



# *Lactobacillus plantarum* Lp3a improves functional constipation: evidence from a human randomized clinical trial and animal model

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**Background:** Functional constipation (FC) is a common gastrointestinal (GI) disorder characterized by symptoms of constipation without a clear physiologic or anatomic cause. Gut microbiome dysbiosis has been postulated to be a factor in the development of FC, and treatment with probiotic regimens, including strains of *Lactobacillus plantarum* (*L. plantarum*), has demonstrated efficacy in managing symptoms. To further understand the role of *L. plantarum* in GI health, we conducted an animal study and a randomized, double-blind, placebo-controlled clinical trial to evaluate the effect of a specific sub-strain, Lp3a, on FC.

**Methods:** For the animal study, male Kunming mice were treated with doses of *L. plantarum* Lp3a ranging from 0.67 to 2.00 g/kg or an equivalent amount of placebo for 15 days prior to the induction of constipation via 20 mL/kg of 25% diphenoxylate solution. GI motility parameters including intestinal motion and stool amount were then assessed. In the human study, 120 patients with FC were randomized to treatment [*L. plantarum* Lp3a;  $2 \times 1.0 \times 10^{10}$  (colony forming units; CFU)  $\times 7$  days] or control groups (n=60 each). The primary endpoint was survey information on FC signs/symptoms. Participants and observers were blinded to group allocation. A subset of 20 Lp3a treated patients underwent pre- and post-treatment 16 s ribosomal ribonucleic acid (rRNA) gene sequencing. Whole genome sequencing (WGS) of *L. plantarum* Lp3a was also performed.

**Results:** Lp3a-treated mice showed significantly improved intestinal motion, reduced time to first defecation, and increased stool amounts. Similarly, patients in the treatment group (n=59) reported significant improvements in FC signs/symptoms compared to controls (n=58; all  $P < 0.05$ ). Although 16 s rRNA sequencing revealed no significant variations between pre- and post-treatment samples, WGS of Lp3a itself revealed several biological pathways that may underlie the relief of FC symptoms in animals and humans, including methane and fatty acid metabolism and bile acid biosynthesis.

**Conclusions:** We found that the use of the novel probiotic sub-strain, *L. plantarum* Lp3a, led to clinically significant improvements in FC in both mice and humans, and identified the potential biological mechanisms underlying this activity.

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## Introduction

Functional constipation (FC), which is defined as constipation without a known anatomical or physiological cause, is a common disorder with an incidence rate of 1.9–40.1% in adults (1). Although the cause of FC remains elusive, it likely involves a constellation of factors including neurologic, psychosomatic, or nutritional components. The symptoms of FC primarily include straining during defecation, hard stools, incomplete evacuation, a sensation of an anorectal obstruction, and the need for manual maneuvers to facilitate defecation. FC is distinct from irritable bowel syndrome (IBS); thus, the diagnosis of FC requires exclusion of IBS (2,3). In addition to the health concerns related to chronic constipation, particularly recurrent pain, FC takes a severe toll on the mental health of those affected, and places an economic burden on hundreds of millions of patients worldwide (4,5).

Generally, the primary pathogenic mechanism characteristic of FC is dysfunctional gastrointestinal (GI) tract motility evidenced by prolonged gut transit time, reduced stool frequency, and fewer bowel movements (6). Thus, the effectiveness of treatments targeting FC are typically judged on the basis of improvements in these measures (4). Treatment modalities with demonstrated effectiveness in FC are multifarious, and include diet changes (particularly, increased fiber intake), exercise (aerobic and pelvic floor), bulking agents, stool softeners, over-the-counter (OTC) or prescription laxatives, probiotics, and biofeedback therapies. In cases that are refractory to the aforementioned medical therapies and severely debilitating, surgical interventions, such as laparoscopic subtotal resection, may even be indicated (2,6-9). Unfortunately, as many as 47% of FC patients have reported dissatisfaction with their current treatment regimens (e.g., OTC or prescription laxatives) due to their ineffectiveness or delayed effectiveness, lack of predictability, adverse effects, taste, and relatively high costs (10).

Given the pervasive effect of FC on the global population, there is an urgent need for the development

of novel treatment approaches that provide rapid and consistent relief with minimal side effects at an affordable cost. Probiotics, which function by influencing the composition and/or activity of the gut microbiome, have been proposed as one such therapy. Indeed, the gut microbiome, which consists of microorganisms that colonize the surface of the GI tract and influence host nutritional, metabolic, immunologic, endocrine, and neural processes, has been thought to be altered in the setting of FC. In infants and children, several studies have demonstrated elevated levels in feces of pathogenic bacteria such as *Proteobacteria* (11).

Additionally, others have shown that compared to healthy controls, patients with FC have significant variations in gut microbial composition, particularly reduced colonization by so-called “beneficial bacteria” and low species abundance (12-14). Consistently underrepresented bacterial taxa in FC patients include *Bifidobacteria*, *Lactobacillus*, *Bacteroides*, and *Prevotella* (13,15,16). Notably, *Bifidobacteria*, *Bacteroides*, and *Prevotella* are potent producers of short-chain fatty acids (SCFAs), while the *Lactobacillus* species produce lactate via fermentation (17-19). These metabolites are important regulators of host physiology and are known to reduce gut transit time (4,6). Thus, replenishing or rebalancing these species through probiotic supplementation may be effective at establishing healthy microbial diversity and treating FC. In fact, several independent studies have demonstrated the efficacy of various *Lactobacillus* and *Bifidobacterium* species in the relief of FC symptoms in animal models of constipation and human FC patients (20-22). Moreover, pretreatment with *Sargassum plagiophyllum* as a prebiotic was effective in increasing fecal *Bifidobacterium* counts and water content, ultimately reducing gut transit time in a mouse model of FC (23).

One species of *Lactobacillus* that demonstrated particular efficacy is *L. plantarum*, a microorganism distributed throughout the healthy GI tract (24-28). In this study, we sought to evaluate the effect of a new strain of *L. plantarum* (i.e., Lp3a) in an animal model of constipation and in human FC patients via a randomized, double-blind,

placebo-controlled clinical trial. Notably, the constipation-related signs and symptoms improved significantly in the animals and humans treated with Lp3a. Whole genome sequencing (WGS) of the bacterial *L. plantarum* Lp3a species revealed significantly enriched biological pathways, including methane and fatty acid metabolism, which are associated with GI motility and might underlie the clinical benefit. To our knowledge, this is the first study to explore the effect of *L. plantarum* Lp3a supplementation on FC. By obtaining data from both a mouse model of constipation and human FC patients, we have produced powerful results demonstrating a conserved ability of Lp3a to relieve the impaired GI motility of constipation. Compared to previous studies of *L. plantarum* species, our analysis also included exploratory gene analysis through WGS that provides novel insight into the mechanism by which this probiotic can influence constipation. We present the following article in accordance with the ARRIVE and CONSORT reporting checklists (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-458/rc>).

