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Effect of Complex Prebiotics on the Intestinal Colonization Ability of *Limosilactobacillus fermentum* DALI02

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Abstract: Intestinal colonization is beneficial to the role of probiotics, and prebiotics can promote the adhesion and colonization of probiotics in the intestine. This study optimized the combination of complex prebiotics that could improve the growth ability and adhesion ability of *Limosilactobacillus fermentum* (*L. fermentum*) DALI02 to Caco-2 cells in vitro and determined the effect of its colonization quantity and colonization time in the immunocompromised rat model. The results showed that all five prebiotics (fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin, stachyose, and xylo-oligosaccharides (XOS)) significantly promoted the growth and adhesion of *L. fermentum* DALI02. It was found that 0.5% (*w*/*w*) inulin had the best growth promotion effect, and 0.5% FOS had the strongest adhesion promotion (the adhesion rate was increase by 1.75 times). In addition, 0.05% FOS, 0.20% GOS, 0.30% inulin, 0.20% stachyose, and 0.30% XOS could significantly improve the adhesion rate of *L. fermentum* DALI02 from 1.72% to 3.98%. After 1 w of intervention, the quantity of colonization in the fermented broth with prebiotics group was significantly higher than that in the fermented broth group. The intervention time was extended from 1 d to 4 w, and the amount of colonization of *L. fermentum* DALI02 in the fermented broth with prebiotics group increased significantly from 4.32 lgcopies/g to 5.12 lgcopies/g. After the intervention, the serum levels of lipopolysaccharide (LPS) and D-lactic acid in rats were significantly reduced, and the most significant was in the fermented broth with prebiotics group, with LPS and D-lactic acid levels of 74.11 pg/mL and 40.33 μmol/L, respectively. Complex prebiotics can promote the growth and adhesion of *L. fermentum* DALI02 and significantly increase the quantity of colonization and residence time of the strain in the intestine, which helps the restoration of intestinal barrier function and other probiotic effects.

Keywords: probiotic; prebiotic; adhesion; intestinal colonization; rats

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1. Introduction

Probiotics are live microorganisms that provide health benefits to the host when given in appropriate amounts [1]. They have probiotic functions, such as improving the structure of the host intestinal flora as well as antioxidants, anti-obesity, and immune enhancement [2]. Adhesion and colonization in the intestinal tract will help the strain resist intestinal peristalsis and food erosion, thus allowing probiotics to play a healthy role [3]. Colonization implies a prolonged residence time of probiotics in the intestine and an increased opportunity to interact with the host. On the one hand, it is beneficial for probiotics to reduce the amount of harmful microorganisms in the gut by seizing colonization sites. On the other hand, it can regulate probiotic metabolites, such as short-chain fatty acids, which serve as key signaling molecules to maintain intestinal health, thus benefiting host health [4].

Prebiotics are indigestible substances that provide health benefits to the host, which are selectively utilized by microorganisms in the gut [5]. They can enhance the viability and probiotic properties of probiotics, such as promoting their reproduction, metabolism, and tolerance to the gastrointestinal tract [6–8]. In addition, prebiotics can enhance the adhesion ability of probiotics [9] by changing their surface film properties. In addition, the enhancement effect is specific [10]. Prebiotics can also modulate the repulsion of probiotic cell membranes to intestinal epithelial cells by increasing the proportion of unsaturated fatty acids, thus improving the adherence of the strain to HT-29 [11]. Prebiotics, as a carbon source, can enhance the adhesion to Caco-2 by highly expressing the gene, encoding its adhesion protein during the growth of *Bifidobacterium* DNG6 [12].

Many studies have been conducted to investigate the effect of prebiotics on the adhesion ability of probiotics in vitro through the Caco-2 cell model [13], HT-29 cell model [14], or Caco-2 and HT-29 symbiosis model [15]. However, colonization in the intestine is very complex. The peristalsis of the intestine, moving of food, and intervention of other flora can affect the initial adhesion and long-term colonization of strains. Although the ability of the strains to adhere to intestinal epithelial cells is an important criterion to screen probiotics in vitro [16], it is crucial to explore the colonization ability of strains in vivo. At the same time, considering the practical application of probiotics, it is of more practical significance to explore the colonization ability of strains in the model of intestinal injury. Therefore, this study investigated the complex prebiotics that can improve the growth quantity and the adhesion ability of *L. fermentum* DALI02 to Caco-2 cells. The complex prebiotics (fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin, stachyose, and xylo-oligosaccharides (XOS)) on the colonization ability of the strain was investigated by evaluating the effect of the fermentation broth with prebiotics on the quantity and time of intestinal colonization of *L. fermentum* DALI02 in immunocompromised model rats.

