



Lotus seed oligosaccharides at various dosages with prebiotic activity regulate gut microbiota and relieve constipation in mice

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ABSTRACT

The aim of this study was to evaluate the effects of lotus seed oligosaccharides (formulation consisting of LSO2, LSO3-1, LSO3-2 and LSO4; relative ratios are 1.107:0.554:0.183:0.443, m/m/m/m) at dosages of 0.42, 0.83 g/d/kg bw and 2.49 g/d/kg bw on the microbiota composition and the propulsion of intestinal contents in the gut of mice. The results showed that fecal water content increased in treated mice; there was less gut microbiota diversity than in other groups; and there was a large number of fauna in the cecum of the mice. At the same time, the number of short-chain fatty acid (SCFA) bacterial producers increased after feeding with oligosaccharides; Lotus seed oligosaccharides (LOS) also enhanced the concentration of SCFAs in the intestine, which also increased the concentration of cytokines in the serum of mice. In conclusion, these findings suggest that LOS or combination with resistant starch has a better effect on relieving constipation.

1. Introduction

Constipation is a common chronic clinical gastrointestinal symptom, with high morbidity and complex pathology (Bharucha et al., 2013). Currently, an osmotic or secretory laxative is often used to relieve constipation, but it can easily cause damage to the colonic nervous system and cause additional symptoms. The gut microbiota is an important “microbial organ” of the human body, and its metabolites can regulate human health. Studies have shown that changes in the composition of gut microbiota may also increase the risk of constipation (Consortium, 2012). However, the composition of the intestinal microbiota can be modulated by the consumption of probiotics or prebiotics, which may help prevent or treat gastrointestinal diseases caused by dysbiosis of the microbiota (Rupa and Mine, 2012). Carbohydrate intake is an important factor regulating the growth of gut microbiota. In recent years, there has been a tendency to use prebiotics instead of probiotics because prebiotics have greater resistance to cross the digestive tract barrier, they are more the cost effective and are associated with reduced safety issues (Lu et al., 2019; Shang et al., 2017).

Prebiotics are defined as carbohydrates that are non-digestible by

the host in the absence of gut microbiota. Prebiotics (such as fructo-oligosaccharide (FOS), isomalto-oligosaccharide (IMO) and galacto-oligosaccharide (GOS)) or compound prebiotics have an important effect on gut microbiota. They can promote the growth of some gut bacteria such as *Lactobacillus* and *Bifidobacterium*, in addition to inhibiting the growth of harmful microbiota (De Filippo et al., 2010). Some studies have used oligosaccharides and probiotics to study their effects on constipation in mice (Wang et al., 2017b).

Lotus seeds are now used both as medicine and food in China. Lotus seeds have been used as food for regulating gastrointestinal functions in Japan, China, India and other countries for thousands of years (Guo et al., 2019). Previous studies have shown that lotus seeds contain high contents of oligosaccharides (Lu et al., 2017a). Oligosaccharides with prebiotic potential include LSO2 (Gal-β-1→4-α-Glc), LSO3-1 (Man-α-1→6-Glc-α-1→2-α-Fru), LSO3-2 (Man-α-1→6-Man-α-1→6-Glc-α/β) and LSO4 (Man-α-1→6-Man-α-1→6-Glc-α-1→2-α-Fru) (Lu et al., 2017b). However, the mechanisms of lotus seed oligosaccharides (LOS) on intestinal regulation *in vivo* are still not fully understood.

It is assumed that oligosaccharides can relieve constipation by stimulating the growth of beneficial bacteria or other microbiota in the

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intestinal tract of the host. But the mouse model of constipation induced by drugs generally cannot reflect the real microecological environment of mice under normal physiological conditions. Therefore, this study used different doses of highly purified lotus seed oligosaccharides (LOS) (mixed oligosaccharides with LSO4: LSO3-2: LSO3-1: LSO2 (relative ratios: 1.107:0.554:0.183:0.443, m/m/m/m) prepared from dried lotus seed by using medium pressure liquid chromatography) or mixed lotus seed-resistant starch (LRS3) to feed healthy mice to determine the changes in the gut microbiota and the effects of LOS on the propulsion of intestinal contents in mice. Studies have shown that *Bifidobacteria* species have different preferences for LOS and LRS3 (Lu et al., 2017b). Therefore, there are several aims in this study: 1) To determine whether LOS affected body weight, food intake, fecal water content, ink propulsion and production of SCFAs of BALB/c mice, 2) which oligosaccharide was most effective in relieving constipation. 3) To evaluate the relationship between composition and relative abundance of different microbiota on the serum factor and related protein expression; 4) To determine possible pathways of LOS and microbiota in relieving constipation. The results can provide possible improvement and treatment for symptoms of constipation.

2. Materials and methods

2.1. Materials

Fresh lotus seeds were obtained from Green Acres (Fujian) Food Co., Ltd., Fujian, China. α -Amylase (10,000 U/mL) was purchased from ANKOM (New York, USA). Glucoamylase (300 U/mL) was acquired from Aladdin Reagent Co., Ltd. (Shanghai, China). Acetic acid, formic acid, and butyric acid were purchased from Aladdin Reagent Co. (Shanghai, China). Fructooligosaccharide (Meiologo-P[®]) was purchased from Meiji Seika Kaisha Ltd. (Tokyo, Japan) and contained 30% 1-kestose, 57% nystose, and 13% 1F- β -fructosylmaltose. All other chemicals and reagents used in the experiment were analytical grade.

2.2. Lotus seed oligosaccharide preparation

Based on the method described by Lu et al. (2017b) of collecting each monomer component of LOS (LSO4 LSO3-2, LSO3-1, LSO2) and mixing them in the original ratio. The oligosaccharide solutions were freeze-dried after rotary evaporation (Model fd-4c-80, Beijing Fuyikang Instrument Company, Beijing, China) to obtain LOS powder.

2.3. Preparation of lotus seed-resistant starch (GP-LRS3)

According to the methods reported by Zeng et al. (2015). Lotus seed starch was fixed with citric acid-disodium hydrogen phosphate buffer, followed by the addition of a high temperature-resistant α -amylase at 90 °C in an orbital incubator shaker (SHA-C, Guo Hua Electric Appliance, Changzhou, China) at 128 rpm for 2 h. After adjusting the pH to 4.5 with a citric acid solution (4 mol/L), glucoamylase (300 U/ml, obtained from Sigma, St. Louis, USA) was added (5000 U/g of starch) and incubated at 60 °C for 1 h in an orbital incubator shaker at 128 rpm. The samples were centrifuged at 3000 g for 15 min, and the supernatant was discarded. The resulting precipitate was washed with distilled water, and then the above processes were repeated twice, and then the sample was dried at 50 °C and crushed and sifted through a 185- μ m mesh screen. Finally, high-purity LRS (GP-LRS3) was obtained.

2.4. Animal feeding trials

Prior to running the experiments the animal rooms, cages and water bottles were disinfected. The temperature of the animal room stayed at 25 ± 1 °C, the relative humidity was $60 \pm 5\%$, and the artificial lighting period was 12 h/day (6:00–18:00 every day). Every night, the basic diet, water and disinfected bedding were replaced. Table 1 shows

Table 1
Composition of the experimental diets.

Ingredient	Quality ratio (%)	Ingredient	Quality ratio (%)
Corn Starch	64	Lard	4.85
Casein	12	Lysine	0.92
Maltodextrin	4	Methionine	0.62
Cellulose	3	Sucrose	0.15
Soy Protein	7	Choline Chloride	0.1
NaCl	0.2	Microelement Premix	0.12
CaHPO ₄	1.8	Vitamin Premix	0.04
CaCO ₃	1.08		

Microelement Premix: including FeSO₄·7H₂O, CuSO₄·5H₂O, MnSO₄·H₂O, ZnSO₄·7H₂O, Na₂SeO₃, KI.

Vitamin Premix: including VE, VB₁, VB₆, VB₅, VB₁₁, biotin and other vitamins.

that the ingredients of the feed met the GB 14924.3–2010 standard of the People's Republic of China. Each mouse was housed separately to allow free eating and to ensure the accuracy of fecal collection. For identification purposes each mouse was numbered with chrysolepic acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) in order. To acclimatize the mice the animals were fed, a basic ration (specific ingredients are listed in Table 1, Jiangsu Xietong Organism Engineering Co Ltd., Nanjing, China) and water ad libitum for 1 week. Subsequently each mice was assigned to a particular diet for the experiment n = 14/group. All of the animal experiments used in this study were performed in compliance with the *Guidelines for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication 85–23, 1996).

The mice in different groups were given the above solutions at 9:00 a.m. every morning over 15 consecutive days by gastric perfusion. The gastric perfusion volume of each mouse was 0.2 mL/10 g bw. According to the content of the Chinese Food and Drug Administration Grant No. (G20040234), the recommended dietary allowance of stachyose was 5 g/d in each adult (60 kg (bw)). The RDA of mice that weighed 20 ± 2 g was equivalent to 0.083 g/d/kg bw for humans. The study divided mice into 3 dose groups that were 5, 10 and 30 times the intake of the stachyose RDA of adult humans.