## Methods

### Probiotics

The probiotic supplement used in both the animal and human studies was obtained from New-Bio. Tech. (Jiangsu Province, China), and packed as 2 g per bag containing  $1.0 \times 10^{10}$  colony forming units (CFU) of *L. plantarum* Lp3a (Lot# 2018041501 for the animal study, and Lot# 2019011501 for the clinical trial).

### Animal study

#### Animals

Specific pathogen free (SPF) male Kunming mice [body weight (BW): 18–22 g; 8–10 weeks old; n=100] were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Certificate No: 211002300046431). The animals were housed individually in an SPF environment for 5 days to allow acclimation under standard diet and environmental conditions (Temperature: 20–26 °C; Relative Humidity: 40–70%; 12-hour light/dark cycle). The animal study were approved by the Institutional Animal Care and Use Committee of Nantong University (No. 2110836), in compliance with guidelines for the care and use of laboratory animals as described by the U.S. National Institute of Health, 8<sup>th</sup> edition (29).

## Experimental design

### Animal grouping, treatment, and induction of constipation

The mice (n=100) were randomly divided into the following groups based on outcome measure assessed: (I) the intestinal motion test group (n=50); (II) the defecation test group (n=50). Each group was randomly subdivided into five subgroups based on treatment. Three of the five groups received varied doses of the Lp3a probiotic supplement [dosing groups (DGs); high-dose/30 times of the human daily dose =2.00 g/kg/BW of the supplement solution in phosphate-buffered saline (PBS), middle-dose/20 times of the human daily dose =1.33 g/kg/BW, and low-dose/10 times of the human daily dose =0.67 g/kg/BW], one group served as the negative (healthy) control group (NCG), and one group served as the constipation control group (CCG), each receiving 2.00 g/kg/BW of the placebo solution in PBS. The mice treated with their respective probiotic or placebo solution received one dose of 20 mL/kg/BW per day for 15 consecutive days via oral gavage. At the conclusion of the 15-day treatment period, the animals were fasted for 16 hours, at which point, the animals randomized to the constipation groups (DG or CCG) were gavaged with 20 mL/kg/BW of diphenoxylate solution (25%) to induce constipation. The healthy control (NCG) animals were gavaged with an equal volume of pure water.

### Intestinal motion test

Thirty minutes following the induction of constipation or the administration of the control solution, the animals were administered 20 mL/kg of BW India Ink solution (Biotech Grade for Biological Stains; Phygene #PH1714) to assess their rate of intestinal motility. The purpose of the ink gavage was to label and monitor the rate of movement of the forming bolus through the GI tract. The bolus was allowed to travel for 25 minutes after labeling, after which the mice were sacrificed via cervical dissection, and the intestines were dissected from the pylorus to the ileocecal valve. The excised intestines were then straightened and measured to determine the total length of the small intestine (the pylorus to the ileocecal region), and the length of ink propulsion (the pylorus to the furthest distance of ink). The ink propulsion rate was defined as the length of ink propulsion (cm)/total length of the small intestines (cm).

### Defecation test

The mice randomized to the defecation test were similarly treated with India Ink via oral gavage following a 16-hour fasting period to label the forming feces. The mice were then returned to individual housing with free access to food and water. The time from the oral gavage to first defecation

of the labeled feces was recorded, and the amount (number of grains) and weight (grams) of the stool produced within 5 hours of the first defecation of the labeled feces were also recorded as measurements of GI motility.

### **Clinical trial**

#### **Study population**

Screening was performed to identify patients complaining of symptoms related to FC, including infrequent defecation (<3 times/week) and hard stool. These potential patients were interviewed and those that met the diagnostic parameters for FC according to the Rome Criteria IV (30) and did not meet the exclusion criteria (see below) were enrolled in the study (n=120). Patients were excluded from the study if they met any of the following exclusion criteria: (I) unable to consume oral medication; (II) had non-specific complaints; (III) had a poor physical condition; (IV) had experienced surgery-related constipation in the past month; (V) had known organic causes of constipation (e.g., colon cancer, bowel obstruction, or inflammatory bowel disease); (VI) had strained defecation with pain; (VII) had acute GI disorders in the past 30 days; (VIII) were currently pregnant or menstruating; (IX) had significant comorbidities (e.g., cardiovascular, hepatic, renal, hematologic, or severe systematic diseases); (X) were currently undergoing treatment for other diseases; and/or (XI) had recently used gastrointestinal active drugs. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The clinical trial in this study was reviewed and approved by the Institutional Ethics Committee of the Central Hospital of Xianyang, Shanxi Province (No. XYSZXY20190702). Informed consent was obtained from all the participants before their enrolment in the clinical trial. The study was performed in compliance with all federal guidelines and institutional policies.

#### **Study design**

Patients were recruited for this randomized, placebo-controlled, double-blind trial from July 5 to August 16, 2019 at the Central Hospital of Xianyang. All the patients were randomly divided into 2 groups with an allocation ratio of 1:1, and stratified by age, sex, and daily diet. Neither subjects nor researchers involved in data collection were aware of the group to which individuals were assigned. The treatment group was instructed to take the above described Lp3a probiotic supplement (2 g,  $1.0 \times 10^{10}$  CFU per bag; 2 bags per day), and the control group was instructed to take

the placebo (2 g per bag; 2 bags per day) for 7 consecutive days. Probiotic and placebo treatments were similar in appearance and composition. The primary endpoint of this study was the information obtained regarding patients' symptoms 7 days before treatment and throughout the duration of the study: (I) stool frequency (number per week); (II) defecation condition on a scale from 0 to 3 (on which 0 represented normal defecation, 1 represented the sensation of distention only, 2 represented obvious distention, or strained defecation, but rare abdominal pain or a burning sensation in the anus, and 3 represented obvious abdominal pain or a burning sensation in the anus); (III) stool form [scored 1–7 based on the Bristol Stool Form Scale (31)]; (IV) daily diet, especially fiber intake; (V) adverse events, such as nausea, diarrhea, and abdominal distention, and pain.

The following clinical parameters were also assessed as secondary endpoints throughout the trial: (I) vital signs, including blood pressure and pulse; (II) routine testing of blood, urine, and stool; (III) hepatic and renal function; (IV) plain chest X-ray, electrocardiogram, and abdominal ultrasonography. This clinical data was collected at the Central Hospital of Xianyang. The subjects were instructed not to change their diet or their daily exercise regimen for the duration of the trial. Between and within group comparisons of each of the variables were conducted.

#### **Bioinformatics analyses**

Fecal samples from the 20 probiotic-treated patients were collected before and after treatment. A16s ribosomal ribonucleic acid (rRNA) gene analysis was conducted to characterize the gut microbial composition of the samples. A WGS analysis of *L. plantarum* Lp3a was also conducted to identify the encoded proteins, functions, and biological pathways relevant to GI motility and/or constipation.