2. Materials and Methods

2.1. Microorganism and Cell Strains

L. fermentum DALI02 was isolated from traditional fermented dairy products by Key Laboratory of Dairy Biotechnology and Safety Control and stored in China General Microbial Strain Conservation Management Center (No. CGMCC 16064). The lyophilized strain was cultured in de Man Rogosa and Sharpe (MRS) media at 37 °C for 24 h.

Caco-2 cells (Procell CL-0050, Procell Life Science & Technology Co., Ltd., Wuhan, China) were maintained in modified Eagle's medium (MEM, Life Technologies Corporation, New York, NY, USA) with high glucose supplemented with 20% fetal bovine serum (FBS, PAN-Biotech, Bavaria, Germany), 1% nonessential amino acids (NEAA, Life Technologies Corporation, New York, NY, USA), 1% pyruvate (Life Technologies Corporation, New York, NY, USA), 1% GlutaMAX (Life Technologies Corporation, New York, NY, USA), and 1% penicillin/streptomycin/ neomycin (PSN, Thermo Fisher Scientific Inc., New York, NY, USA) and incubated at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific Inc., New York, NY, USA).

2.2. Preparation of Whey Hydrolysate and Prebiotics

Whey protein powder (Fonterra Brands (NZ) Ltd., Auckland, New Zealand) was adjusted with NaOH (0.1 mol/L, China Pharmaceutical Group Co., Ltd., Beijing, China) at 10% (*w*/*w*), pH 7.00, and papain (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 6000 U/g was added, hydrolysis temperature was 65 °C, and enzymatic digestion time was 2 h. Control of the degree of hydrolysis was 20%, high temperature inactivation was performed, and the supernatant was extracted after centrifugation at 6500 r/min for 15 min, heat-treated at 105 °C for 10 min, and placed at 4 °C.

Different weights of prebiotics (FOS, GOS, Inulin, Stachyose, and XOS, Jinfuyuan Biotechnology Co., Ltd., Shenzhen, China) were weighed and dissolved in sugar-free MRS and whey hydrolysate, respectively, sterilized at 105 °C for 10 min, and then prepared for use.

2.3. The Effects of Probiotics on L. fermentum DALI02

Activated *L. fermentum* DALI02 was inoculated at 3% (*v*/*v*) in the prebiotic content of 2% (*w*/*w*) MRS-Lactose medium, MRS-FOS medium, MRS-GOS medium, MRS-Inulin medium, MRS-Stachyose medium, MRS-XOS medium, as well as sugar-free MRS and incubated at 37 °C for 24 h. The OD_{600} was measured by enzyme marker (Thermo Fisher Scientific Inc., New York, NY, USA).

The activated *L. fermentum* DALI02 was inoculated into enzymatic skim milk with a prebiotic content of 0.5% (*w*/*w*) at 3% (*v*/*v*) inoculum for 24 h at 37 °C. The number of viable bacteria and their ability to adhere to Caco-2 cells were determined by dilution-plate method.

2.4. Experimental Design, Modelling, and Optimization

According to the results of the pre-experiment (Figure S1), the five prebiotics at different concentrations in this experiment had a significant effect ($p < 0.05$) on the adhesion capacity of *L. fermentum* DALI02. Therefore, a combination of five levels of quadratic regression orthogonal rotation combination design (QRORCD) and response surface methodology (RSM) were used to optimize the addition of complex prebiotics for the high adhesion capacity of *L. fermentum* DALI02. The coded and natural values of the independent variables X_1 (FOS addition, %), X_2 (GOS addition, %), X_3 (Inulin addition, %), X_4 (Stachyose addition, %), and X_5 (XOS addition, %) are shown in Table 1. The QRORCD included 10 replicate centroids and a set of axial points selected to allow for rotatability, which ensured that the variance of the model predictions was constant at all points equidistant from the design center. The experimental runs were randomized to minimize the effect of unexpected variation in the observed responses.

Table 1. Codes and levels of factors.

Code X	Extrusion Condition				
	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (%)	X_5 (%)
−2	0.00	0.00	0.00	0.00	0.00
−1	0.05	0.15	0.10	0.10	0.30
0	0.10	0.30	0.20	0.20	0.60
1	0.15	0.45	0.30	0.30	0.90
2	0.20	0.60	0.40	0.40	1.20

2.5. Adhesion Ability to Caco-2 Cell Lines

The cultured Caco-2 cells (Procell CL-0050, Procell Life Science & Technology Co., Ltd., Wuhan, China) were carried out and the concentration was adjusted to 2×10^5 cell/mL, and then the cells with the adjusted concentration were placed in a 24-well plate (Costar, Corning Inc., New York, NY, USA) and continued to be cultured until they grew into monolayer cells. The samples were diluted to a viable count of 10^6 CFU/mL added to each well and continued to be incubated at 37 °C in a 5% CO_2 incubator for 2 h. The fermented broth was discarded, and the cells were washed twice with PBS solution and then scraped off and resuspended in sterile phosphate buffered saline (PBS) (pH 7.2; Sangon Biotech Co., Ltd., Shanghai, China) to count the viable count. The equation of adhesion rate is shown as follows:

Adhesion rate = number of live bacteria after 2 h of adhesion/number of live bacteria before adhesion × 100%.