Eighty-four BALB/c mice (SPF level, 4 weeks of age, weight approximately 16.2–17.8 g, animals certificate number: 2015000524782, SLAC Laboratory Animal Co., Shanghai, China) were randomly divided into six groups of 14 animals (50:50 males:females) as follows: (1) blank control group: consumed sterile distilled water daily; (2) low dose LOS group: consumed 0.42 g/kg bw of lotus seed oligosaccharides; (3) medium dose LOS group: consumed 0.83 g/kg bw of lotus seed oligosaccharides; (4) high dose LOS group: consumed 2.49 g/kg bw of lotus seed oligosaccharides; (5) medium dose of both LOS and LRS3 control group: consumed 0.21 g/kg bw of lotus seed oligosaccharides and were fed ad libitum with a diet of 5% resistant starch on the same day (after crushing ingredients for the basic ration shown in Table 1, the same amount of GP-LRS3 was provided instead of the 5% corn starch, followed by extrusion molding and drying to a steady mass at 50 °C); and (6) FOS control group: consumed 0.83 g/kg bw of FOS. The various oligosaccharides were dissolved at the corresponding dose in sterile water (high-pressure moist heat sterilization at 121 °C for 15 min).

Fresh fecal samples were collected and placed in a dried sterile test tube every 24 h immediately prior to gastric perfusion. Feeding of mice was stopped to retain fasting for 12 h before the mice were killed. The normal chow were given to ensure adequate contents in the cecum and colon, as well as to record food intake. All animal tests were according to the guidelines of and approved by the Animal Health and Use Committee of the Experimental Animal Center of Fujian Medical University (Facility license number: SCXK (Min) 2012–0001; animal ethics review table number: 2017–020).

2.4.1. Determination of food intake and weight gain

During the experiment, mice were given adequate amounts of basic rations. According to the determination of the total feed, daily surplus feed and weight, the following formula was used to calculate daily intake and weight gain. The mice were weighed, and the daily feces were weighed by wet weight (m_1). Feces were dried at 80 °C for 10 h and weighed to determine the dry weight (m_2). The fecal water content (FWC) was calculated by formula (1). In addition, the daily feed intake (DFI) of each group of mice was calculated according to formula (2).

$$FWC = \frac{(m_1 - m_2)}{m_2} \times 100\% \quad (1)$$

$$DFI(\text{g/group}) = \text{The total feed on one day(g/group)} + \text{The surplus feed on one day(g/group)} - \text{The surplus feed on next day(g/group)} \quad (2)$$

2.4.2. Sample collection

2.4.2.1. Fecal collection. During the experiment, the feces of a mouse from a single cage in each group were separately transferred to 10 mL sterile centrifuge tubes. The samples were mixed in each group and stored immediately at -80 °C in an ultralow temperature freezer (Thermo Fisher Scientific, Waltham, MA).

2.4.2.2. Blood collection. The blood of the mice was collected by eyeball extirpation on the 15th day. In addition, 2 mL of arterial blood was centrifuged at 4 °C (Heraeus Biofuge Stratos Centrifuge; Thermo Fisher Scientific, Inc), and the serum collected. The blood levels of motilin, gastrin, endothelin, substance P, somatostatin, acetylcholine enzyme and vasoactive intestinal peptide in the serum of mice were tested using the following radioimmunoassay kits: motilin ELISA kit, gastrin ELISA kit, endothelin ELISA kit, somatostatin ELISA kit, acetylcholinesterase ELISA kit, substance P ELISA kit and vasoactive intestinal peptide ELISA kit (Beijing Puer Weiye Biotechnology Co., Ltd., Beijing, China). The tests were performed following the manufacturer's instructions.

2.4.3. Intestinal content collection

Each mouse was euthanized by dislocation of the cervical vertebrae. The abdominal cavity was dissected under sterile conditions to intercept the cecum and colon. The contents were placed in the 1.5 mL sterilized centrifuge tubes with a sterile cotton swab and then weighed immediately, followed by immediate storage in a -80 °C ultralow temperature freezer.

2.4.4. Determination of short-chain fatty acids (SCFAs) and lactic acid in the feces of mice

Quantitative analysis was performed by the gas chromatography method according to the method reported by Feria-Gervasio et al. (2011). The methodological validation of the established GC method is based on the standards of the Food and Drug Administration (FDA) for bioanalytical method validation. The lowest limit of detectability (LLOD) for each analyte was equal to 5 times the concentration (peak area) of each analyte, which was relative to the noise signal. Therefore, the minimum concentration of a standard analyte was determined, which was added to 5 blanks. (Each standard analyte was divided to 10 concentration gradients, and each concentration was measured three times in parallel.) By means of determining the recovery rate to ensure the accuracy of the method, the recovery rate was calculated by formula (3):

$$RR\% = \frac{c_1}{c_2} \times 100\% \quad (3)$$

where c_1 is the tested concentration, and c_2 is the known concentration of the standard sample added to the fecal culture. Inter-assay precision is the relative standard deviation percentage (% RSD), which was used

to analyze the determination of each analyte from the same sample (fecal culture) on different days. Each sample was measured independently 5 times. The results showed that concentration was the horizontal coordinate and the peak area was the longitudinal coordinate for plotting a standard curve to ensure that the RSD of the standard analyte was under 5%, to determine the appropriate concentration ranges. The standard curve and the corresponding concentration ranges of the test are shown in Supplementary Table 1.

For analysis, 0.5000 g feces was put in a 5 mL centrifuge tube. After cooling in an ice bath for 10 min, 4 mL of sterilized deionized water was added and then stirred by magnetic rotation for 2 min or longer until the feces was completely dissolved. The fecal suspension was centrifuged at 5000 × g (4 °C) for 20 min. The resulting supernatant was centrifuged again. The second centrifuged supernatant was collected and then injected into a 1.5 mL sample bottle after filtering through a 0.45 μm mesh. Three independent replicates were carried out for each sample. The content of SCFAs in the feces was determined under the same GC-MS conditions. The total SCFA concentration was the sum of the concentrations of acetic acid, lactic acid, propionic acid and butyric acid in feces.

2.4.5. Extraction of DNA from fecal microbiota of mice and analysis of PCR products by denaturing gradient gel electrophoresis (DGGE)

According to the methods reported by Zhou et al. (2014).

2.4.6. Analyses of diversity, homogeneity and principal components

The DGGE profiles were analyzed by QUANTITY ONE (version, 4.3.0, Bio-Rad, Laboratories, Inc, USA) for quantitative analysis of the number of electrophoretic bands and the density of the bands. The results were used for analyses of diversity and principal components of intestinal and fecal microbiota. The Shannon-Weiner index (H), abundance (S) and the valence index (E) were used to compare the diversity of different treated bacteria. The specific algorithms are shown in equations (4)–(6):

$$H = - \sum_{i=1}^s \frac{n_i}{N} \ln \frac{n_i}{N} = - \sum_{i=1}^s p_i \ln p_i \quad (4)$$

$$E_H = \frac{H}{H_{\max}} = \frac{H}{\ln S} \quad (5)$$

$$N = \sum n_i \quad (6)$$

where H is the Shannon-Weiner index ($0 < H < \ln S$); E_H is the evenness index; p_i is the ratio of the grayscale of a single band to the total strength of all the bands of the sample; n_i is the abundance of the i -th band; and S is the sum of all the bands in the sample. The major components of the microbiota structure were analyzed according to the brightness and position of the different bands by CANOCO software.

Subsequently, the sequences were submitted to the GenBank database. The Blast procedure in GenBank was used to compare homologies and obtain the 16S rDNA sequences of the most similar strains. The neighbor-joining method by MEGA version 5.0 software was used to construct phylogenetic trees, and the bootstrap number was 1000.

2.5. Evaluation of the promotion of the fecal excretion function in mice

2.5.1. Preparation of ink and loperamide hydrochloride solution

The experiment was performed according to the “verification method of laxative function” of the “Technical specifications of health food inspection and evaluation, version 2003” from China. Accurately weighed 100 g gum arabic was mixed with 800 mL water and boiled until the solution became transparent. Activated carbon (powder) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was weighed, and 500 g was mixed with the above solution. The solution was boiled 3 times, and distilled water was added up to 1000 mL when the solution was cold. Then, the solution was stored in the refrigerator at 4 °C and

shaken well before using.

2.5.2. The excretion time of black feces

Ninety-eight mice from the same source were divided into seven groups and subjected to gastric perfusion as described below. Mice of each group were injected with 10% ink by gastric perfusion at 9 o'clock every morning from the seventh day up to the 15th day; all mice were injected with ink by gastric perfusion after 24 h of fasting. The volume of perfusion of each mouse was 0.2 mL/10 g bw. The blank control group were injected with the same mass of gum arabic (0.2 mL/10 g bw) without ink. The model group (MOG) were injected with the same volume of loperamide hydrochloride solution by gastric perfusion. The mice in each group were used to determine the time of first excretion of black feces. Finally, the mice were euthanised and autopsied after the gastric perfusion of ink.