#### **16s rRNA-analysis**

Total bacterial genomic DNA was extracted from fecal samples, and the integrity and size of the DNA was validated by gel electrophoresis using a 1% agarose gel (Majorbio Bio-pharm Tech., Shanghai, China) Universal forward (319F: 5'-ACTCCTACGGGAGGCAGCAG-3') and reverse (806R: 5'-GGACTACHVGGGTWTCTAAT-3') primers were used for the polymerase chain reaction (PCR) amplification of the V3–V4 hypervariable regions of the bacterial 16s rRNA gene to define the bacterial composition and abundance (32). The PCR products were purified and quantified by QuantiFluor™-ST (Promega). Libraries, which were constructed via the TruSeq™ DNA Sample Prep Kit (Illumina), were sequenced on the Illumina Miseq



**Table 1** The effects of probiotics on intestinal motion in mice

Groups	Ink propulsion rate (%)	P value
High-dose group	64.00±17.36	0.012*
Middle-dose group	61.39±16.51	0.037*
Low-dose group	65.39±18.77	0.009*
CCG	41.08±9.74	0.000 <sup>#</sup>
NCG	87.05±8.59	–

\*, compared to CCG; <sup>#</sup>, compared to NCG. CCG, constipation control group; NCG, negative (healthy) control group.

platform. Paired-end reads were generated and merged to a single sequence using the Fast Length Adjustment of Short reads (FLASH; 1.2.0) (33). Quality control was continuously monitored by filtering for bases with Phred scores <20. The remaining high-quality, non-repetitive sequences were clustered into Operational Taxonomic Units (OTUs) at a 97% sequence identity threshold for clustering using Usearch (version 7.0 <http://drive5.com/usearch/>) (34). Chimeras were checked and removed during the clustering process using UCHIME (35). Sequence alignments were carried out with MAFFT (v7.427), and OTUs were assigned to the lowest possible taxonomic level using the Ribosomal Database Project (RDP) Classifier Version 2.2 at a 70% bootstrap value threshold to obtain species classification information. Alpha and beta diversity indices were computed using QIIME (36). Taxa abundances at the phylum and genus levels were analyzed using the Metastats function in Mothur and compared via ANOSIM (37). Between sample differences in specific microbiota abundance were identified via the Student's *t* test and/or Wilcoxon rank-sum test.

### WGS

High-molecular-weight genomic DNA was extracted from *L. plantarum* Lp3a (Tianjin Biochip Co., China), and evaluated for purity, quantity, and size through UV-Vis (NanoDrop, ThermoFisher, USA), fluorometric (Qubit, ThermoFisher), and Pulsed Field Gel Electrophoresis assays. DNA (5 g) was used to prepare 20 kb SMRTbell libraries in accordance with the manufacturer's (PacBio's) directions. Libraries were size-selected using the BluePippen instrument to deplete short inserts and impurities before sequencing on the PacBio Sequel System using 1.2.1 chemistry and a 360-minute movie length. *De novo* genomes were assembled using the hierarchical genome assembly process of HGAP4.0 from original genomic data obtained

from the PacBio sequel (38). After the validation of the obtained genome, advanced annotations were performed, including analyses of Clusters of Orthologous Groups (COGs) of proteins, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEEG). Secreted proteins were identified by SignalP 5.0 (<http://www.cbs.dtu.dk/services/SignalP/>) and the TMHMM 2.0 online server (<http://www.cbs.dtu.dk/services/TMHMM/>).

### Statistical analyses

The data obtained from the animal and clinical studies were statistically analyzed using IBM SPSS Version 21.0. A P value <0.05 was considered statistically significant. The distribution of the continuous variables was assessed for normality using the Kolmogorov-Smirnov test. For the animal study, the normally distributed data were analyzed using a one-way analysis of variance. For the clinical trial, the normally distributed data are presented as the mean ± standard deviation. Between group comparisons were made using two-sample *t* tests, and within group (pre- *vs.* post-treatment) comparisons were made using paired *t* tests. The categorical data were assessed using the Chi-square or Fisher's exact tests as appropriate.

## Results

### Animal studies

#### The effects of probiotics on body weight

To assess the effect of the probiotic treatment on overall metabolism, health, and wellbeing, the mice were weighed before and after the 15-day treatment period. No significant difference was observed in the body weight of the mice in the three treated (DG) and the non-treated (CCG) constipation groups compared to the healthy control group (NCG). This suggests that neither the probiotic treatment nor the induction of constipation significantly affected body mass (see [Table S1](#)).

#### Intestinal motion test

The ink propulsion rate was calculated to determine the effect of the probiotic treatment on intestinal motility (see [Table 1](#)). Compared to the NCG, the CCG had a significantly lower ink propulsion rate (87.05%±8.59% *vs.* 41.08%±9.74%; *P*<0.001), which was consistent with the expected effect of constipation on GI motility. The ink propulsion rate was significantly lower in the probiotic-

**Table 2** The effects of probiotics on defecation in mice

Groups	Time to first defecation (min)	P value	Grains in stool	P value	Weight of stool (g)	P value
High-dose group	111.9±36.8	0.765*	13.7±4.2	0.045*	0.332±0.081	0.010*
Middle-dose group	113.9±43.0	0.833*	10.6±3.2	0.770*	0.251±0.099	0.618*
Low-dose group	125.3±48.8	0.999*	10.1±4.1	0.916*	0.233±0.097	0.904*
CCG	128.7±43.3	0.030 <sup>#</sup>	8.9±2.4	0.004 <sup>#</sup>	0.206±0.066	0.001 <sup>#</sup>
NCG	78.6±29.5	–	15.4±6.0	–	0.365±0.099	–

\*, compared to CCG; <sup>#</sup>, compared to NCG. CCG, constipation control group; NCG, negative (healthy) control group.

treated mice in the high-dose (64.00%±17.36%), middle-dose (61.39%±16.51%), and low-dose (65.39%±18.77%) groups than those in the CCG (each DG *vs.* CCG; all  $P<0.05$ ). This suggests that probiotic treatment reduces constipation-induced delays in GI motility.

### Defecation test

To determine the effect of the probiotic treatment on defecation, we next assessed the time to first defecation following oral gavage with ink, as well as the weight and number of grains of stool produced within 5 hours (see *Table 2*). Constipation led to a delay in the average time to first defecation (128.7±43.3 *vs.* 78.6±29.5 min;  $P=0.030$ ) and a reduction in the amount (15.4±6.0 *vs.* 8.9±2.4 grains;  $P=0.004$ ) and weight (0.365±0.099 *vs.* 0.206±0.066 g;  $P=0.001$ ) of stool in the CCG *vs.* the NCG mice. The significant increase in time to defecation and the reduction in the amount and weight of stool between the CCG and NCG confirmed the successful establishment of the constipation model (39). Treatment with high-dose probiotic supplementation ameliorated the effect of constipation on stool weight (0.332±0.081;  $P=0.010$ ) and the amount (13.7±4.2;  $P=0.045$ ), but did not significantly reduce the time to first defecation (111.9±36.8;  $P=0.765$ ) compared to the CCG. There was no significant difference between these parameters in low- and middle-dose groups compared to the CCG.

