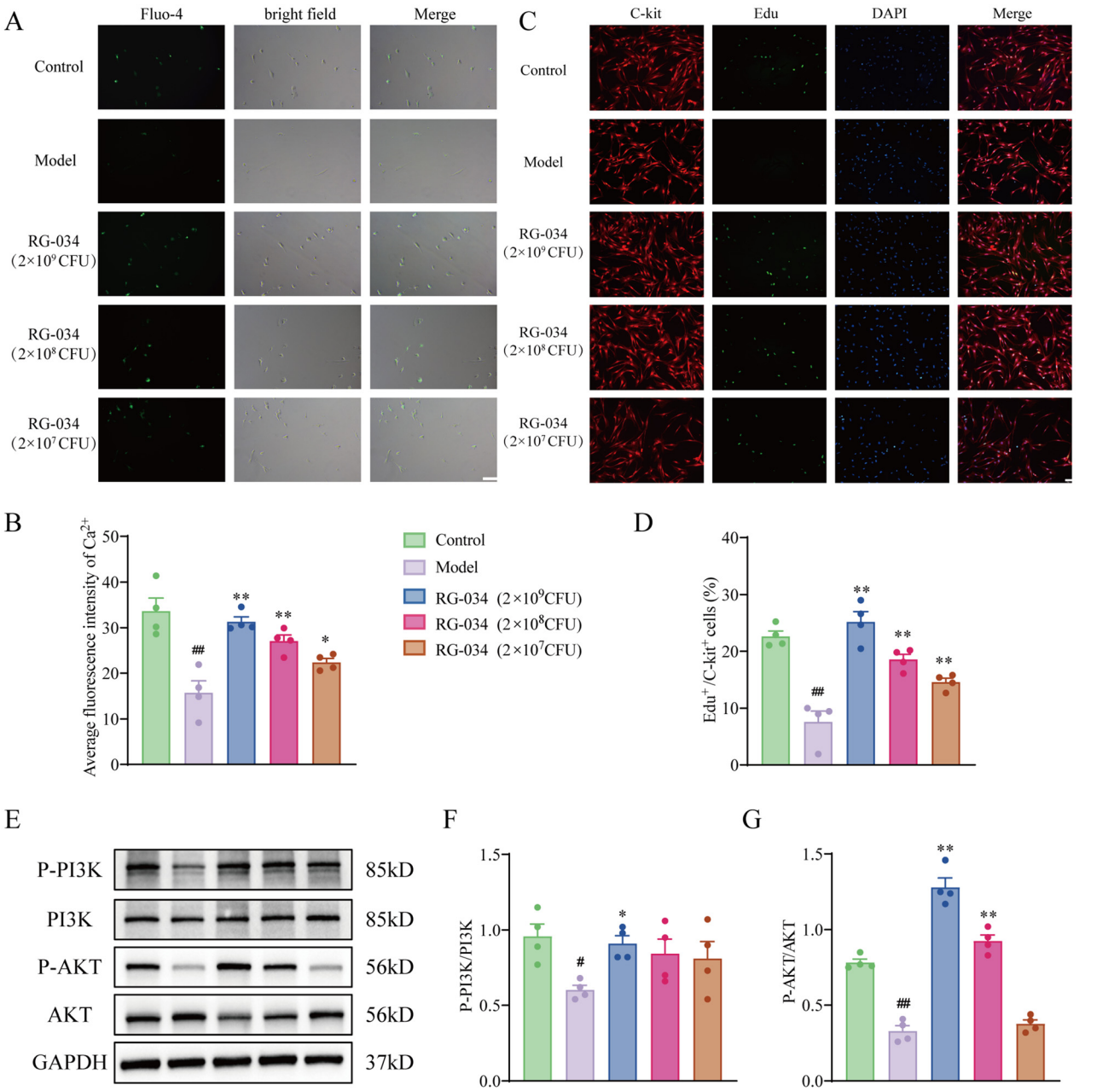

Fig. 5 RG-034 promotes the proliferation and Ca^{2+} uptake of primary ICCs (mean ± SEM, n = 4). (A) The effect of RG-034 on the Ca^{2+} uptake of the ICCs, scale bar = 100 μm. (B) Statistical analysis of the intracellular calcium ion concentration of the ICCs. (C) The effect of RG-034 on the proliferation of the primary ICCs, scale bar = 50 μm. (D) Statistical analysis of immunofluorescence. (E) The effect of RG-034 on the phosphorylation levels of PI3K and AKT in the primary ICCs. (F) Statistical analysis of the PI3K phosphorylation level. (G) Statistical analysis of the AKT phosphorylation level. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* the control; $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* the model.