2.5.3. Small intestine ink propulsion experiment

Mice were euthanised by dislocation of the cervical vertebrae after gastric perfusion of ink. The abdomen was dissected and the mesentery of the mouse was separated. In addition, the whole intestine from the pylorus to the ileocecal was clipped. The whole small intestine was straightened into a line on absorbent paper to measure the length of the intestine, which was the total length of the small intestine. The length from the pylorus to the front of the ink was the "ink propulsion length". By measuring the ink propulsion length in the small intestine, the propulsion rate was calculated according to formula below (7).

$$\text{Small intestine propulsion rate (\%)} = \frac{\text{Moving distance of ink}}{\text{Length of small intestine}} \times 100\% \quad (7)$$

2.5.4. Determination of reverse transcription PCR (RT-PCR)

Total RNA was extracted from tissue by Trizol (Invitrogen, Carlsbad, CA, USA). After determining the purity by ultraviolet radiation, the concentration of total RNA was adjusted to the same level in each group. The same amount of RNA (2 µg) from the sample was added to 1 µL oligodT18, RNase, and dNTP and 10 µL 5 × buffer MLV enzyme (Promega, Wisconsin, USA). The cDNA was synthesized in 10 µL body fluids for 120 min at 99 °C, for 4 min at 4 °C, and for 3 min at 37 °C. Reverse transcription and amplification were performed for the target genes c-Kit, stem cell factor, glial cell line-derived neurotrophic factor, transient receptor potential vanilloid 1, and nitric oxide synthase (Supplementary Table S2). Quantitative real-time RT-PCR was performed using an Applied Biosystems Sequence Detection System 7900 (ABI Prism 7900HT, Applied Biosystems, Foster City) with 10 mL of a mixture comprising Power SYBR GREEN PCR Master Mix (Applied Biosystems), 500 nmol of each primer and 300 ng of the complementary

DNA template. The reaction conditions were denaturation at 95 °C for 5 min, annealing at 58 °C for 50 s, extension at 72 °C for 90 s and cycling the above steps 40 times. Finally, the sample was extended at 95 °C for 10 min. Then, the expression of the final product was determined by 2% agarose gel electrophoresis. qRT-PCR and data collection were performed by the ABI PRISM 7900HT sequence detection system.

2.5.5. Determination by western blots

According to the methods reported by Li et al. (2017), protein lysate was added to the tissues, and the sample was washed with precooled PBS three times. The resulting mixture was decomposed at 4 °C and centrifuged (9000 g/min) for 15 min. Then, the supernatant containing protein mixed with buffer was extracted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A primary antibody was placed in the solution after SDS-PAGE and moved to a polyvinylidene fluoride (PVDF) membrane. The protein was kept at 4 °C overnight. The horseradish peroxidase-conjugated secondary antibodies were incubated with protein at room temperature. Finally, the immunoreactive protein was tested by an enhanced chemiluminescence assay kit (GE Healthcare, Uppsala, Sweden), and β-actin was the internal reference. The bands were displayed by an enhanced chemiluminescence system (ECL, Thermo Fisher Scientific, MA, USA) and LAS image software (Fuji, New York, NY, USA).

2.5.6. Statistical analysis

The figures were drawn by the software Origin Pro 9.0 (Origin Lab Corporation, USA). All statistical analyses were performed using Data Processing System software (DPS, V9.05, Science Press, Beijing, China). One-way ANOVA, followed by Duncan's post-hoc test, was used to determine the significant differences between treatment groups. $p < 0.05$ was considered statistically significant; all tests were two-sided, and no corrections were applied for multiple significance testing. All the experiments were repeated at least three times. All distributions are presented as the mean value ± standard deviation.

3. Results and discussion

3.1. Effects of lotus seed oligosaccharides on mouse weight

The effects of oligosaccharide and resistant starch on mice weight are shown in Fig. 1A. During the experiment, the mice were healthy. Their fur was smooth and did not fall out, the defecation was regular and no mice unexpectedly died or became ill. As the experiment progressed, the weights of mouse increased. After the 15th day, the weight gain sequence was the blank control group > low dose of LOS group > medium dose of LOS group > FOS control group > high dose of LOS group > medium dose of both LOS and LRS3 control

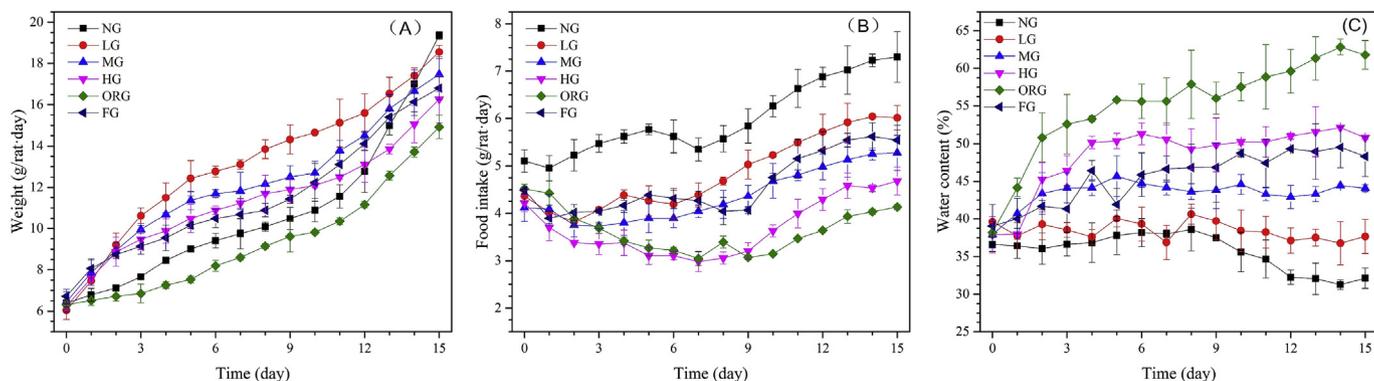


Fig. 1. Effects of the intake of LOS, FOS, LRS3, or their mixture, for two weeks, on mice weight (A), food intake (B), and fecal water content (C). Data in the figure represent the mean ± SD of 14 mice in each group. NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group.

group.

Compared to the NG, the weight gain of mice injected with increasing doses of lotus seed oligosaccharides by gastric perfusion was significantly reduced. In the first 11 days, the weight gain of mice fed LOS was higher than that of the blank control group. Feeding LOS and FOS was beneficial for keeping the mouse weights stable. This result was in accordance with the result obtained by Caroline Thum et al. (2016). Feeding with caprine milk oligosaccharides and GOS may help to increase the weight in early stages and extend the growth cycle and muscle mass of the weaning period in mice. In the medium term of 30 days, the main form of metabolism slowly turned to carbohydrate metabolism. Oligosaccharides increased short-term weight gain and produced insulin resistance through intestinal intake, and the glucose transport protein expression pathway was changed. These differences persisted into adulthood (Thum et al., 2016). Mice fed with both LOS and LRS3 had the lowest weight gain. This may be related to satiety and low food intake caused by feeding with oligosaccharides and resistant starches. Dietary fiber and some FOS were proven to limit fat storage. This is mainly reflected by the inhibition of short-term food intake.

The influence of food intake of mice is shown in Fig. 1B. After 15 days, the sequence of food intake in different groups was the blank control group > low dose of LOS group > FOS control group > medium dose of LOS group > high dose of LOS group > medium dose of both LOS and LRS3 control group, which was similar to the tendency of weight change in the mice. However, compared to the mouse feces from the FOS positive or blank control groups, feeding with high doses of LOS and LOS-LRS3 mixture helped mice to reduce weight gain and food intake. After that, the intake of mice in all treatment groups increased slowly. This phenomenon showed that food intake was an important factor on the mouse weight change.

The feces water content of all animals sustained a high water content (Fig. 1C). Functional oligosaccharides and resistant starch induce intestinal microbiota to produce SCFAs to acidify the contents of the intestinal tract. This results in stimulating the intestinal peristalsis and increasing the moisture content of feces. If the fecal water content was high, the fecal propulsion was so good that it reflected good gastrointestinal motility. Additionally, the possibility of constipation was reduced in these mice (Thum et al., 2016).

3.2. Microbiota structure of diversity results in mice

PCR-DGGE technology is widely used to monitor microbial community diversity and population dynamics. The different bands represent different bacterial families in each lane. The structure and relative quantity of the bacteria in the intestinal track and feces of the LOS-treated mice is shown in Fig. 2. The letters and numbers represent different lanes and band numbers, respectively. Different positions of the lanes represent different gut microbial species. The more bands present was the more abundant number of microbial families or genus in the sample. The brighter the highlight, the greater the number of corresponding strains represented by the bands. It was shown by the gray value of the band 2B that the bacteria in each band were well separated. Thirty-two clear bands were identified from positions in seven lanes. The standard was the blank control group in which the number of families was 100%. The next highest number of families was in the FOS control group (72%) and cecum groups with a medium dose of LOS treatment by gastric perfusion. The lowest microbial diversity was in the colon group with a medium dose of LOS treatment by gastric perfusion. The dose effect was not obvious in the low dose group, medium dose group and high dose group most likely because high doses of LOS would inhibit the growth of some species (The relative abundance of families: medium dose group > low dose group > high dose group). From the specific distribution of the profiles, the blank and FOS control groups were similar to the low dose of LOS group, and there were little differences between the colon and cecum samples of mice from the mice of medium dose of LOS groups, the high dose of LOS

group and the medium dose of both LOS and LRS3 control group. This indicated that there was no difference in the structure of the bacterial community among these samples. It also showed that a low dose of LOS had little effect on the gut microbiota of mice. The relative abundance of families in the colon and cecum of mice was similar and in this regard the microbiota in the mouse intestinal tract differs from that of humans. The human intestinal fermentation area is located in the colon, while the microbial fermentation in mice is located mainly in the cecum (Campbell et al., 1997). Therefore, microbial abundance of the cecum was highest as evidenced by the higher number of electrophoretic bands.