### Clinical trial

#### Participants and baseline characteristics

A total of 120 participants at the Central Hospital of Xianyang who met the diagnostic (inclusion) criteria for FC were enrolled in this study between July 5 and August 16, 2019. Subjects were randomly allocated to either the treatment with the probiotic ( $n=60$ ) or placebo ( $n=60$ )

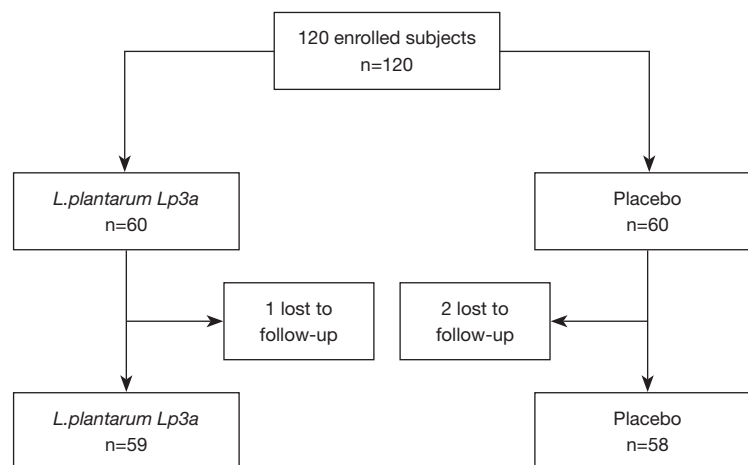
groups. At the conclusion of this study, 3 patients had been lost to follow-up, 1 in the treatment group, and 2 in the placebo group (see *Figure 1*). The baseline demographic and clinical characteristics, including age, gender, daily fiber intake (%), and stool frequency and form, did not differ significantly between the enrolled FC participants in each group (see *Table 3*). No abnormalities warranting subject exclusion were detected by the baseline clinical tests, which included chest X-ray, electrocardiogram, abdominal ultrasonography, and urine, and stool analyses.

#### Safety of *L. plantarum* Lp3a in humans

To ensure the safety of the *L. plantarum* Lp3a supplement, we used clinical tests to identify any potential physiologic or biochemical disturbances in the 117 patients before and after treatment. No obvious changes were detected by routine blood tests (see *Table S2*), biochemical parameters (see *Table S3*), and vital signs (see *Table S4*). No adverse effects or allergic reactions were observed or reported during this trial. These data indicate that the supplementation of *L. plantarum* Lp3a is generally safe for FC patients at the administered dosage.

#### *L. plantarum* Lp3a could alleviate FC symptoms

As described above, the baseline FC symptoms before the trial were indistinguishable between the two groups, indicating that these participants were drawn from a similar population and successfully randomized. Following the 7-day treatment period, patients randomized to the *L. plantarum* Lp3a group reported significant increases in stool frequency (2.75±0.94 *vs.* 1.61±0.49;  $P<0.001$ ), a reduction in defecation difficulty (1.03±0.96 *vs.* 1.64±1.06;  $P<0.001$ ), and more normal form according to the Bristol Stool Scale (0.53±0.57 *vs.* 0.93±0.76;  $P<0.001$ ; see *Table 4*). Conversely, individuals randomized to the placebo group did not report significant improvements in these symptoms



**Figure 1** Flow chart demonstrating the design of the clinical trial.

**Table 3** Baseline characteristics between the treatment and control groups

Variables	<i>L. plantarum</i> Lp3a (n=59)	Placebo (n=58)	P value
Female/male	43/16	35/23	0.150 <sup>#</sup>
Age (years)	45.80±12.03	43.07±9.36	0.174*
Stool frequency (times/week)	1.61±0.49	1.62±0.49	0.908*
Defecation condition	1.64±1.06	1.66±1.07	0.955*
Stool form (Bristol scale)	0.93±0.76	0.97±0.82	0.820*
Daily fiber intake (%)	27.99±4.52	28.56±3.56	0.447*

\*, data compared by *t*-test; <sup>#</sup>, data compared by chi-square test.

after the treatment period. To determine if biased dietary changes between these 2 groups confounded the results, we compared their daily fiber intake percentage, and found that the change in symptoms was not clearly influenced by dietary changes. We also observed no significant difference in the fiber intake of the probiotic-treated individuals before and after treatment, or any difference compared to the controls. These results extend those of our animal study, confirming that *L. plantarum* Lp3a is both biologically and clinically active against constipation.

### 16s rRNA gene analysis of stool samples

To identify alterations in the microbiome composition related to probiotic supplementation, we performed a 16s rRNA gene analysis on the fecal samples obtained from 20 participants before and after Lp3 treatment. A total of 1,679,233 high-quality sequences containing 691,231,074 bases and ranging in length from 401–450 bp

were generated from 40 (20 pre-treatment and 20 post-treatment) fecal samples (see Figure S1A). These sequences clustered into 639 OTUs (582 after the trial, 575 before the trial, of which 518 were common) at 97% sequence identity (see Figure S1B). Shannon index and rank-abundance curves were computed to demonstrate the overall microbial species richness and evenness between the pre- and post-treatment fecal samples. The Shannon index curve, which plots out levels against the number of reads, demonstrated a plateau at 5,000 reads, indicating that the samples were even and sufficiently rich in species diversity (see Figure S1C). Similarly, the extended smooth lines plotted in the rank-abundance curve demonstrated high species richness and evenness in both the pre- and post-treatment samples (see Figure S1D).

To compare the diversity of species represented in the gut microbial colony between the samples, a beta diversity analysis was performed. Using the ANOSIM method, we

**Table 4** Comparison of constipation symptoms and diet before and after treatment

Variables	<i>L. plantarum</i> Lp3a (n=59)	Placebo (n=58)	P value
Stool frequency (#/week)			
Before trial	1.61±0.49	1.62±0.49	0.908*
After trial	2.75±0.94	1.66±0.55	0.000*
Margin	-1.14±0.75	-0.03±0.53	0.000*
Comparison within group (t, P)	-11.579, 0.000 <sup>#</sup>	-0.497, 0.621 <sup>#</sup>	
Defecation condition			
Before trial	1.64±1.06	1.66±1.07	0.955*
After trial	1.03±0.96	1.66±1.12	0.002*
Margin	0.61±0.49	0.00±0.50	0.000*
Comparison within group (t, P)	9.528, 0.000 <sup>#</sup>	0.000, 1.000 <sup>#</sup>	
Stool form (Bristol scale)			
Before trial	0.93±0.76	0.97±0.82	0.820*
After trial	0.53±0.57	0.95±0.83	0.002*
Margin	0.41±0.56	0.02±0.40	0.000*
Comparison within group (t, P)	5.572, 0.000 <sup>#</sup>	0.331, 0.742 <sup>#</sup>	
Daily fiber intake (%)			
Before trial	27.99±4.52	28.56±3.56	0.447*
After trial	28.10±4.07	27.74±4.19	0.643*
Margin	-0.11±5.86	0.82±4.72	0.347*
Comparison within group (t, P)	-0.150, 0.881 <sup>#</sup>	1.314, 0.194 <sup>#</sup>	