normal except for a slight fluctuation in MCHC. The difference in MCHC values between the RG-034 group and the control group was small, and no difference in MCH levels was observed, confirming that RG-034 did not induce hematotoxicity. Biochemical analysis indicated transient decreases in CREA, TGL and BUN, while liver function markers such as ALT, AST, and CHOL remained normal. Further evaluation of renal function in rats administered RG-034 revealed urine parameters within normal ranges and no detectable urinary protein, confirming normal renal function. Two rats exhibited positive urine ketones, likely due to depletion of glucose and glycogen reserves from fasting, leading to lipolysis producing abundant free fatty acids. The liver converted these fatty acids into ketone bodies, representing a physiological, transient elevation. Pathological examination also confirmed normal liver and kidney tissue structures. Overall, these abnormal indicators may lack significant clinical relevance, and RG-034 demonstrated acceptable safety at the tested dose.

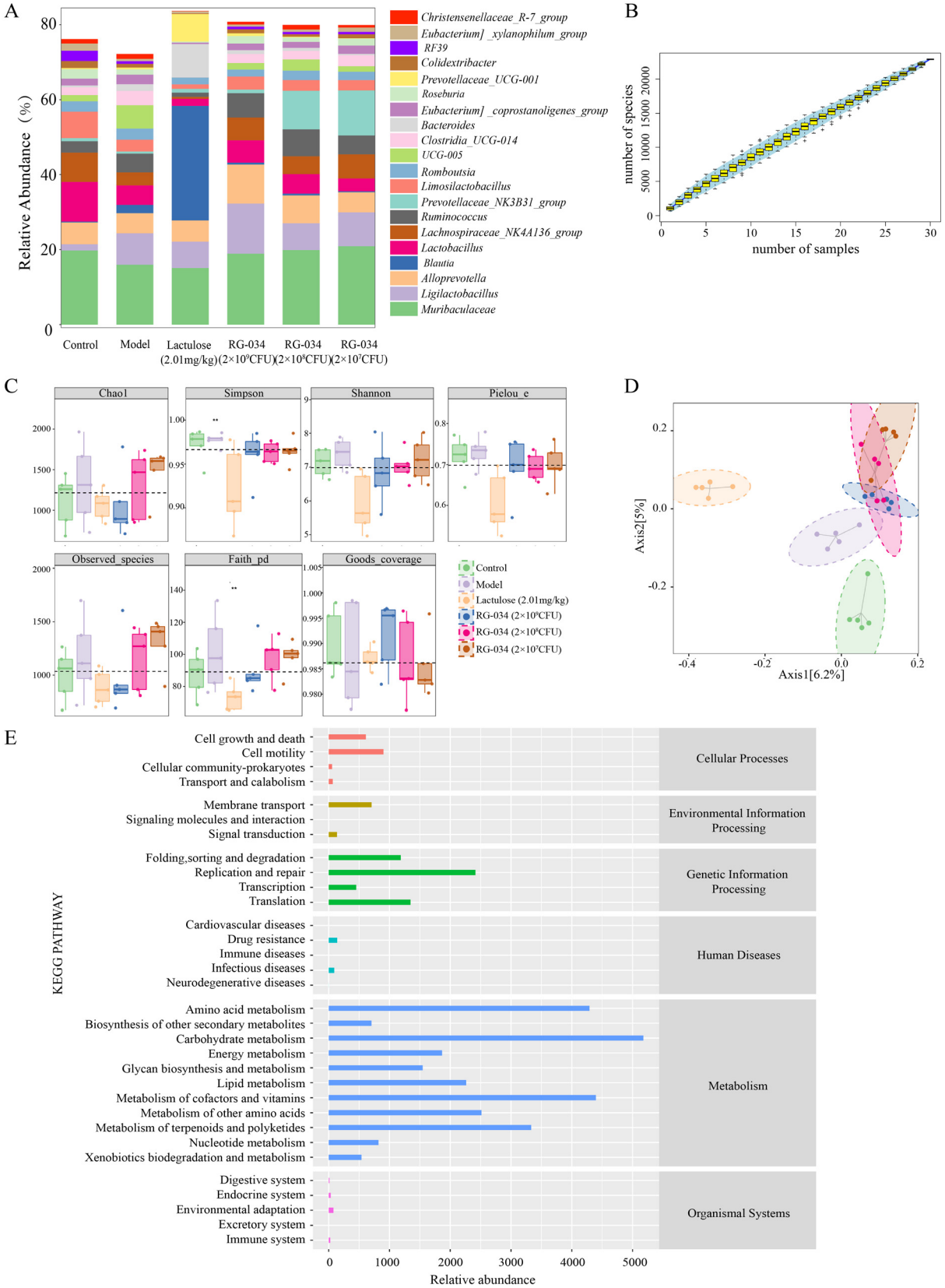

Fig. 6 RG-034 alters the distribution of the gut microbiota in rats with functional constipation (n = 5). (A) Bar chart of species composition at the genus level. (B) Species accumulation curve. (C) Alpha diversity of microbiomes. (D) PCoA analysis of microbiomes. (E) KEGG analysis of the RG-034 (2×10^9 CFU) group compared with the model group.