2.6. Adhesion Parameters in Rat Model

2.6.1. Experimental Design Based on the Animal Model

To investigate the effect of gavage time on the number and residence time of *L. fermentum* DALI02 intestinal colonization, 36 Wistar male mice (Yangzhou University Medical Animal Experiment Center, Yangzhou, China), aged 4 weeks and weighing 140–160 g were

studied. They were housed under a 12 h light/12 h dark cycle in a controlled room with a temperature of 24 ± 2 °C and a humidity of 50 ± 10%. All rats were allowed free access to food and water. All 5 rats were placed in a cage and were fed a diet (flour 20%, rice flour 10%, corn 20%, drum skin 26%, soy material 20%, fish meal 2%, and bone meal 2%). All rats were randomly divided into 4 groups (n = 15): control group, model group, fermented broth group, and fermented broth with prebiotics group. The immunocompromised rat model was established by intraperitoneal injection of 80 mg/kg of CTX for 3 consecutive days. During the next 4 weeks, all rats received the following treatments by gavage: control group and model group—whey hydrolysate (1 mL/100 g), fermented broth group—*L. fermentum* DALI02 fermented broth without prebiotics (1 mL/100 g, 1×10^9 CFU/mL), and fermented broth with prebiotics group—*L. fermentum* DALI02 fermented broth with prebiotics (1 mL/100 g, 1×10^9 CFU/mL). At the end of the 4th week, rats underwent 12 h of fasting prior to being anaesthetized and dissected. All rats were euthanized at the anestrus period following anesthesia under 1% sodium pentobarbital.

2.6.2. Colonization of *L. fermentum* DALI02

The ability of *L. fermentum* DALI02 to colonize the rat intestine was determined by measuring the number of *L. fermentum* in rat feces. Mouse fecal DNA was extracted using the Fecal Genomic DNA Extraction Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions (F: GACCAGCGCACCAAGTGATA; R: AGCGTAGCGTTCGTGGTAAT) and PCR-amplified using primers specific to *L. fermentum* from the existing literature [17]. The total volume was 10 µL and was run on StepOnePlus™ Real-Time PCR System with Tower (Applied Biosystems, Inc., New York, NY, USA) using SYBR Premix Ex Taq II (Takara Bio Inc., Tokyo, Japan). All measurements were performed in triplicate.

Standard curves were constructed using PCR products of *L. fermentum* DALI02 and the 16S rRNA gene of E. coli, and then the obtained PCR products were cloned into the T vector (Takara Bio Inc., Tokyo, Japan) and transfected into DH-5α and incubated at 37 °C for 6 h (100–140 rpm) as standard samples. The recombinant plasmids were used for real-time fluorescent quantitative PCR (qPCR), and 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, and 10^{10} copies of each plasmid were used for calibration for each reaction. The copy number K = (p*6.02*1023)/Mw of DALI02 was calculated using the following metric, where p (g/L) and Mw (base pair) are the plasmid DNA concentration and relative molecular mass, respectively. Three standard curves were generated from at least five 10-fold plasmid dilutions, and Ct values were obtained by StepOne Plus software, with the Ct value as the y-axis and the logarithm of the copy number as the x-axis (Figure S2) [18]. The quantity of colonized *L. fermentum* DALI02 in the intestine of different groups of rats was expressed by CT values.

2.6.3. Residence Time of *L. fermentum* DALI02 in the Intestine

After gavage, rat feces were picked up every 12 h in sterile EP tubes and stored at −80 °C for storage. Using *L. fermentum* specific primers, the amount of strains in the fresh feces of rats was monitored at different times after cessation of the intervention until the quantity of colonization in the intervention group was not significantly different from that in the blank group, which was the time of colonization.

2.7. Determination of the Integrity of the Intestinal Barrier in Rats

Rat intestinal barrier integrity was determined by measuring lipopolysaccharide (LPS) and D-lactic acid levels in rat serum using an endotoxin assay kit (Shanghai Hualan Electronic Co., Ltd., Shanghai, China) and a D-lactic acid assay kit (Shanghai Hualan Electronic Co., Ltd., Shanghai, China).

2.8. Statistical Methods

Statistical analysis was performed using GraphPad Prism 9 (San Diego, CA, USA). The results are presented as the means and standard deviations, and comparisons among different groups were assessed by analysis of variance (ANOVA) with the Tukey post hoc test (one-way ANOVA-Tukey). Values of $p < 0.05$ were considered statistically significant. The graphics were created using GraphPad Prism 9 (San Diego, CA, USA).