\*, data compared by independent *t*-test; <sup>#</sup>, data compared by paired *t*-test.

failed to identify a significant difference in the microbial composition between the pre- and post-treatment samples at the phylum and genus levels (see *Figure 2A,2B*). At the phylum level, *Firmicutes* (55.4%), *Actinobacteria* (28.5%), *Proteobacteria* (8.7%), and *Bacteroidetes* (5.8%) were predominant in the pre-treatment samples. A seemingly similar composition was observed in the matched post-treatment samples: *Firmicutes* (52.0%), *Actinobacteria* (29.0%), *Proteobacteria* (12.5%), and *Bacteroidetes* (4.9%; see *Figure 2C*). Similarly, at the genus level, 5 genera predominated; that is, *Bifidobacterium* (pre-treatment =23.8% vs. post-treatment =25.3%), *Romboutsia* (7.9% vs. 4.8%), *Escherichia-Shigella* (7.8% vs. 10.5%), *Blautia* (6.6% vs. 4.8%), and *Subdoligranulum* (6.1% vs. 7.8%; see *Figure 2D*). We failed to identify any specific microbiota that were significantly enriched or depleted at the genus and species levels using the Wilcoxon rank-sum test (see

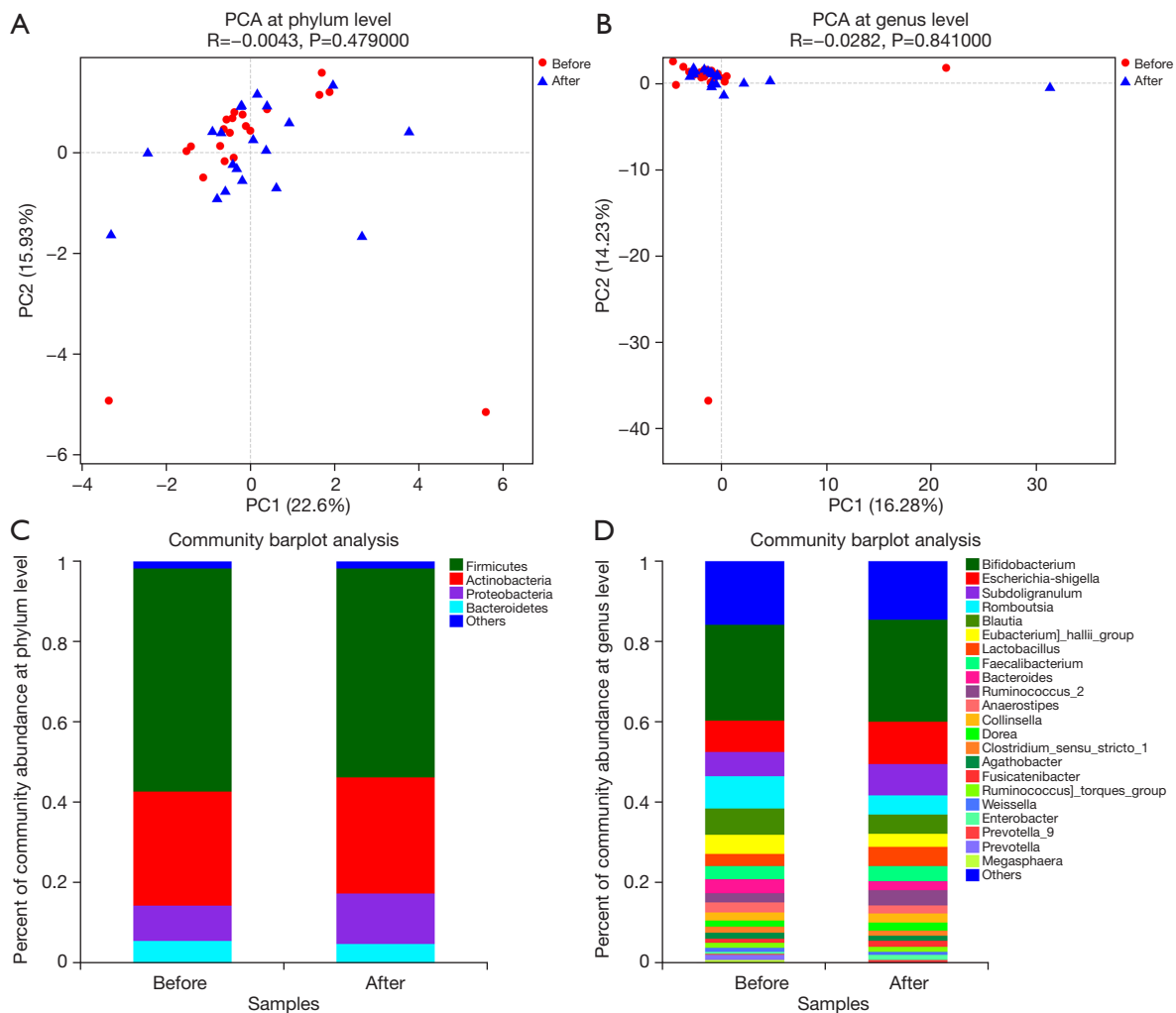
*Figure 3A,3B*). Overall, our 16s rRNA sequencing analysis revealed no significant difference in microbiome composition, which suggests that the observed effect of *L. plantarum* Lp3a on constipation is not directly mediated by microbial diversity.

### WGS analysis

After failing to identify a treatment-related change in microbial diversity, we sought to determine if the biological activity inherent to *L. plantarum* Lp3a by virtue of its encoded proteins might underlie the effect on GI motility via WGS. This analysis provides a high-throughput pipeline for identifying protein-coding genes and biologically active pathways that are relevant to the various metabolic and physiologic functions of the sequenced organism.