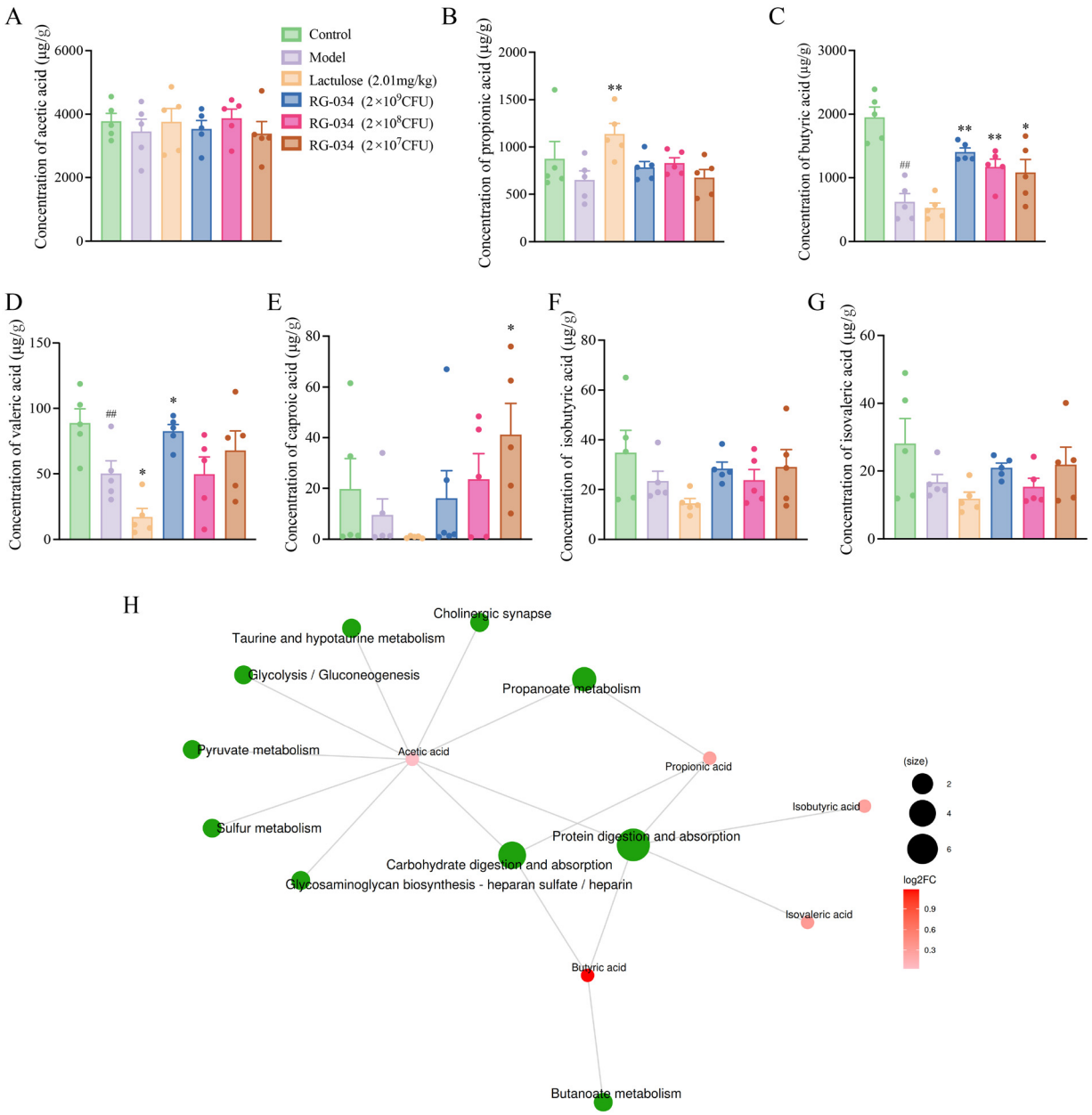

Fig. 7 RG-034 increases the intestinal SCFAs in rats with FC (mean ± SEM, n = 5). (A) The effect of RG-034 on the concentration of acetic acid. (B) The effect of RG-034 on the concentration of propionic acid. (C) The effect of RG-034 on the concentration of butyric acid. (D) The effect of RG-034 on the concentration of valeric acid. (E) The effect of RG-034 on the concentration of caproic acid. (F) The effect of RG-034 on the concentration of isobutyric acid. (G) The effect of RG-034 on the concentration of isovaleric acid. (H) KEGG metabolite molecular network diagram of the RG-034 (2×10^9 CFU) group compared with the model group. $^{\#\#}P < 0.01$ *vs.* the control; $^{*}P < 0.05$ *vs.* the model.

The therapeutic efficacy of probiotics depends on their ability to colonize the intestine.[34] Safety evaluation studies have demonstrated that RG-034 exhibits colonization in the colon following a 28-day administration. Pharmacodynamic experiments revealed that colonic colonization concentrations further increased after 14 days of treatment with *L. plantarum* RG-034 colonic soluble capsules. Furthermore, treatment with RG-034 induced a progressive, dose-dependent alleviation of constipation: fecal defecation increased by day 3, stool consistency improved by day 7, and fecal water content increased by day 14.

Histopathological hallmarks of constipation, including reduced thickness in the intestinal muscularis external layer, loss of goblet cells in mucosa and obvious oedema and hyperaemia in the longitudinal muscle layer,[35,36] were consistent with our observations. Treatment with RG-034 (2×10^9 CFU) markedly attenuated these colonic injuries and morphological alterations. We further evaluated intestinal motility, revealing