The bacterial structure of the diversity analysis results is shown in Table 2. The diversity index was used to evaluate the diversity of bacteria. In addition, evenness was used to indicate the average levels of distribution of species. The result shows that compared to the blank control group, there was no significant difference between evenness of different samples. The results showed that the distribution of the microbiota in the intestinal tract and feces was uniform before and after gastric perfusion of LOS, mixture and FOS. However, the diversity index and abundance of the gastrointestinal and feces samples of oligosaccharides treated mice were higher. These results showed that oligosaccharides could reduce the diversity of bacterial composition in the gastrointestinal of mice after digestion. This is probably because the bacteria from different species respond differently to the polysaccharides injected. The species with strong tolerance to high doses of oligosaccharides survived while the viability of families such as with poor tolerance reduced. As a result, there was reduced diversity of the microbiota in those groups fed oligosaccharides and resistant starch. In addition, these results may also be related to the impact of some groups of bacteria on each other such as antagonism and growth promotion. Compared with the colon groups and medium dose of LOS group, the diversity index of cecum and medium dose of LOS groups were higher. It showed that the microbial diversity increased as fecal propulsion progressed in the intestinal tract, while the medium dose of both LOS and LRS3 control group had a lower diversity. It was shown that the addition of LRS3 was helpful to the formation of dominant families in the gut microbiota of mice.

According to the UPGMA algorithm, a similarity analysis of sample bands was performed. The genetic similarity Jaccard index was used to represent the genetic relationships among bands. From Fig. 3A, the microbiota of colon and cecum groups were most similar, (Jaccard index reached 0.84). The next was the high dose of LOS group and the FOS control group. The results showed that the high dose of LOS could change the gut microbiota composition of mice, which was similar to the effect of FOS. The Jaccard index of the medium dose of both LOS and LRS3 control group and the previous two groups was 0.70. The gut microbiota of the high dose of LOS group and medium dose of both LOS and LRS3 control group may have as many bacterial structures as the FOS group in the intestinal tract of mice. Fig. 3B shows further differences in the composition of intestinal and fecal bacterial structures in mice with different treatments. The closer the test points on the PCA distribution figure, the greater the similarity of the bacterial composition. The medium dose of both LOS and LRS3 control group was mainly distributed in the first quadrant; The high dose of LOS group and the FOS control group were mainly distributed in the second quadrant; The blank control group, low dose of LOS group, and medium dose of LOS group were distributed in the third quadrant; and the colon and cecum groups were mainly distributed in the fourth quadrant. In different quadrants, the bacterial diversity of treatment groups was similar. The results of the PCA analysis were consistent with the clustering analysis of bacterial community structure. Except for the low dose of LOS group, the bacterial structure of the mice in different dose groups was different to that of the blank control group. This indicated that the intake of oligosaccharides is the main factor affecting the microbiota. The medium dose of both LOS and LRS3 control group, colon and cecum groups clustered together. The results showed that the structure of the

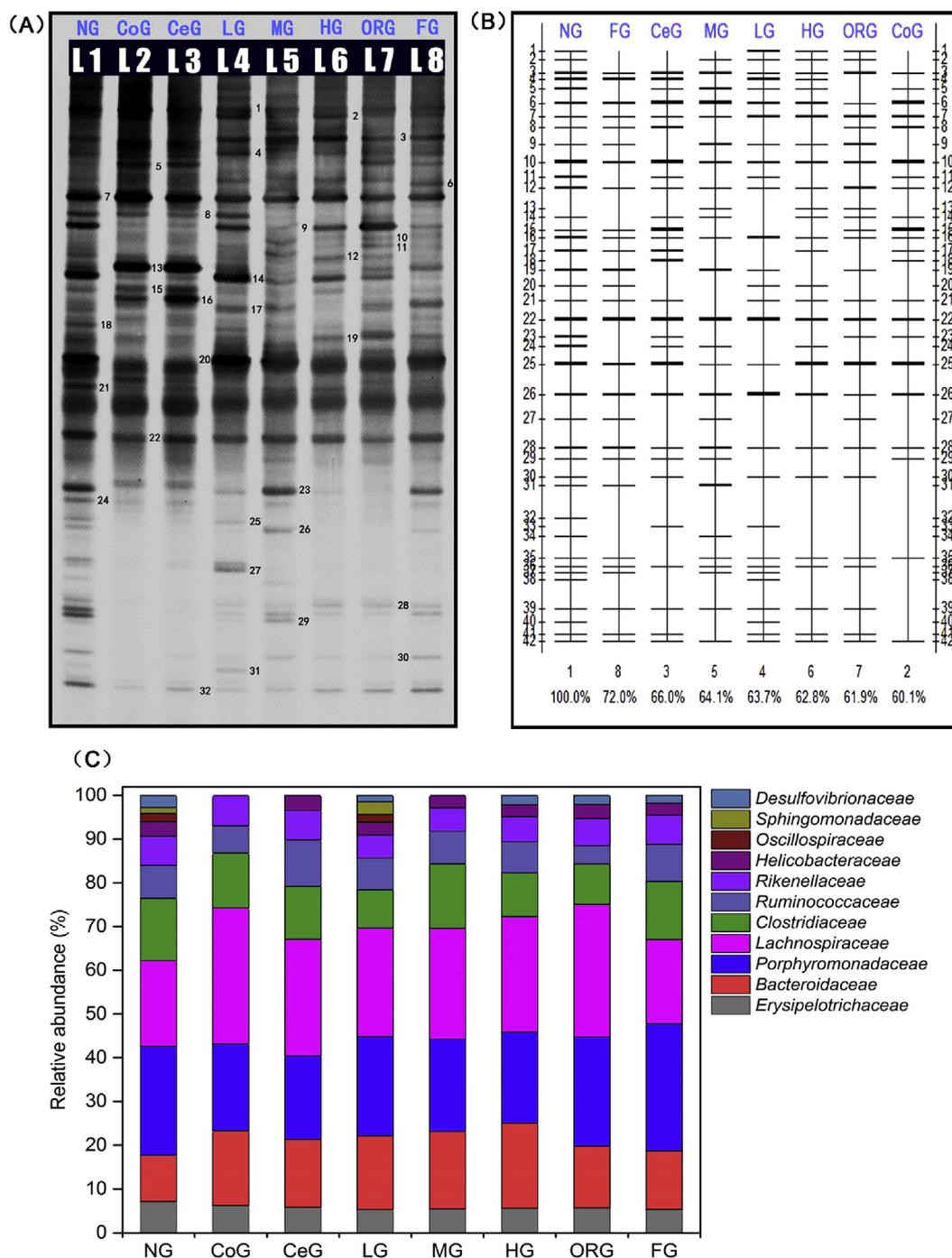


Fig. 2. DGGE profiles (A) and quantitative level (B) of bacteria in the intestinal tract and feces of mice, (C) Structural comparison of gut microbiota at the family level.

NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group. CoG: Colon samples of mice from MG; CeG: Cecum samples of mice from MG.

carbon source fed and the animal tissue areas might be important factors affecting the quantity and composition of gut microbiota.

3.3. DGGE gel band-recovered sequence analysis results

The bands were sequenced by gel-cutting, and homogeneity analysis was performed on the NCBI website. Exact analysis results are shown in Table 3. Bands were selected for gel sequencing. The results were compared in the NCBI website and used to perform phylogenetic analysis. Compared with data from Table 3 and Fig. 2, the sequencing

results showed that compared with the blank control group, groups given oligosaccharides or resistant starches, even with increasing doses of LOS, had significantly decreased numbers of Erysipelotrichaceae in the intestinal tract of mice (band 1). The decrease in Erysipelotrichaceae numbers have been reported in some patients with metabolic disorders such as obesity or steatosis or in an animal model of colorectal cancer, but the causative links are not established (Zhu et al., 2014b).

Bands 2, 3 and 8 belonged to the Bacteroidaceae family, which had a greater number in the colon and cecum groups, which was slightly

Table 2
Alpha diversity analysis in mice by diet.

Diet	Shannon-Wiener Diversity index (H')	Evenness(E)	Richness(S)
NG	3.46	0.990	33
CoG	3.06	0.989	22
CeG	3.18	0.988	25
LG	3.14	0.988	24
MG	3.24	0.994	26
HG	3.11	0.992	23
ORG	3.07	0.995	22
FG	3.16	0.995	24

NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group. CoG: Colon samples of mice from MG; CeG: Cecum samples of mice from MG.

increased in the high dose of LOS group, the medium dose of both LOS and LRS3 control group and the FOS control group. *Bacteroides acidifaciens* (band 2) was high in the high dose of LOS group, and *Bacteroides oleiciplenus* (band 8) was high in the low dose of LOS group. *Bacteroides* can degrade carbohydrates and metabolic intermediates. At the same time, they can produce SCFAs, such as acetic acid, lactic acid, propionic acid, and other fatty acids. The increased number of *Bacteroides acidifaciens* has been shown to significantly reduce body weight and body fat content in mice. It has the potential to treat traditional metabolic diseases such as diabetes and obesity (Yang et al., 2017). The carbohydrates promoted the growth of *Bacteroides graminsolvans*. *Bacteroides oleiciplenus* is an obligate anaerobic bacterium, which was isolated from human feces. It can metabolise glucose into acetic acid, succinic acid, formic acid and lactic acid (Watanabe et al., 2010). High concentrations of LOS can inhibit its growth in mouse feces in this study.