The whole genome size of *L. plantarum* Lp3a was 3,214,487 bp with a guanine-cytosine (GC) content of





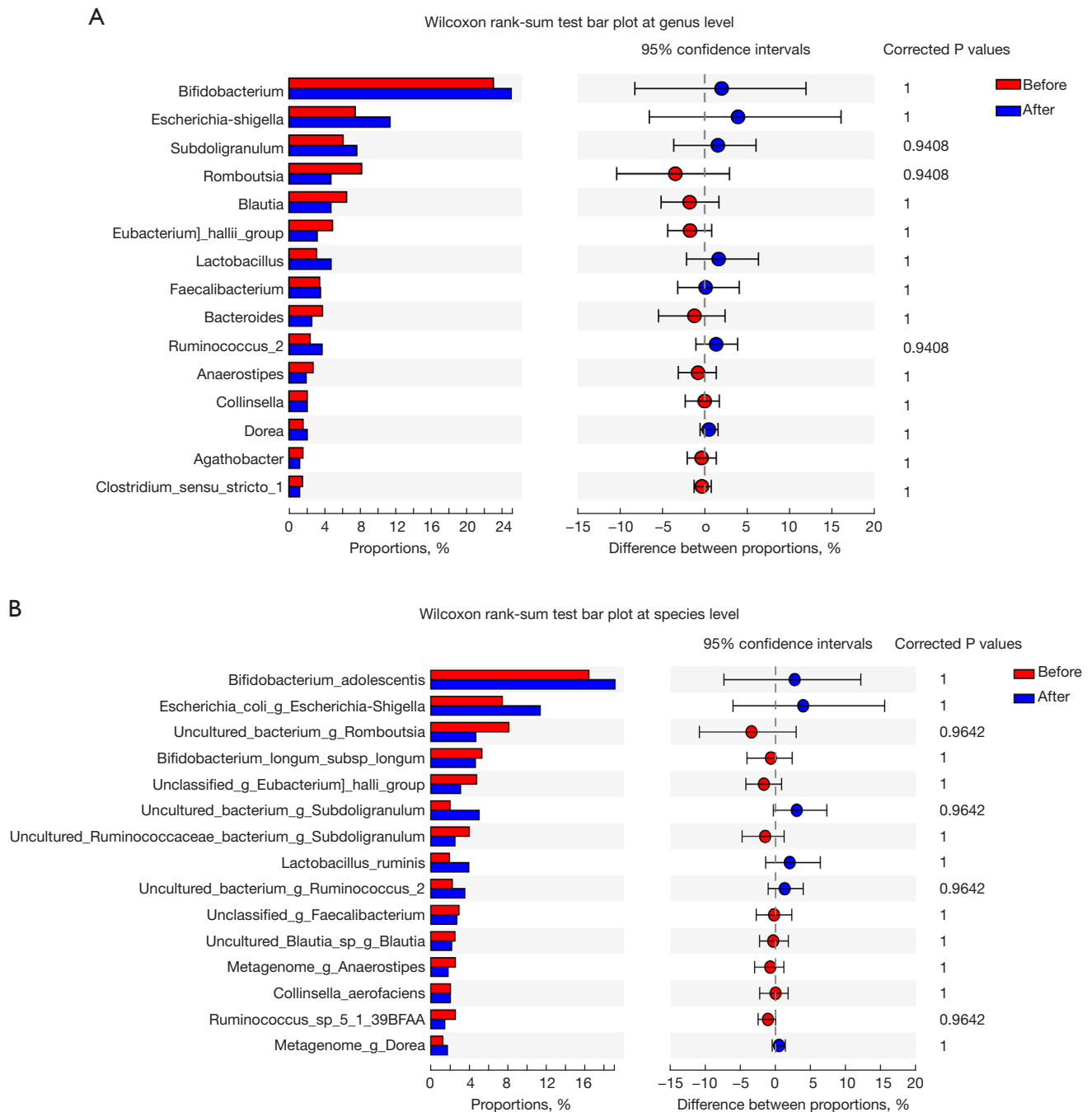
**Figure 2** Analyses of microbiome composition in pre- and post-treatment fecal samples by 16S rRNA sequencing: (A) The principal component analysis (PCA) revealed no difference in composition between the pre- and post-treatment fecal samples at the phylum level ( $P=0.479$ , ANOSIM). (B) The PCA analysis revealed no difference in composition between the pre- and post-treatment fecal samples at the genus level ( $P=0.841$ , ANOSIM). (C) The relative abundance of microbial phyla between the pre- and post-treatment samples was indistinguishable ( $P=0.479$ , ANOSIM analysis). (D) The relative abundance of microbial genera between the pre- and post-treatment samples was indistinguishable ( $P=0.841$ , ANOSIM).

44.6%. Open reading frames (ORFs) accounted for 83.9% of the genome, and a total of 3,076 genes were identified with an average length of 876 bp.

COG-based annotation analyses are designed to classify proteins from completely sequenced genomes based on the ortholog concept. Around 20 COG functional categories were discovered in the genome of *L. plantarum* Lp3a (see Figure 4A). The most highly enriched clusters were “General function prediction only” (484 unigenes), “Function unknown” (352 unigenes), “Carbohydrate transport and

metabolism” (235 unigenes), “Amino acid transport” (199 unigenes), and “metabolism and transcription” (198 unigenes). The GO functional classifications identified 31 subclasses, 10 of which were related to molecular function, 11 to biological processes (BPs), and 10 to cellular components (see Figure 4B).

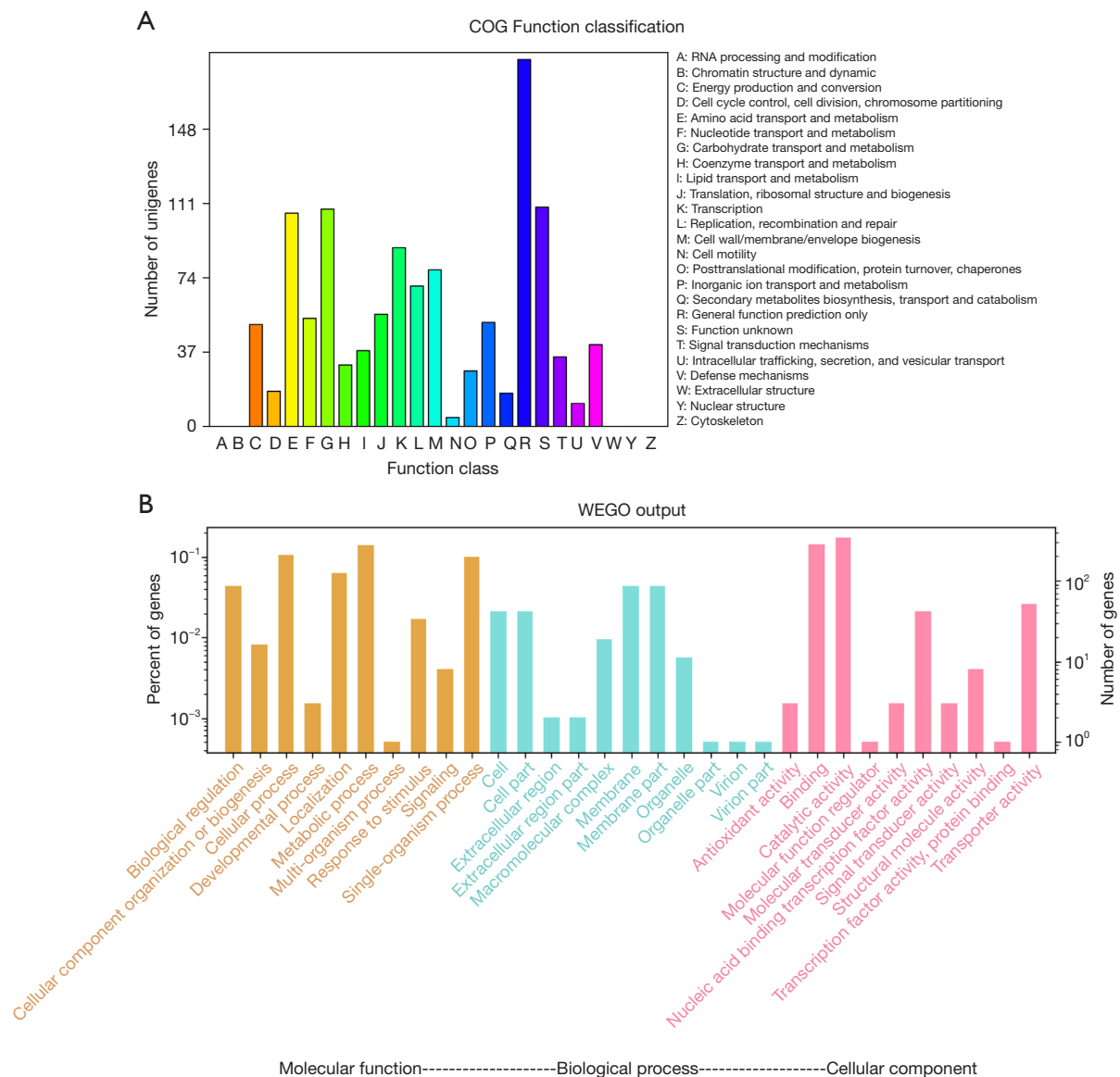
The functional pathway analysis identified 149 unique biological pathways, among which “metabolic pathways” (ko:01100) prevailed (415 unigenes). Other highly enriched pathways included the “biosynthesis of secondary