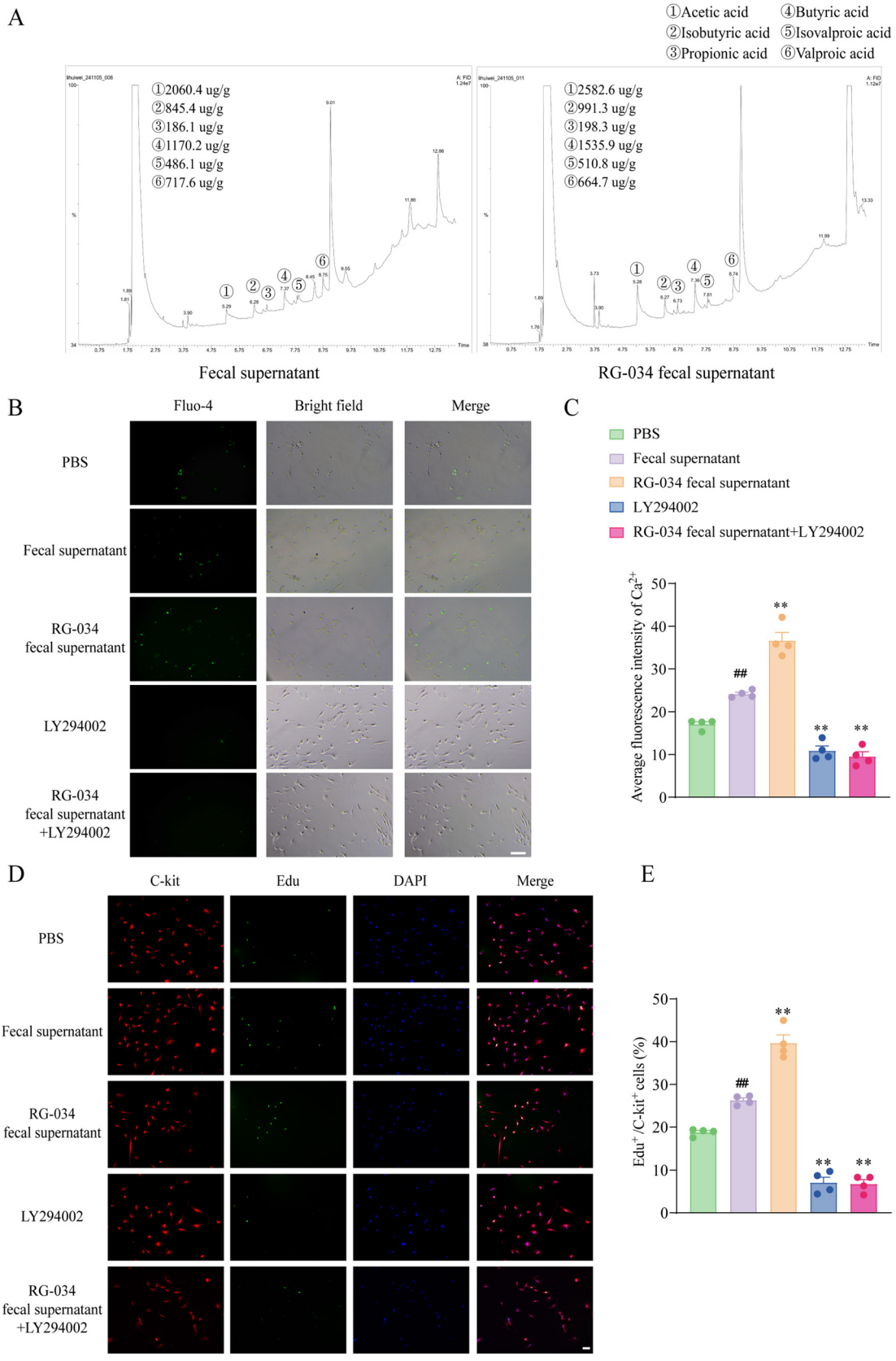

Fig. 8 RG-034 promotes ICC proliferation and Ca^{2+} uptake by upregulating SCFAs through the PI3K/AKT pathway (mean ± SEM). (A) The chromatogram of the fecal supernatant, n = 1. (B) The effect of the RG-034 fecal supernatant on the Ca^{2+} uptake of the ICCs, n = 4, scale bar = 100 μm. (C) Statistical analysis of the intracellular calcium ion concentration of the ICCs, n = 4. (D) The effect of RG-034 on the proliferation ability of the primary ICCs, n = 4, scale bar = 50 μm. (E) Statistical analysis of immunofluorescence, n = 4. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* PBS; $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* the fecal supernatant.

that RG-034 shortened the gut transit time and increased the intestinal transit rate in constipated rats. In addition, contraction and relaxation tension of isolated colon segments increased significantly after administration. Similarly, reduced ileal tension in the constipated rats was restored by administration of 8×10^{10} cells per mL *L. plantarum* nF1, an effect potentially linked to diminished release of pro-inflammatory cytokines associated with constipation.[35] Multiple *L. plantarum* strains have similar anti-inflammatory activity. For instance, *L. plantarum*-derived extracellular vesicles promoted differentiation of human monocytic THP1 cells towards the anti-inflammatory M2 phenotype,[37] while exopolysaccharides from *L. plantarum* R301 decreased nitric oxide production and IL-6 expression.[38] These parallels suggest that RG-034 may possess inherent anti-inflammatory activity, warranting further investigation to elucidate its mechanisms.