3. Results

3.1. Effects of Probiotics on the Growth and Adhesion of L. fermentum DALI02

The growth curves of *L. fermentum* DALI02 in MRS medium with different prebiotics as the sole carbon source are shown in Figure 1A. The OD_{600} value in the sugar-free medium was always 0.21, indicating that a carbon source is necessary for the growth of *L. fermentum* DALI02. In addition, all five target prebiotics were utilized by *L. fermentum* DALI02, and the growth curves under 2% lactose, FOS, and GOS were more closely matched, with growth better than that of stachyose, inulin, and XOS.

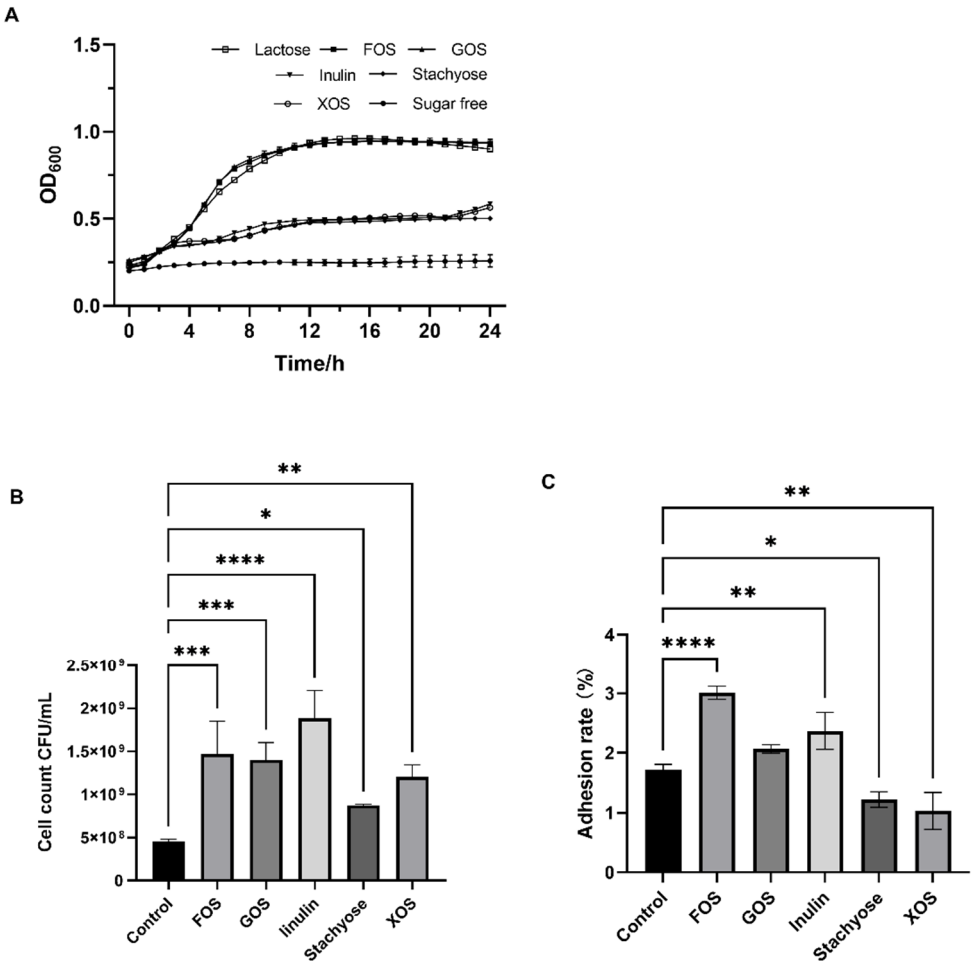


Figure 1. Effects of probiotics on growth, number of living bacteria, and adhesion rate of *L. fermentum* DALI02. (**A**) Effects on growth condition; (**B**) Effects on living bacteria; and (**C**) Effects on adhesion rate. * indicates a significant difference between groups ($p < 0.05$), ** indicates a significant difference between groups ($p < 0.01$), *** indicates a significant difference between groups ($p < 0.001$), **** indicates a significant difference between groups ($p < 0.0001$).

The viable bacterial counts of *L. fermentum* DALI02 in enzymatic whey supplemented with an additional 0.5% (w/w) prebiotics after 24 h are shown in Figure 1B. The addition of all five prebiotics significantly increased the viable bacterial counts of *L. fermentum* DALI02

($p < 0.05$), with inulin being the most significant, with the viable count increasing from $4.92 \times 10^8 \pm 0.91$ CFU/mL to $1.88 \times 10^9 \pm 0.32$ CFU/mL, up to 3.8-fold, followed by FOS, GOS, and XOS. The potentiation effect of different prebiotics on the in vitro adhesion of *L. fermentum* DALI02 is shown in Figure 1C. FOS and inulin were able to significantly increase the adhesion of *L. fermentum* DALI02 to Caco-2 cells ($p < 0.01$), and the adhesion rate increased from 1.72% ± 0.09 (control group) to 3.01% ± 0.11 and 2.37% ± 0.31, respectively, but stachyose and XOS significantly decreased the adhesion rate ($p < 0.05$).