**Figure 3** Analyses of microbiome composition differences in major genera and species between the pre- and post-treatment fecal samples by the Wilcoxon rank-sum test: (A) bar plots demonstrating no significant changes in major genera abundance between the pre- and post-treatment (red and blue, respectively) fecal samples (all  $P > 0.05$ , Wilcoxon rank-sum test); (B) bar plots demonstrating no significant difference in abundance of major species between the pre- and post-treatment fecal samples (all  $P > 0.05$ , Wilcoxon rank-sum test).

metabolites” (ko:01100, 216 unigenes), “biosynthesis of amino acids” (ko:01230, 111 unigenes), “microbial metabolism in diverse environments” (ko:01120, 111 unigenes),

“carbon metabolism” (ko:01200, 70 unigenes), “ABC transporters” (ko:02010, 68 unigenes), “purine metabolism” (ko:00230, 57 unigenes), “phosphotransferase system”



**Figure 4** Clusters of Orthologous Group (COG) and Gene Ontology (GO) classifications of functional proteins in the genome of *L. plantarum* L p3a: (A) protein-coding genes were clustered into 20 COG functional categories, including *general function prediction only* (484 unigenes), *function unknown* (352 unigenes), *carbohydrate transport and metabolism* (235 unigenes), *amino acid transport* (199 unigenes), and *metabolism and transcription* (198 unigenes); (B) protein-coding genes were clustered into 31 major GO subclasses, including *molecular function* (10 unigenes), *biological process* (11 unigenes), and *cellular component* (10 unigenes).

(ko:02060, 53 unigenes), ribosome (ko:03010, 50 unigenes), “glycolysis/gluconeogenesis” (ko:00010, 46 unigenes), and “pyrimidine metabolism” (ko:00240, 44 unigenes). Notably, less abundant pathways directly related to GI motility and constipation were also identified, such as “methane metabolism” (ko:00680, 25 unigenes), “fatty acid metabolism” (ko:01212, 20 unigenes), “primary bile acid

biosynthesis” (ko:00120, 4 unigenes), and “secondary bile acid biosynthesis” (ko:00121, 4 unigenes) (40).

As the probiotic supplementation did not directly affect composition, we took particular interest in the secreted and surface membrane proteins through which *L. plantarum* Lp3a could interact with the host gut microenvironment. A total of 224 surface and secreted proteins were identified,

138 of which were located in the bacterial membrane. The most abundant protein classes were “hypothetical” (19.2%), followed by “extracellular proteins” (17.0%), “cell surface protein and its precursor” (12.9%), “transport system substrate-binding protein” (9.4%), such as amino acid ABC transporter, phosphate ABC transporter, glutamine ABC transporter and sugar ABC transporter, and “lipoprotein and its precursor” (8.0%). Ectoenzymes, such as cell surface hydrolases and extracellular zinc metalloproteinases, accounted for a small but significant part of the predicted proteome. Overall, our WGS analysis identified important encoded proteins and biological pathways through which *L. plantarum* Lp3a may interact with the gut microenvironment to effect GI motility and relieve constipation.

## Discussion

The role of the gut microbiome in GI and other systemic diseases is becoming increasingly apparent in the scientific literature. Numerous preclinical and clinical trials have explored the effect of probiotics in adult and pediatric patients with FC (20,22,28,41). Unfortunately, variability in study design, the included populations, the composition and dosage of the probiotic formulation, and, importantly, the diagnostic criteria for FC have resulted in inconsistencies across these studies. For example, in their meta-analysis of 14 randomized, placebo-controlled trials, Dimidi *et al.* demonstrated that probiotics significantly reduced gut transit time by 12.4 (95% CI: 22.3–22.5 hours) and increased stool frequency by 1.3 bowel movements per week (95% CI: 0.7–1.9 bowel movements/week) in adults (4). However, a similar analysis of randomized controlled trials, which included pediatric patients, failed to demonstrate that probiotics provided any clinical benefits in the relief of FC (16). It is clear that future research evaluating the characteristics of probiotics, particularly specific species, strains, and genome-encoded features, and their effects on FC need to be conducted to produce targeted formulations for this debilitating disease.

A promising species that has been explored in FC studies is *L. plantarum*, a lactic acid bacterium (LAB) that is widespread in multiple ecological niches, and widely used in the fermentation of food products, including cheeses, meats, vegetables, and beverages (42). Given their extensive role in food production, lactic acid bacteria, such as *L. plantarum*, have already been designated as “Generally Recognized As Safe” by the United States Food and Drug Administration,

and thus are obvious targets for probiotic development (43). Additionally, these LAB species have well described BPs that help to stabilize the gut microenvironment, including the synthesis of SCFAs and the production of bacteriostatic substances (i.e., lactocins), and thus confer pathogen resistance (44,45). Many studies have investigated the effectiveness of various strains of *L. plantarum* on disease processes, such as dyslipidemia (46), diarrhea (47), constipation (24–28), IBS (48,49), and inflammatory bowel disease (50) in both animals and humans. Strains of *L. plantarum* have been shown to have an effect in animal models of constipation, including NCU116 (24), YS2 and YS3 (25,26), CQPC01, and CQPC02 (51,52). The relief of constipation induced by CQPC01 and CQPC02 is thought to be mediated by antioxidant activities and an increase in the levels of GI neuropeptides, such as motilin (51,52). In humans, *L. plantarum* SN35N and SN13T have shown promise in relieving constipation, reducing low-density lipoprotein cholesterol, and improving hepatic function (28). Further the *L. plantarum* LRCC5193 strain has been proven to be effective at ameliorating constipation-related symptoms in both a rat model (53) and human FC patients (27). Interestingly, *L. plantarum* has been showed to be effective in improving gut motility in a loperamide-induced model of constipation indicating that efficacy is not dependent on viability of the bacterium. Our current study adds to this growing body of literature by demonstrating the beneficial effect of an additional *L. plantarum* strain (i.e., Lp3a) on constipation symptoms in both mice and humans.