Bands 4, 11, 19, 20, 23, 29, 30 and 32 belonged to the Porphyromonadaceae family. It is one of the dominant families in human feces. Porphyromonadaceae is generally involved in complex carbohydrate and protein fermentation (Wang et al., 2016). Except for bands 23 and 29, compared with the blank control group, the relative proportion of Porphyromonadaceae decreased significantly after LOS treatment by gastric perfusion. The relative abundance of Porphyromonadaceae decreased at high carbohydrate concentrations. If the number of Porphyromonadaceae increased to a certain extent, it may have led to cognitive deficits (Wang et al., 2017b). However, the presence of *Parabacteroides distasonis* reduces the possibility of colitis. Resistant starch contributed to increasing the number of *Parabacteroides*

distasonis (Martínez et al., 2010). This may be one of the reasons that a high abundance of *Parabacteroides distasonis* was in the medium dose of both LOS and LRS3 control group. In addition, high doses of LOS gastric perfusion also help to enhance the abundance of *Parabacteroides distasonis*.

The Lachnospiraceae family (bands 5, 7, 12, 14, 15, 16, 17 and 31) contains *Butyrivibrio*, *Lachnospira* and *Roseburia* species. and was detected at high relative abundance in the colon and cecum of mice. These genus are associated with the fermentation of a variety of carbohydrates, but it cannot metabolise amino acids. Lachnospiraceae is abundant in the digestive tract of many mammals. This family is associated with the production of butyric acid in the gastrointestinal of humans, which is an important for both for the growth of microbiota and host epithelial cells. Some studies have shown that it may play a role in the prevention of obesity and colon cancer in humans (Meehan and Beiko, 2014). *Butyrivibrio* species were associated with bands 12 and 14. and correlated with the high dose of LOS and ORG group. This species is known to produce SCFA such as lactic, acetic and butyric acids.

The Clostridiaceae family was identified from bands 6, 22, 24 and 26 LOS by gastric perfusion significantly decreased the relative abundance of a number of Clostridiaceae. Clostridiaceae colonizes the gastrointestinal of infants in addition to the strict anaerobic bacterium such as Bifidobacteriaceae a few days after birth. Until the age of 1, Clostridiaceae dominates the microbiota (Wegh et al., 2017). *Clostridium thermosuccinogenes* (band 6) produced acetic acid, lactic acid, succinic acid, and H₂ through the fermentation of inulin. The metabolites of *Clostridium sporosphaeroides* (band 22) were a large amount of acetate and butyrate, and a small amount of *Clostridium xylanovorans* (band 26) can ferment raffinose, sucrose, xylan, mannitol, cellobiose, galactose, mannose and melibiose to produce formate, acetate, butyrate, H₂, CO₂, isopropyl butyrate and ethanol (Mechichi et al., 1999). *Clostridium grantii* (band 24) can ferment alginate, cellobiose, glucose, maltose, mannose and fructose to produce acetate, ethanol, formate, and carbon dioxide (Rainey et al., 2015).

Bands 13 and 21 were both Ruminococcaceae, belonging to the Firmicutes phylum. The ratio Firmicutes and Bacteroidetes phyla has an important relationship to human obesity. It has been shown that obese subjects had approximately 20% more Firmicutes than those who were thinner. At the same time, obese subjects had approximately 90% less Bacteroidetes than the thinner subjects. Bacteroidetes phylum of Ruminococcaceae was also one of the bacteria producing SCFAs that inhabited the cecum and colon of mammals. Ruminococcaceae is responsible for the degradation of various polysaccharides and fibers. In addition, the relative proportions of Ruminococcaceae is related to the

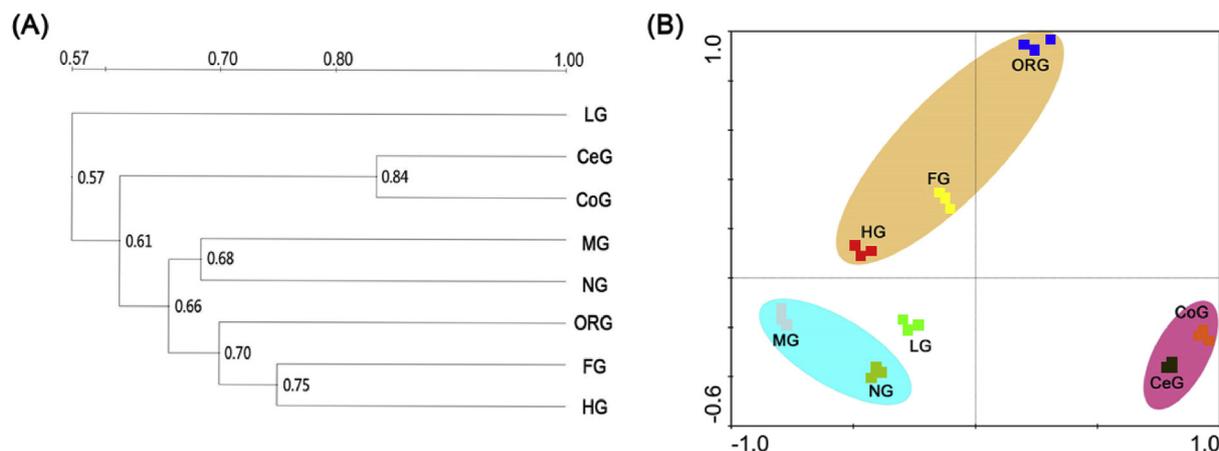


Fig. 3. The cluster analysis (A) and the principal component analysis (PCA) (B) results of the bacteria in the DGGE spectra.

NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group. CoG: Colon samples of mice from MG; CeG: Cecum samples of mice from MG.

Table 3
DGGE Band identification to family/genus level using bacterial V3 primers.

Band Number	Similar strain	Accession number	Similarity	Classification
Band 1	<i>Erysipelothrix rhusiopathiae</i> str. Fujisawa	NR_074878	87	<i>Erysipelotrichaceae</i> ; <i>Erysipelothrix</i>
Band 2	<i>Bacteroides acidifaciens</i>	NR_112931	100	<i>Bacteroidaceae</i> ; <i>Bacteroides</i>
Band 3	<i>Bacteroides graminisolvens</i>	NR_113069	96	<i>Bacteroidaceae</i> ; <i>Bacteroides</i>
Band 4	<i>Parabacteroides merdae</i>	NR_041343	93	<i>Porphyromonadaceae</i> ; <i>Parabacteroides</i>
Band 5	<i>Acetatifactor muris</i>	NR_117905	99	<i>Lachnospiraceae</i> ; <i>Acetatifactor</i>
Band 6	<i>Clostridium thermosuccinogenes</i>	NR_119284	91	<i>Clostridiaceae</i> ; <i>Clostridium</i>
Band 7	<i>Stomatobaculum longum</i>	NR_117792	99	<i>Lachnospiraceae</i> ; <i>Stomatobaculum</i> .
Band 8	<i>Bacteroides oleiciplenus</i>	NR_113070	97	<i>Bacteroidaceae</i> ; <i>Bacteroides</i>
Band 9	<i>Alistipes putredinis</i>	NR_113152	99	<i>Rikenellaceae</i> ; <i>Alistipes</i>
Band 10	<i>Alistipes finegoldii</i>	NR_102944	90	<i>Rikenellaceae</i> ; <i>Alistipes</i>
Band 11	<i>Porphyromonas gingivicanis</i>	NR_113087	86	<i>Porphyromonadaceae</i> ; <i>Porphyromonas</i>
Band 12	<i>Butyrivibrio proteoclasticus</i>	NR_102893	91	<i>Lachnospiraceae</i> ; <i>Butyrivibrio</i>
Band 13	<i>Anaerotruncus colihominis</i>	NR_027558	97	<i>Ruminococcaceae</i> ; <i>Anaerotruncus</i>
Band 14	<i>Butyrivibrio proteoclasticus</i>	NR_102893	92	<i>Lachnospiraceae</i> ; <i>Butyrivibrio</i>
Band 15	<i>Stomatobaculum longum</i>	NR_117792	99	<i>Lachnospiraceae</i> ; <i>Stomatobaculum</i>
Band 16	<i>Lachnoanaerobaculum umeaense</i>	NR_116814	97	<i>Lachnospiraceae</i> ; <i>Lachnoanaerobaculum</i>
Band 17	<i>Roseburia hominis</i>	NR_074809	97	<i>Lachnospiraceae</i> ; <i>Roseburia</i>
Band 18	<i>Helicobacter pullorum</i>	NR_116595	100	<i>Helicobacteraceae</i> ; <i>Helicobacter</i>
Band 19	<i>Barnesiella intestinihominis</i> YIT 11860	NR_041668	90	<i>Porphyromonadaceae</i> ; <i>Barnesiella</i>
Band 20	<i>Barnesiella intestinihominis</i>	NR_113073	89	<i>Porphyromonadaceae</i> ; <i>Barnesiella</i>
Band 21	<i>Anaerofilum pentosovorans</i>	NR_029313	95	<i>Ruminococcaceae</i> ; <i>Anaerofilum</i>
Band 22	<i>Clostridium sporosphaeroides</i>	NR_044835	92	<i>Clostridiaceae</i> ; <i>Clostridium</i>
Band 23	<i>Parabacteroides distasonis</i>	NR_074376	87	<i>Porphyromonadaceae</i> ; <i>Parabacteroides</i>
Band 24	<i>Clostridium grantii</i>	NR_026131	95	<i>Clostridiaceae</i> ; <i>Clostridium</i>
Band 25	<i>Oscillibacter valericigenes</i>	NR_074793	97	<i>Oscillospiraceae</i> ; <i>Oscillibacter</i>
Band 26	<i>Clostridium xylanovorans</i>	NR_028740	88	<i>Clostridiales</i> ; <i>Lachnospiraceae</i>
Band 27	<i>Novosphingobium nitrogenifigens</i>	NR_043857	99	<i>Sphingomonadaceae</i> ; <i>Novosphingobium</i>
Band 28	<i>Desulfovibrio desulfuricans</i> subsp. <i>desulfuricans</i> str. ATCC 27774	NR_074858	96	<i>Desulfovibrionaceae</i> ; <i>Desulfovibrio</i>
Band 29	<i>Dysgonomonas capnocytophagoides</i>	NR_113133	89	<i>Porphyromonadaceae</i> ; <i>Dysgonomonas</i>
Band 30	<i>Parabacteroides distasonis</i>	NR_074376	88	<i>Porphyromonadaceae</i> ; <i>Parabacteroides</i>
Band 31	<i>Marvinbryantia formatexigens</i>	NR_042152	97	<i>Lachnospiraceae</i> ; <i>Marvinbryantia</i>
Band 32	<i>Barnesiella viscericola</i>	NR_041508	88	<i>Porphyromonadaceae</i> ; <i>Barnesiella</i>