Given the well-documented role of probiotics in modulating gut microbiota, we performed 16S rRNA sequencing to analyze the microbial composition. In the model group, SCFA-producing bacteria, including *Muribaculaceae*, *Lactobacillus* and *Lachnospiraceae_NK4A136_*group were decreased, disrupting the balance of the intestinal microenvironment. Treatment with RG-034 restored this imbalance by increasing the relative abundance of *Muribaculaceae*, *Ligilactobacillus*, and *Alloprevotella* in the gut contents of constipated rats. These bacteria are associated with potential gut health benefits. For instance, *Muribaculaceae* has been identified as a "next-generation probiotic" as it can produce SCFAs and regulate intestinal barrier function and the immune response.[39] Similarly, multiple *Ligilactobacillus* strains exhibit probiotic properties,[40] and *Alloprevotella* exhibits antioxidant and anti-inflammatory activities.[41] Alpha diversity analysis revealed a non-significant trend toward reduced microbial diversity after RG-034 treatment compared to the model group, potentially due to competitive exclusion of other bacteria during probiotic colonization. However, there has always been controversy over the impact of probiotics on the diversity of the intestinal microbiota. Some studies report increased alpha diversity following probiotic intake,[42] whereas certain *L. plantarum* strains have been shown to decrease diversity.[43] Other studies have found that probiotics have no significant effect on the diversity,[44,45] highlighting the need for further experimental validation.