3.2. Response Criteria for the RSM Analysis

The results of the single-factor experiment (Figure S1) show that the optimal concentration of prebiotics added varied among the different prebiotics, with the highest adhesion rates reached at 0.5% FOS, 1.5% GOS, 1.0% inulin, 1.0% stachyose, and 3.0% XOS, respectively, with 3.01%, 2.83%, 2.89%, and 1.91%, and 3.12%.

The experimental values obtained for the 36 experimental runs of the five-factor quadratic regression orthogonal rotational combination design are shown in Table S1. The parameter coefficients for each term in the mathematical model are presented in Table 2, which shows the specific parameters for the effects of linear, quadratic, and interactive effects of the five independent variables (FOS, GOS, inulin, stachyose, and XOS) on the adhesion rate of *L. fermentum* DALI02. The models exhibited non-significant misfit values ($p > 0.05$) and sufficient precision, which indicated that the model equations adequately described the effects of the independent variables on the final results. As shown in Table 2, the coefficient $R^2 = 0.7793$, indicating that the response values can be explained by the independent variables.

$$Y = 2.75632 - 15.02972\ X_1 + 5.90181\ X_3 + 1.06449\ X_5 - 88.62500\ X_1X3 + 26.29167\ X_1X_5 + 28.37500\ X_2X_4 \quad (1)$$

Table 2. Analysis of variance of regression equation for adhesion rate of *L. fermentum* DALI02.

	Sum of Squares	df	Mean Square	F Value	*p* Value	Significance
Model	28.98	20	1.45	2.65	0.0296	*
X_1	5.39	1	5.39	9.85	0.0068	**
X_2	0.0070	1	0.0070	0.0128	0.9114	
X_3	2.99	1	2.99	5.46	0.0337	*
X_4	0.0570	1	0.0570	0.1043	0.7512	
X_5	2.66	1	2.66	4.86	0.0435	*
X_1X_2	0.4456	1	0.4456	0.8146	0.3810	
X_1X_3	3.14	1	3.14	5.74	0.0300	*
X_1X_4	0.2730	1	0.2730	0.4991	0.4907	
X_1X_5	2.49	1	2.49	4.55	0.0499	*
X_2X_3	0.2525	1	0.2525	0.4616	0.5072	
X_2X_4	2.90	1	2.90	5.30	0.0361	*
X_2X_5	0.1871	1	0.1871	0.3420	0.5674	
X_3X_4	0.8327	1	0.8327	1.52	0.2363	
X_3X_5	0.6281	1	0.6281	1.15	0.3009	
X_4X_5	0.0297	1	0.0298	0.0544	0.8187	
X_1^2	0.0966	1	0.0966	0.1766	0.6803	
X_2^2	1.38	1	1.38	2.52	0.1334	
X_3^2	1.41	1	1.41	2.58	0.1290	
X_4^2	2.14	1	2.14	3.92	0.0665	
X_5^2	1.67	1	1.67	3.06	0.1007	
Residual	8.20	15	0.5470			
Lack of fit	1.13	6	0.1884	0.2398	0.9519	
Pure error	7.07	9	0.7860			
Cor total	37.18	35				

* indicates a significant difference between groups ($p < 0.05$), ** indicates a significant difference between groups ($p < 0.01$).

According to Equation (1), the effects of these five prebiotics on the adhesion rate of *L. fermentum* DALI02 were in the order of FOS > inulin > XOS > stachyose > GOS. The optimal formulations were obtained as 0.05% FOS, 0.20% GOS, 0.3% inulin, 0.20% stachyose, and 0.30% XOS. The theoretical adhesion rate of the strain was 4.04%, and the actual adhesion rate was 3.98 ± 0.12%, as verified by the experiment, which was not significantly different from the predicted value.

3.3. Effect of Intragastric Administration on Colonization Quantity

The content of *L. fermentum* in the feces of rats at 1 d, 1 w, 2 w, 3 w, and 4 w of intervention was measured, and the results are shown in Figure 2. The results showed that the fermented broth group and the fermented broth with prebiotics group were always significantly higher than the model group as well as the blank group ($p < 0.05$), and the fermented broth with prebiotics group was significantly higher than the fermented broth group for the remainder of the intervention time, except for 1 d of the intervention. This indicates that the presence of prebiotics can significantly increase the number of colonized strains ($p < 0.05$) and that a certain time is needed for this effect to take place. In addition, there was no significant difference in the time of intervention on the colonization of the fermented broth group ($p > 0.05$). The quantity of colonization was significantly higher after 1 w of the fermentation broth with prebiotics intervention ($p < 0.05$), but the quantity of colonization reached the maximum after 2 w and was not significantly different from 3 w and 4 w ($p > 0.05$).