prevention of alcohol hepatitis, nonalcoholic hepatitis, hepatic encephalopathy, and increases of intestinal permeability (Shang et al., 2016). DGGE results showed that LOS could significantly increase the number of *Anaerotruncus colihominis* in the mouse colon and cecum (band 13); mixed prebiotics and FOS could also increase the number of this bacteria in feces.

Bands 9 and 10 were Rikenellaceae and *Alistipes*, a genus in the Rikenellaceae family. If the relative proportion of Rikenellaceae increases to a certain extent, it may induce the body's systemic infectious diseases (Boente et al., 2010). After gastric perfusion of LOS, the abundance of Rikenellaceae decreased significantly. In addition, the relative proportions of Rikenellaceae in the colon and cecum were not high. This may occur because oligosaccharides inhibit the adhesion of pathogens to the surface of the epithelial cells of the host (Kunz et al., 2000).

Band 18 belonged to the *Helicobacter* genus. *Helicobacter* is commonly associated with other bacteria in a symbiotic relationship in the gastrointestinal mucus. (Zhang et al., 2006). *Helicobacter* inhabit the colon, where high numbers it can adversely affect the intestinal health (Muyzer et al., 1993). Compared to the blank control group, the relative proportion of *Helicobacter* was significantly reduced ($p < 0.05$) after gastric perfusion of LOS. Dietary carbohydrates, such as the oligosaccharides, 2-fucosyllactose and 3-fucosyllactose from breast milk, and polysaccharides such as laminaran and alginate had been shown to inhibit *Helicobacter* in mice (Nakata et al., 2016; Weiss et al., 2014).

Band 25 belonged to the Oscillospiraceae family, which was present at low relative abundance in the low dose of LOS group. Oscillospiraceae are anaerobic bacteria and are involved in the production of leptin and butyrate, and some bacteria have the capacity to utilize glucuronic acid (Gopha et al., 2017). Band 27 belonged to the Sphingomonadaceae family. Similar to band 25, the abundance was high in the low dose of LOS with gastric perfusion. Sphingomonadaceae are found in fresh cut fruit and vegetable products (Gorni et al., 2015).

Therefore, it may be derived from the daily routine of the mouse's diet. In addition, the bacteria were also found in the intestinal tract of healthy and elderly people (Yu et al., 2015). Band 28 belonged to the Desulfovibrionaceae family; compared to the blank control group, the relative abundances of members of this family in the high dose of LOS group, the medium dose of both LOS and LRS3 control group and the FOS control group were decreased significantly. The genome of *Desulfovibrio desulfuricans* contains pathways from choline to the choline utilization gene and cluster of trimethylamine. Thus, it is an intestinal microbiota that may promote atherosclerosis in humans (Matsumoto et al., 2017).

In summary, medium to high doses of LOS and mixed prebiotics could enrich the growth of families that produce SCFAs, such as Bacteroidaceae, Lachnospiraceae, Clostridiaceae in mice. They can also reduce the number of other families such as Erysipelotrichaceae, Porphyromonadaceae, Rikenellaceae, Desulfovibrionaceae and *Helicobacter*. The acetate and butyrate in SCFAs have an important effect on intestinal mucosal biology (Bultman and Jobin, 2014). Therefore, it may have a potential positive effect on the proliferation of human epithelial cells and mucosal immune response.

3.4. Effects of LOS on SCFAs in mice feces

The total SCFA content in mice feces treated with LOS is shown in Fig. 4. The SCFA content was higher than that of the blank control group after oligosaccharide gastric perfusion ($p < 0.05$), while that of the medium and high dose of LOS groups had no significant differences ($p > 0.05$). There was no difference between the high dose of LOS group and the FOS control group. In addition, there was no difference between the medium dose of both LOS and LRS3 control group and the medium dose of LOS group, and they showed no greater ability to produce SCFAs. Oligosaccharides can promote the proliferation of probiotics in the cecum, while resistant starch can only significantly

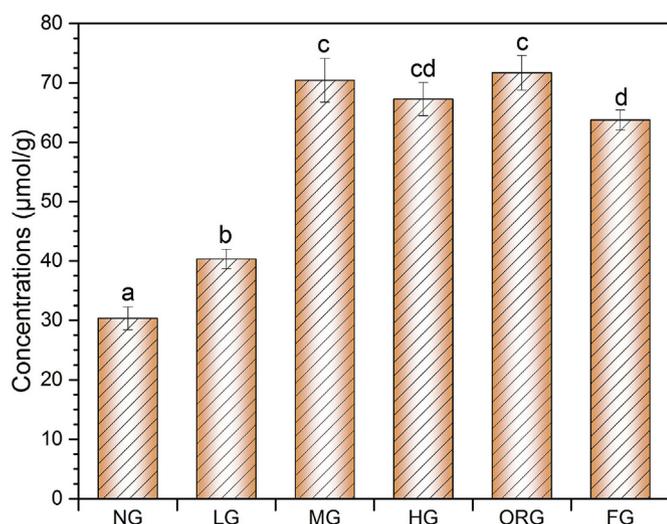


Fig. 4. Effects of LOS on the concentration of SCFAs in mouse feces. Data in the figure represent the mean \pm SD of 14 mice in each group. Different letters in the same chart represent significant differences between different treatments according to Duncan's multiple range test. ($p < 0.05$). NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group.

enhance the proliferation of probiotics in the colon (Rodríguez-Cabezas et al., 2010). When the dose of LOS and LRS3 were basically the same, the total SCFA content of the medium dose of both LOS and LRS3 control group was almost unchanged. A high dose of LOS can increase the number of Bacteroidaceae, Lachnospiraceae, Clostridiaceae, Ruminococcaceae and other bacteria.

However, the specific families were different, and the bacterial families were more diverse. LOS and LRS3 mixed prebiotics increased the relative proportions of families such as Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae. The primary bacteria were the butyric acid-producing bacteria Lachnospiraceae. Bacteroidaceae and Ruminococcaceae mainly produce acetic acid and lactic acid. These bacteria were the main SCFA-producing bacteria. SCFAs were beneficial to reduce intestinal pH in mice, which inhibited the growth of gut pathogenic microbiota.