The mechanisms through which probiotics affect GI physiology and might improve FC symptoms are multifaceted and remain poorly understood. Perhaps most obviously, probiotics can directly modulate the GI microbial composition through direct colonization, the promotion of beneficial bacterial species, and/or the antagonism of pathogens (6). For example, Botelho *et al.* demonstrated that a multispecies probiotic supplement was capable of attenuating increases in the abundance of two bacterial species (i.e., *Blautia faecis* and *Ruminococcus torques*) that are known to be enriched in the microbiome of chronic constipation patients. This attenuation was associated with a significant reduction in symptoms of constipation, including incomplete defecation and the sensation of blockage (54). However, a lack of changes in gut microbial composition is not indicative of probiotic failure. Indeed, Kim *et al.* reported that the intake of a probiotic containing various species of *Bifidobacterium* and *Lactobacillus* in addition to *Streptococcus thermophilus* for 2 weeks improved constipation

symptoms (i.e., increased defecation frequency) without a concomitant change in the GI flora (55).

The failure of our 16s rRNA analysis to identify significant differences in the gut microbiome structure between the probiotic- and placebo-treated patients indicated that the effect of the treatment on FC symptoms must be mediated by more indirect mechanisms. Such mechanisms could include the metabolism of nutrients into metabolically active substances, and the interaction of these metabolites or surface-bound proteins with the GI tract, central or enteric nervous system, endocrine system, or immune system (6). In a study of rats, Kunze *et al.* showed that supplementation with *Lactobacillus reuteri* for 9 days enhanced the excitability of enteric neurons by inhibiting the calcium-dependent potassium channel opening (56). These findings describe a neural pathway through which probiotics might affect GI motility and pain. To determine whether the effect of *L. plantarum* Lp3a on intestinal motility and constipation symptoms in mice and humans is mediated via the interaction of secreted and/or surface proteins with the host metabolism, we analyzed the genome of Lp3a using WGS. From the whole genome, which measured 3.2 Mb, consistent with other *L. plantarum* strains (57), we identified a number of enriched pathways that are directly correlated with GI motility. For example, encoded proteins related to methane metabolism might function to enhance gut transit rates by degrading methane, a metabolite associated with delayed transit times (58-60). Our WGS analysis also revealed the enrichment of the primary and secondary bile acid biosynthesis pathways in the *L. plantarum* Lp3a. Taken together with evidence that bile acids serve as physiological laxatives (6,61), this suggests a role for the modulation of bile acid metabolism as an additional putative mechanism of Lp3a in FC.

The WGS analysis also predicted a number of membrane-bound and secreted proteins in *L. plantarum* Lp3a that support its use as a probiotic for GI diseases, and potentially, for general health. The secretion of transport system substrate-binding proteins by Lp3a, which are associated with the uptake of sugar, amino acids, phosphate, and glutamine, may allow this microbiota strain to compete with pathogenic flora and thrive within the gut microenvironment (62). Extracellular zinc metalloproteinase, another secreted protein, might also facilitate the successful colonization of *L. plantarum* Lp3a in the digestive tract (63). Additionally, the production of lipoproteins and/or precursors by *L. plantarum* Lp3a might explain its contribution to protection

against hyperlipidemia reported previously (42). Lp3a supplementation was not observed to have an effect on total cholesterol or triglycerides; however, a future analysis of a selected patient population with hyperlipidemia might reveal the potential role of Lp3a in cardiovascular health. Overall, the predicted pathways and secreted or membrane-bound proteins identified in this study support its role in alleviating constipation, and bolster its credibility as a clinical probiotic.

It is important that we recognize the limitations of this study. First, the sample size in the clinical trial was relatively small and was drawn from a single center, which introduces a risk of selection bias and limits the cross-geographic generalizability of the results. The small sample also prevented us from conducting subgroup analyses among the subtypes of FC and/or phenotypes of the gut microbiome before treatment. The latter is particularly limiting, as previous research has shown that the baseline composition of the microbiome is an important predictor of probiotic responses (64). Additionally, as we only enrolled adults in this study, we were unable to examine the role of *L. plantarum* Lp3a in children. This represents an obvious avenue for future research given the burden of FC in pediatric patients. For these reasons, future multicenter randomized controlled trials that include pediatric patients need to be conducted to validate the effectiveness of Lp3a, among other formulations, for FC. Similarly, animal studies remain important in elucidating the mechanisms by which probiotics affect host physiology.

Despite these limitations, this study made an important contribution to the growing body of literature on the use of probiotic therapy to treat GI and other systemic diseases, including FC. To our knowledge, this study was the first to investigate and validate the effect of the Lp3a strain of *L. plantarum* in both an animal model and human patients. Additionally, the combination of the 16s rRNA and WGS analyses allowed us to identify the diverse mechanisms by which Lp3a might affect the gut microenvironment, including numerous BPs and protein clusters relevant to GI motility. This provided an unprecedented understanding of the biological functions of this probiotic agent, which could inform the treatment of FC and other pathological, such as cardiovascular disease.

In conclusion, *L. plantarum* Lp3a effectively relieved features of constipation in both a mouse model and adult FC patients. Taken together, the mouse and human data suggest that this agent relieves FC symptoms by enhancing intestinal motility, and putatively modulating methane metabolism and



bile acid synthesis. Larger studies need to be conducted to confirm its effectiveness in clinically relevant subgroups, including children and those with various subtypes of FC.

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## Footnote

**Reporting Checklist:** The authors have completed the ARRIVE and CONSORT reporting checklists. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-458/rc>

**Trial Protocol:** Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-458/tp>

**Data Sharing Statement:** Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-458/dss>

**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-458/coif>). KM, GW, RW are from Jiangsu Biodep Biotechnology. The other authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The animal study in this study was reviewed and approved by the Institutional Animal Care and Use Committee at Nantong University (No. 2110836), in compliance with guidelines for the care and use of laboratory animals as described by the U.S. National Institute of Health, 8th edition. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The clinical trial in this study was reviewed and approved by the Institutional Ethics Committee of the Central Hospital of Xianyang, Shanxi Province (No. XYSZXYY20190702). Informed consent was obtained from all the participants before their enrolment in the clinical trial.

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