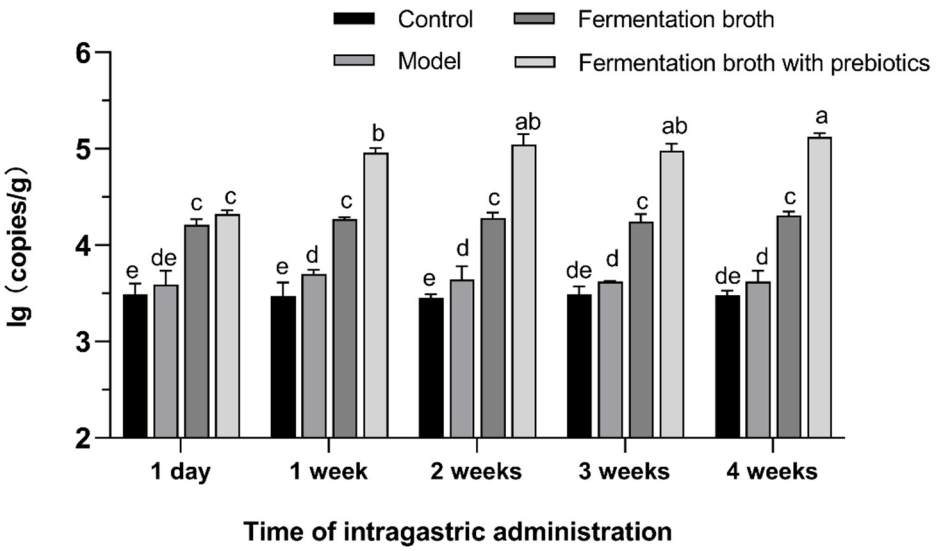


Figure 2. Effect of intervention time on colonization quantity of *L. fermentum* DALI02. a, b, c, d, e indicates a significant difference between groups ($p < 0.05$).

3.4. Effect of Intragastric Administration Time on Intestinal Residence Time

Gavage was stopped after 1 d, 2 w, and 4 w of intervention, and feces were collected at 0 d, 0.5 d, 1 d, 2 d, 3 d, 5 d, and 7 d, and the content of *L. fermentum* was measured (Figure 3). The intestinal residence of the strains at 1 d of intervention was approximately equal to that at 2 d. The residence time was longer in the fermented broth with prebiotics group than in the fermented broth group. After 2 w of intervention, the initial quantity of colonization was significantly higher in the fermented broth with prebiotics group (5 d) and fermentation broth group (3 d) than in the rest of the groups, and the residence time was extended. The residence time at 4 w of intervention was 7 d for the fermented broth with prebiotics group and 5 d for the fermented broth group. The results showed that with the extension of the intervention time, the intestinal residence time was also gradually extended, and the fermented broth with prebiotics group was always better than the fermented broth

group, that is, the addition of complex prebiotics could increase the adhesion capacity and intestinal residence time of *L. fermentum* DALI02.

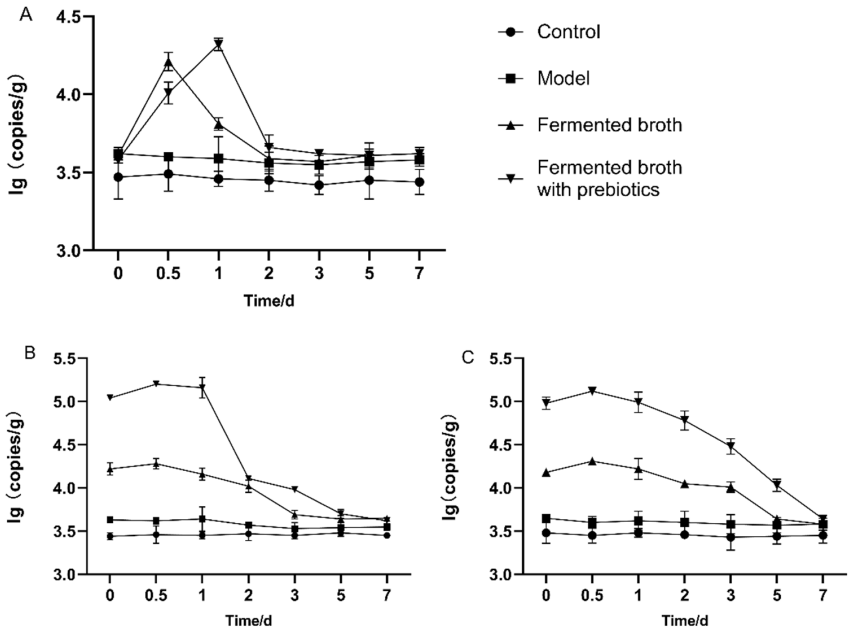


Figure 3. Effect of intragastric administration time on intestinal residence time. (**A**) 1 day; (**B**) 2 weeks; and (**C**) 4 weeks.