3.5. Effect of LOS on the intestinal transit in mice

Constipation can cause abdominal pain and bloating, which is due to reduced defecation, caused by feces that are difficult to pass through the intestinal tract. Most often, constipation occurs after feces slowly passed through the digestive tract. Constipation is caused by many reasons, including medications, defecation habits, low fiber diet, abuse of laxatives, hormonal imbalance, and other diseases in different parts of the body. Constipation causes a large amount of water in the feces to be absorbed by the intestinal track during the slow movement, leading to feces that are hard and dry. Fecal status, food intake, water consumption and defecation time are important criteria for the study of constipation. In this study, a mouse constipation model was established as a positive control group by administering loperamide hydrochloride. The results of the small intestine peristalsis test showed that the ink propulsion rate in mice treated with high doses of LOS and LOS-resistant starch mixture was significantly higher than that of mice in the constipation control group (Fig. 5, $p < 0.05$). The results of the medium dose of LOS group and the FOS control group were similar. It was shown that LOS and LRS3 can promote intestinal peristalsis, and intestinal contents move toward the colon during defecation. By measuring the defecation time of the first black feces in mice, it was found that the defecation time of mice in the high dose of LOS group and the

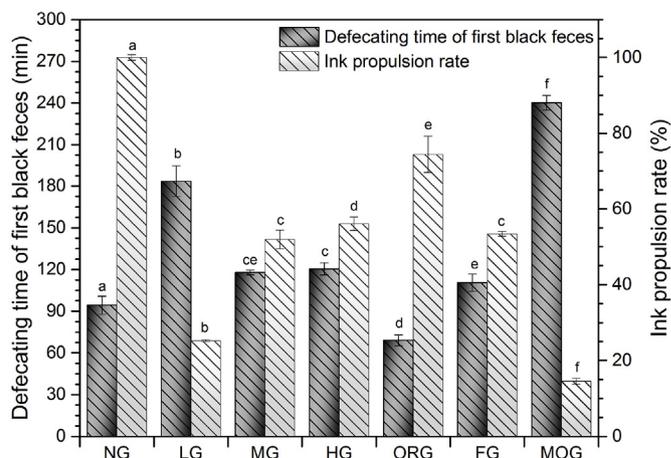


Fig. 5. Defecating times of the first black feces and the ink propulsion rates in the small intestine of LOS-treated mice and constipation control mice. Data in the figure represent the mean \pm SD of 14 mice in each group. Different letters in the same chart represent significant differences between different treatments according to Duncan's multiple range test. ($p < 0.05$). NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group; MOG: Model group.

medium dose of both LOS and LRS3 control group were significantly shorter than that of the constipation control mice ($p < 0.05$). This suggested that LOS and resistant starch can significantly shorten the residence time of intestinal contents, thereby relieving constipation. The effects on defecation of the mice of medium dose of LOS group and the FOS control group were similar. The mice in low dose of LOS group were better than the model group. It was notable that after adding the LRS3, LOS greatly improved defecation time. Compared to the blank control group, the defecation time of the first black feces was significantly reduced ($p < 0.05$). However, the ink propulsion rate was not the highest one measured. This may occur because resistant starch can increase fecal volume and water content, and the increased fecal volume slows down the rate of propulsion. However, feces were soft and easily moved to the anus and excreted, shortening the transportation time. Supplementation with LOS had better effects on the prevention of constipation. Other oligosaccharides had similar results. For example, studies in humans showed that the supplement isomaltooligosaccharide increased the frequency of spontaneous defecation and the total mass of feces of constipated elderly subjects (Yen et al., 2011). In addition, FOS and GOS (a FOS preparation with 95% FOS (FOS95); GOS preparation with 90% GOS (GOS90) and IMO) had similar effects (Wang et al., 2017b). Some probiotics, such as *Streptococcus thermophilus*, *Bifidobacterium* sp and *Lactobacillus* sp prevented or treated constipation. Anti-constipation mechanisms of oligosaccharides may be due partly to oligosaccharides remaining in the intestines and increasing the number of beneficial gut microbiota. Oligosaccharides reduce the pH in the colon and inhibit the growth of harmful bacteria. This low pH increases the water content and mass of the feces and contributes to the intestinal peristalsis (MacDonald and Wagner, 2012).

3.6. Motilin, gastrin, endothelin, somatostatin, acetylcholine enzyme, substance P and vasoactive intestinal peptide levels in serum

The results of the related serum factors showed that motilin, gastrin, endothelin, acetylcholine enzyme, substance P and vasoactive intestinal peptide of the model group were significantly lower than those of the normal group ($p < 0.05$). The somatostatin level was significantly higher than that of the blank control group ($p < 0.05$). Compared to the model group, the three doses of LOS were able to increase the motilin, gastrin, endothelin, acetylcholine enzyme, substance P and

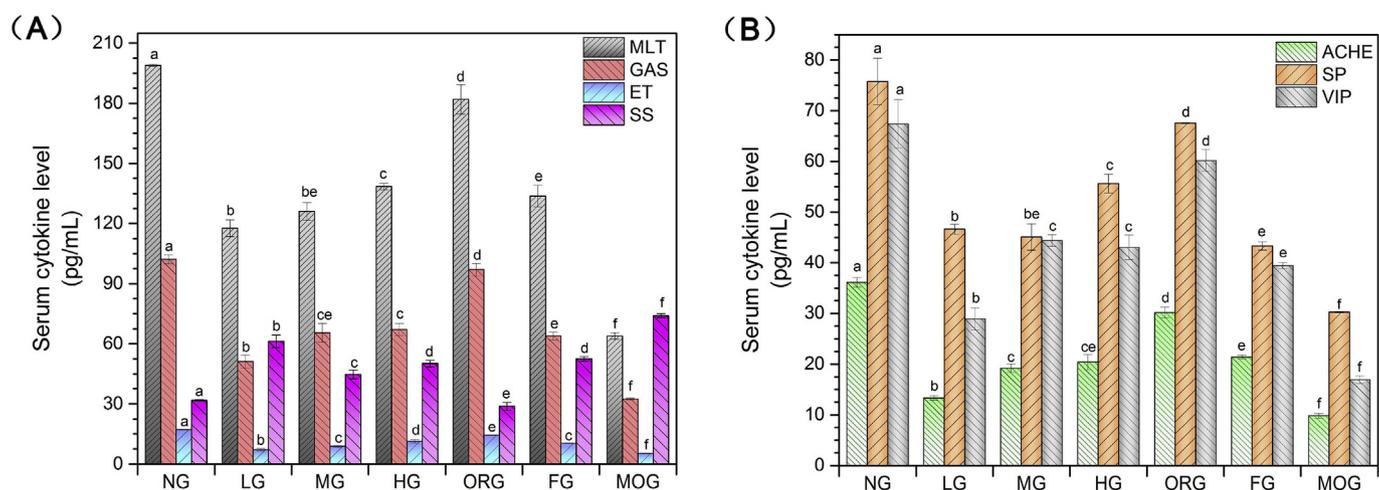


Fig. 6. Effect of various samples on serum MLT, Gas, ET, SS, AChE, SP and VIP levels in mice.

Data in the figure represent the mean \pm SD of 14 mice in each group. Different letters in the same chart represent significant differences between different treatments according to Duncan's multiple range test. ($p < 0.05$). NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group; MOG: Model group. MLT: Motilin; GAS: Gastrin; ET: Endothelin; SS: Somatostatin; AChE: Acetylcholine enzyme; SP: Substance P; VIP: Vasoactive intestinal peptide.

vasoactive intestinal peptide levels in serum disproportionately and reduce somatostatin factor levels. The effect of the high dose of the LOS group was the strongest, and the effect of medium dose of both LOS and LRS3 control group was closest to the blank control group. As shown in Fig. 6, the results showed that different types of LOS and resistant starch could stimulate some hormones, such as motilin, and some neurotransmitter levels in mouse serum. The secretion of somatostatin was reduced, and then stimulation of the intestinal nerve, promotion of intestinal peristalsis and increased intestinal osmotic pressure, maintenance of the secretion of vascular tension, water and digestive juice (gastric acid, pancreatic juice and bile, etc.) led to relaxation of the pyloric sphincter so that it can be used for easy defecation. Probiotic composition may play an important role in neurotransmitter changes in intestinal peristalsis.

3.7. Gene expression and the protein expression of c-kit, stem cell factor, transient receptor potential vanilloid 1, glial cell line-derived neurotrophic factor and nitric oxide synthase

Interstitial cells of cajal are mesenchymal cells between the gastrointestinal nervous system and the smooth muscle in gastrointestinal. They are neurotransmitters between the pacemaker cells and neuromuscular cells during the gastrointestinal motility. They play an important role in regulating the intestinal neural signal in smooth muscle cells. Interstitial cells of cajal receive neurotransmitters through receptors (such as vasoactive intestinal peptide and substance P) and then transfer receptors to adjacent smooth muscle cells through the gap junction to produce colon motility (Wang et al., 2013). C-Kit is a membrane receptor of stem cell factor expressed by interstitial cells of cajal, which has tyrosine kinase activity. C-Kit is a specific marker of interstitial cells of cajal and is interdependent with stem cell factor. Under normal conditions, c-kit is a monomer that exists in the cell membrane and activates cellular signaling pathways, which are essential for normal cell growth and development. The expression of c-Kit mRNA and c-Kit proteins are significantly decreased in colon interstitial cells of cajal of patients with chronic constipation (Yuzawa et al., 2007). As shown in Fig. 7A, Figs. 7B and 8, compared to the model group, the levels of the c-Kit and stem cell factor gene were significantly increased by LOS gastric perfusion ($p < 0.05$). The higher the concentration of LOS provided, the greater increase in c-Kit and stem cell factor expression levels. The effect of medium dose of both LOS and LRS3 control group was the strongest. The expression of mRNA and its

corresponding protein of stem cell factor was also activated. Increased expression of transient receptor potential vanilloid 1 may be a sign of intestinal injury. Nitric oxide synthase is the key to the production of endogenous NO from the esophagus to the anal sphincter. Nitric oxide synthase control can reduce the amount of NO, which is a feasible way to control constipation. Glial cell line-derived neurotrophic factor is an active protein factor that can control the growth and development of nerve cells and protect and repair damaged nerve fibers. Glial cell line-derived neurotrophic factor can enhance the content of factor and then repair the damaged intestinal tract to avoid constipation. Transient receptor potential vanilloid 1, glial cell line-derived neurotrophic factor and nitric oxide synthase genes were important intestinal nerve-related genes. LOS and LRS3 altered the mRNA and protein expression of transient receptor potential vanilloid 1, glial cell line-derived neurotrophic factor, and nitric oxide synthase. After feeding with oligosaccharides and resistant starch, the expression of the mRNA and protein of glial cell line-derived neurotrophic factor was increased significantly ($p < 0.05$) in treated mice, while the expression of transient receptor potential vanilloid 1 and nitric oxide synthase had a contrasting tendency, resulting in inhibiting constipation. In addition, nitric oxide synthase inhibited the release of acetylcholine enzyme. The above three factors maintained a balance of intestinal motility under LOS feeding.