ICCs are known as the "pacemaker cells" of the intestine, and the electrical signals they generate can initiate and coordinate the contraction of digestive muscles, playing a crucial role in regulating intestinal peristalsis and digestive system function. A reduction in ICC numbers is one of the typical pathological features of functional constipation.[46] ICC expression is associated with activation of the PI3K/AKT signaling pathway, which modulates cell cycle regulatory proteins through phosphorylation, thereby promoting cell proliferation.[32] This study observed that RG-034 treatment increased the number of newly generated ICCs in the colons of constipated rats and promoted the proliferation of primary ICCs, while simultaneously activating the PI3K/AKT signaling pathway. Given that ICCs rely on calcium ion influx to initiate calcium transients and maintain anoctamin 1 channel activation during slow-wave depolarization,[47] our results indicated that RG-034 enhanced the intracellular calcium ion concentration in ICCs.

SCFAs play an important role in intestinal health. For instance, acetate participates in cholesterol synthesis, butyrate serves as an energy source for intestinal epithelial cells and regulates the intestine barrier and immunity, and propionate decreases cholesterol synthesis in the liver and improves lipid metabolism.[48] A previous study showed the probiotic combination of *Lacticaseibacillus paracasei* JY062 and *Lactobacillus gasseri* JM1 alleviated the constipation syndrome by increasing the number of ICCs and improving the contents of SCFAs in the cecal contents of mice.[45] SCFA analysis revealed a significant increase in butyric acid and valeric acid levels in intestinal contents following RG-034 intervention. To mimic the *in vivo* environment, this study innovatively treated cells with the supernatant obtained from the co-culture of RG-034 with rat feces. The results showed an increased concentration of butyric acid in the supernatant, which stimulated the proliferation of primary intestinal epithelial cells, accompanied by increased Ca^{2+} influx. However, the aforementioned promoting effects of RG-034 were blocked following the inhibition of the PI3K/AKT pathway using LY294002. In summary, this study confirms that RG-034 promotes SCFA production in the intestine and enhances ICC proliferation and Ca^{2+} influx by activating the PI3K/AKT signaling pathway, thereby improving intestinal function.

This research holds important implications for food science, particularly in developing synbiotics and functional foods targeting gastrointestinal disorders such as constipation. Notably, *L. plantarum* RG-034 has already been approved as a food additive and has obtained a food production license (production license no. SC20144510302355). Due to its enhanced probiotic properties, RG-034 can be incorporated into functional foods to improve intestinal health while delivering therapeutic effects with minimal side effects. Given the increasing demand for natural, non-pharmacological treatments for functional constipation, RG-034 exhibits substantial potential as a food additive or long-term dietary supplement, addressing an unmet medical need.

Conclusion

This study demonstrates that *L. plantarum* RG-034 significantly improves loperamide-induced functional constipation by regulating the intestinal microbiota structure and promoting SCFA synthesis and ICC proliferation. Overall, RG-034 has the potential to be used as a food additive or long-term medication; we hope this research will provide novel insights into further probiotic research and development of *in vitro* models of functional constipation.

Author contributions

F. X.: investigation, conceptualization, writing – original draft, and data curation. Y. Z.: conceptualization, writing – original

draft, investigation, and data curation. Y. L.: resources, conceptualization and validation. Z. W.: investigation and methodology. Z. L.: visualization and investigation. Y. Z.: validation. Y. L.: validation. T. H.: validation. L. G.: software. W. F.: resources, conceptualization, and writing – review and editing.

Conflicts of interest

There are no conflicts of interest to declare.

Ethics approval

The animal study protocol was approved by the Animal Care and Use Committees of China Pharmaceutical University (approval no. 202306008).

Data availability

The raw sequencing data have been submitted to SRA under accession PRJNA1259761. The data will become publicly available on 2027-06-01 and can be accessed at: https://www.ncbi.nlm.nih.gov/sra/PRJNA1259761.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d5fo02451j.

Acknowledgements

The authors thank PhD Erxin Shang from Nanjing University of Chinese Medicine and Assistant Engineer Shaoyan Xu from Guangdong Qiangji Pharmaceutical Co., Ltd. for their technical support.

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