3.5. Intestinal Barrier Integrity in Rats

At the end of 4 weeks of gavage, fresh blood was taken from rats of different groups and their serum levels of LPS and D-lactic acid were measured (Figure 4). The levels of LPS and D-lactic acid in the serum of rats in the model group were 102.14 pg/mL and 51.03 μmol/L, respectively, which were significantly increased compared with the blank group ($p < 0.05$), indicating that the intake of CTX causes some damage to the intestinal barrier integrity of rats. After the intervention of the fermented broth group and the fermented broth with prebiotics group, the serum levels of LPS and D-lactic acid in rats were significantly downregulated compared with the model group ($p < 0.05$), being most significant in the fermented broth with prebiotics group, with LPS and D-lactic acid levels of 74.11 pg/mL and 40.33 μmol/L, respectively.

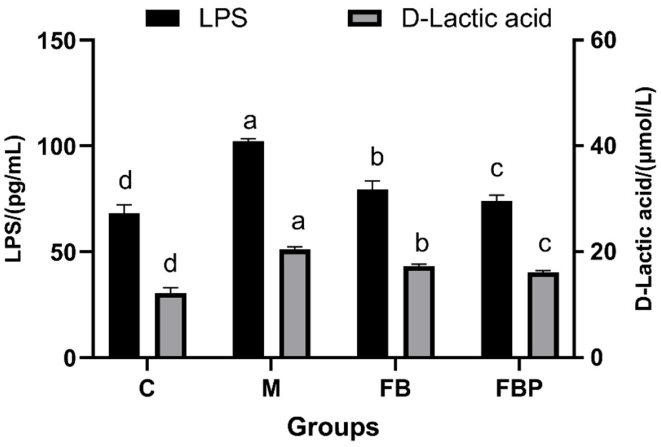


Figure 4. Effect of different intervention groups on the integrity of the intestinal barrier in rats. C: Control; M: Model; FB: Fermentation broth; and FBP: Fermentation broth with prebiotics. a, b, c, d indicates a significant difference between groups ($p < 0.05$).

4. Discussion

It is a key indicator to identify the adhesion in vitro when screening candidate probiotics. Adherence will promote the transient stay of the strain in the intestine and provide the possibility of effective colonization, thus providing sufficient response time for the strains to exert their functional properties in the intestine [19]. The addition of prebiotics is known to enhance the health benefits of probiotics, including the regulation of the structure of the intestinal flora via competing for colonization sites [20]. The number of colonized strains and their residence time are crucial for the realization of probiotic function. In this study, we found that the optimized complex prebiotics could significantly increase the viable number, the adherence in vitro, and colonization in vivo. The continuous intervention for 4 weeks would effectively prolong the colonization quantity and residence time of the strain.

There is variability in the proliferative effect of different prebiotics on strains [21]. Thus, it is a critical first step to explore the utilization of prebiotics by strains, which was usually determined by measuring the ability of strains to grow in a medium with prebiotics as a single carbon source [22]. *L. fermentum* DALI02 in this study was able to utilize all five prebiotics (FOS, GOS, inulin, stachyose, and XOS), but there were significant differences in utilization efficiency. The strain showed the best condition of growth in FOS and GOS, and the growth curve of the strain was close to the situation in lactose (Figure 1A). This is in agreement with the previous results that the strains utilized oligofructose better [23], while the strains utilized inulin less efficiently [24]. GOS and FOS, which were utilized less efficiently in this study, showed better results in previous studies [25]. These results once again suggested that the utilization of prebiotics by strains was specific. This has been revealed by previous studies by comparing genomic differences in transporter proteins, degradation enzymes, and transcriptional regulation of strains, which may be related to operons controlling the strains involved in metabolic enzymes [26]. Such results call for the need for researchers to screen for the selection of suitable prebiotics for specific probiotics.