The medium dose of both LOS and LRS3 control group contained a high concentration of SCFAs, but the intestinal propulsion time was longer and there was a lower expression level of associated protein in the medium and high dose of LOS groups. This differed from the conclusion that the concentration of SCFAs in the intestine can relieve constipation (Wang et al., 2017a). Dietary fiber content may cause differences. It was not found that the higher the content of SCFAs, had a better effect on defecation. High concentrations of butyric acid can inhibit secretion of mucin in intestinal goblet cells or reduce fecal volume by stimulating absorption of water and electrolytes in the colon (Zhu et al., 2014a). This may be due to different compositions between gut microbiota and fatty acids. For example, the medium and high dose of LOS groups, the medium dose of both LOS and LRS3 control group significantly reduced the content of Oscillospiraceae (band 25). The bacteria affected the metabolism of nutrient fiber, which was conducive for defecation (Konikoff and Gophna, 2016). The effects of a high dose of LOS on gut microbiota in mice were similar to those of FOS. This can be attributed to the different structures and compositions of each monomer of LOS (LSO4, LSO3-2, LSO3-1, LSO2; relative ratios are

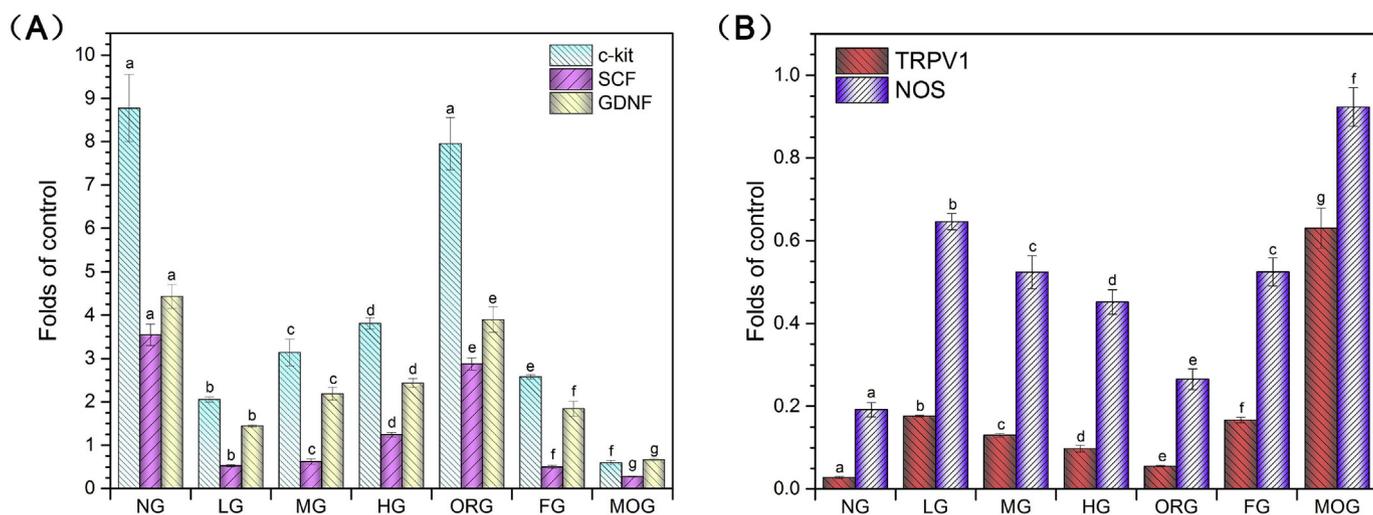


Fig. 7. Effects of LOS on the mRNA expression of c-Kit, SCF, TRPV1, GDNF and NOS in the small intestine. Data in the figure represent the mean \pm SD of 14 mice in each group. Different letters in the same chart represent significant differences between different treatments according to Duncan's multiple range test. ($p < 0.05$). NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group; MOG: Model group; SCF: Stem cell factor; GDNF: Glial cell line-derived neurotrophic factor; TRPV1: Transient receptor potential vanilloid 1; NOS: Nitric oxide synthase.

1.107:0.554:0.183:0.443). Different monosaccharide contents, glycosidic bonds and polymerization degrees may influence the different compositions of gut microbiota. Therefore, relevant work is needed for further study at the next stage. A concurrent reference control group received 2.49 g/kg bw/day of oligosaccharides already used in FOS (a permitted food ingredient), for direct comparison with the high-dose LOS group. There were no treatment-related effects of LOS on survival, clinical observations, body weight, body weight gain, food consumption, food efficiency, hematology, microbial composition. Comparison to several HMOs currently approved as ingredients and infant formula/follow-on formula uses indicates that LOS has the similar safety profile, based on the current studies presented here (Coulet et al., 2013; Phipps et al., 2018; Pitt et al., 2019). No evidence of toxicity was observed and in the absence of compound-related adverse effects in the study. These results may support the use of LOS as a nutritional ingredient for food use.

4. Conclusions

This study showed that ingesting LOS can improve body weight gain and fecal water content, decrease appetite and have no adverse effects on the physiological status by regulating the gut microbiota, SCFAs and the concentrations of serum hormones were shown to produce high levels of c-Kit, stem cell factor, and glial cell line-derived neurotrophic factor and lower expression levels of transient receptor potential vanilloid 1 and nitric oxide synthase mRNA and protein. These results indicated that LOS can shorten the time of defecation and promote the intestinal peristalsis of mice and prevent constipation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

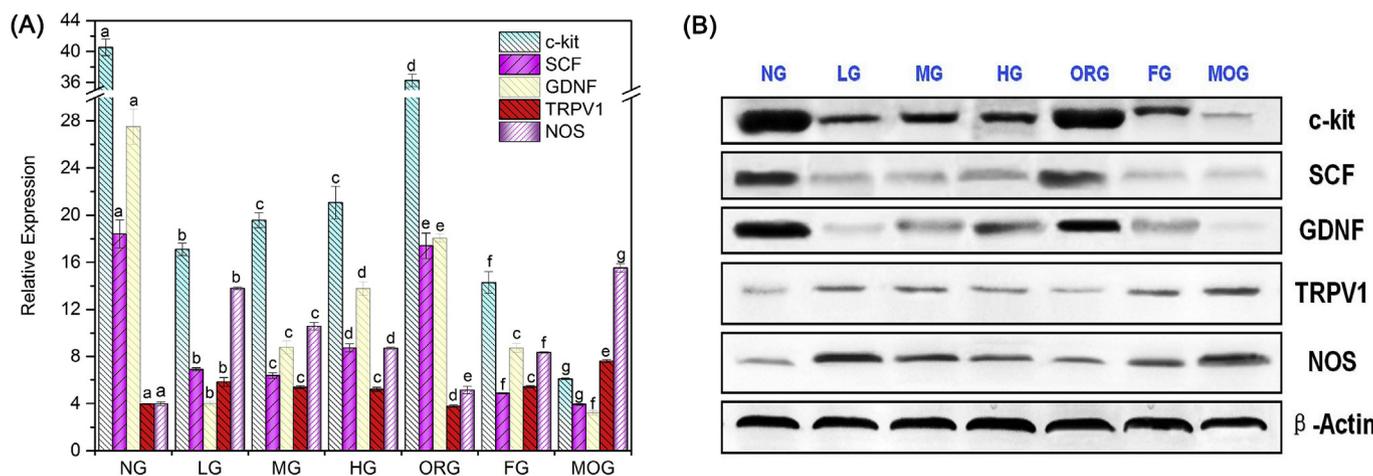


Fig. 8. Effects of LOS on the protein expression of c-Kit, SCF, TRPV1, GDNF and NOS in the small intestine. $n = 14$ mice in each group. NG: Blank control group; LG: Low dose of LOS group; MG: Medium dose of LOS group; HG: High dose of LOS group; ORG: Medium dose of both LOS and LRS3 control group; FG: FOS control group; MOG: Model group; SCF: Stem cell factor; GDNF: Glial cell line-derived neurotrophic factor; TRPV1: Transient receptor potential vanilloid 1; NOS: Nitric oxide synthase.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2019.110838>.

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