It was found that additional prebiotics also significantly increased the number of viable bacteria in the presence of a carbon source (Figure 1B). Stachyose and XOS, which performed poorly in a single carbon source, showed promising results here. This suggested that there was a prioritization of carbon source use by the strains, and there was a synergistic effect among the different carbon sources. However, it was more noteworthy that the addition of different prebiotics positively modulated the adherence of the strains in vitro. The strains showed an increase and decrease in adherence compared with the blank group (Figure 1C). The adhesion of probiotics to intestinal epithelial cells is triggered mainly by the interaction between the mucosal layer secreted by the intestinal epithelium, the surface structures, and secretions of the bacteria. The mucosal layer of intestinal epithelial cells consists mainly of mucoprotein, glycolipid, electrolyte, etc. The substances associated with adhesion on the surface of the strain are mainly lipophosphatidic acid, surface-layer-associated protein, and mucin-binding protein [27]. In this study, prebiotics were involved in the fermentation process of the strain, which would produce a large number of metabolites, such as short-chain fatty acids, which would increase the viability of the intestinal epithelial cells. It would enhance the adhesion ability of the strain [28]. In addition, the structure of prebiotics may have a similar polysaccharide composition to the cell wall of *Limosilactobacillus*. This would also increase the adhesion efficiency of cell wall polysaccharides [13]. Prebiotics may enhance the receptor interaction with intestinal epithelial cells by modulating the ratio of unsaturated fatty acids in the cell membrane of the strain and by regulating the secretion of cell membrane surface proteins [11]. In addition, the decrease in adhesion rate could be due to the dose-dependent effect of prebiotic potentiation [29], which was verified in the subsequent concentration one-way experiments (Figure S1).

The effectiveness of probiotic function is dose-dependent. The concentration of probiotics required to obtain clinical effects is generally considered to require a concentration of 10^6 CFU/mL [30], a value that has become the lowest dose group in many studies exploring

effective doses of probiotic interventions. Many studies have shown that higher dose experimental groups tend to have better results [31]; thus, this study used 10^9 CFU/mL of fermentation broth for the intervention. In this study, we investigated the effect of prebiotics on the colonization of probiotic bacteria in the damaged intestine in an immunocompromised model of rats induced by CTX. The intestinal flora is disturbed and the intestinal mucosa is damaged [32]. In addition, it would affect the adhesion and colonization of exogenous probiotic bacteria. Gavage was performed according to the body weight of the rats at 100 g/mL. While the dose of a single intervention is important, there is, however, a significant effect on the frequency regarding the number of colonized strains and the residence time. It has been shown that the target strains could not be detected after 48 h of single gavage [33]. This is consistent with the results of the present study (Figure 3A), where at 48 h, the intervention groups were all close to the blank group. The residence time of strains also obtained an extension with the increase of the intervention time (intervention 1 d (48 h), intervention 2 w (72 h), and intervention 4 w (96 h)), and the residence time of the fermented broth group with prebiotics also obtained an extension from 72 h to 7 d. The prolongation of the intervention time may lead to some recovery of the already damaged intestinal flora of the rats, thus reaching relative homeostasis. According to the serum levels of LPS and D-lactic acid in rats, after 4 weeks of intervention, the levels in the fermented broth group and the fermented broth with prebiotics group were significantly reduced ($p < 0.05$), indicating that their intestinal barrier function was significantly restored and the intestinal mucosa was repaired, which may provide a basis for intestinal adhesion and colonization of the strain.

In terms of the quantity of colonization, there was no significant difference between the presence or absence of prebiotics after 1 intervention ($p > 0.05$). With the extension of the intervention time, there was no significant effect of the intervention time on the fermented broth group, and the number of *L. fermentum* was significantly higher in the fermented broth with prebiotics group than in the rest of the groups ($p < 0.05$), which indicated that prebiotics had a significant effect on the number of colonized strains ($p < 0.05$). In addition to the multiple reasons mentioned above, the addition of prebiotics would also lead to a sustained proliferation of *L. fermentum* already colonized in the intestinal tract.

5. Conclusions

In the present study, we report that the complex prebiotics can increase the adhesion capacity of *L. fermentum* DALI02 and their colonization parameters in immunocompromised model rats. We found that all five prebiotics (FOS, GOS, inulin, stachyose, and XOS) studied were utilized by *L. fermentum* DALI02, and the addition of all five prebiotics to enzymatically defatted milk increased the viable count of *L. fermentum* DALI02. The optimized fermentation broth with prebiotics increased the adherence of the strains to Caco-2 cells, the quantity of intestinal colonization, and the duration of colonization. Furthermore, the prolongation of the intervention time did not affect the quantity of colonization in the fermented broth group, but it did affect the quantity of colonization in the fermented broth with prebiotics group. The prolongation of the intervention time also prolonged the intestinal colonization time of *L. fermentum* DALI02. These results suggest that the complex prebiotics enhance the intestinal colonization of *L. fermentum* DALI02. This may be a strategy for developing functional foods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9010025/s1, Figure S1: Effect of different prebiotic additions on the adhesion rate of *L. fermentum* DALI02 A FOS B GOS C Inulin D Stachyose E XOS; Figure S2: Standard curve of plasmid of *L. fermentans*; Table S1: Results of quadratic regression rotation orthogonal experiment.

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