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ORIGINAL PAPER

The Effect of *Lactobacillus plantarum* BW2013 on The Gut Microbiota in Mice Analyzed by 16S rRNA Amplicon Sequencing

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Abstract

Lactobacillus plantarum BW2013 was isolated from the fermented Chinese cabbage. This study aimed to test the effect of this strain on the gut microbiota in BALB/c mice by 16S rRNA amplicon sequencing. The mice were randomly allocated to the control group and three treatment groups of *L. plantarum* BW2013 (a low-dose group of 10^8 CFU/ml, a medium-dose group of 10^9 CFU/ml, and a high-dose group of 10^{10} CFU/ml). The weight of mice was recorded once a week, and the fecal samples were collected for 16S rRNA amplicon sequencing after 28 days of continuous treatment. Compared with the control group, the body weight gain in the treatment groups was not significant. The 16S rRNA amplicon sequencing analysis showed that both the Chao1 and ACE indexes increased slightly in the medium-dose group compared to the control group, but the difference was not significant. Based on PCoA results, there was no significant difference in β diversity between the treatment groups. Compared to the control group, the abundance of *Bacteroidetes* increased in the low-dose group. The abundance of Firmicutes increased in the medium-dose group. At the genus level, the abundance of *Alloprevotella* increased in the low-dose group compared to the control group. The increased abundance of *Ruminococcaceae* and decreased abundance of *Candidatus_Saccharimonas* was observed in the medium-dose group. Additionally, the abundance of Bacteroides increased, and *Alistipes* and *Candidatus_Saccharimonas* decreased in the high-dose group. These results indicated that *L. plantarum* BW2013 could ameliorate gut microbiota composition, but its effects vary with the dose.

K e y w o r d s: *Lactobacillus plantarum*, composition, gut microbiota, 16S rRNA amplicon sequencing, BALB/c mice

Introduction

Gut microbiota, a large and complex microbial community in the gastrointestinal tract, is essential to the host's health and well-being (Koh et al. 2016; Liu et al. 2020). Gut microbiota can not only break down the indigestible carbohydrates in food (Schwalm and Groisman 2017), but also produce short-chain fatty acids (SFAs), which can provide nutrition for gut microbiota (Jia et al. 2020). The disturbance of gut microbiota induces inflammation, insulin resistance, diabetes, and osteoporosis (Ma et al. 2019; Bi et al. 2020). Even the novel coronavirus pneumonia was found associated with gut microbiota disturbance (He et al. 2020a).

Probiotics can modulate gut microbiota and cause favorable changes in the gut microbiota structure and functions (Hasan et al. 2019). When given enough dose, probiotics will reach the intestinal tract in an active state, thus improving intestinal microorganisms' balance and producing beneficial effects on the host (Deng et al. 2020). *Lactobacillus casei* ZX633 may ameliorate the infant diarrhea microbiota, thus reducing the rate of infant bacterial diarrhea (Wang et al. 2020b). After treated with mixed lactic acid bacteria, *Staphylococcus*

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aureus infection could be prevented in mice, and the structure of intestinal microbiota could be improved (Ren et al. 2018).

Lactic acid bacteria and *Bifidobacteria* are the most commonly used probiotics (He et al. 2020b). *Lactobacillus plantarum*, a rod-shaped, facultative anaerobic, Gram-positive lactic acid bacterium, can effectively improve the health of the host by decreasing the level of bloodstream cholesterol, managing gastrointestinal disorders, and preventing diarrhea (Liu et al. 2015; Seddik et al. 2017a). Wang et al. (2018) found that *L. plantarum* ZDY2013 remits ulcerative colitis by modifying of intestinal microbiota to regulate both oxidative stress and inflammatory mediators. Some functional activities are strain-specific (Biagioli et al. 2019). Qiu et al. (2018) injected mice with potential probiotic strains, including *L. plantarum* ZDY04 (PLA04) and *L. plantarum* ZDY01 (PLA01). As a result, both serum trimethylamine *N*-oxide and cecal trimethylamine levels was reduced significantly only by *L. plantarum* ZDY04. Li et al. (2019) demonstrated the loss of gut microbiota diversity induced by glycerol monolaurate could be remedied by *L. plantarum* T17, but the same effects were not found in the group of *L. plantarum* T34. *L. plantarum* BW2013 was isolated from fermented Chinese cabbage, and the influence of this strain on the gut microbiota is unknown.

Many methods were used to study the gut microbiota, such as the culture of gut microbiota, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), quantitative real-time polymerase chain reaction (qRT-PCR), 16S rRNA amplicon sequencing (Margiotta et al. 2020; Ling et al. 2020). As a relatively new technology, 16S rRNA amplicon sequencing opens out new potential avenues of research and facilitates in-depth studies exploring microbial populations and their dynamics in the animal gut (Peng and Zhang 2009; Kim and Isaacson 2015). The 16S rRNA technology has been widely used in biomedical research, linking the establishment between microbiota disorders and human disease (Evariste et al. 2019). For example, Zhu et al. (2020) used 16S rRNA amplicon sequencing to study the gut microbiota of ulcerative colitis with different glucocorticoid response types and found that they had different bacterial composition and function, which linked the microbiota disorders and ulcerative colitis. Analysis of 16S rRNA amplicon sequencing of intestinal microbiota found that high-calorie diet and lipopolysaccharide atomization synergistically promoted pneumonia process in rat pups, which is related to changes in the structure of intestinal flora (Bai et al. 2020).

Based on 16S rRNA amplicon sequencing, this study investigated the effects of different doses of *L. plantarum* BW2013 on gut microbiota composition in mice.

Experimental

Materials and Methods

Bacterial strains and cultural conditions. *L. plantarum* BW2013 was isolated from fermented Chinese cabbage and preserved by the China General Microbiological Culture Collection Center (CGMCC NO. 9462). *L. plantarum* BW2013 was grown anaerobically in the Man-Rogosa-Sharpe medium broth for 20 h at 37°C, and then centrifuged at 3,000 g for 15 min. The bacteria were washed twice and resuspended in sterile phosphate-buffered saline (PBS, pH 7.4).

Simulate gastrointestinal digestion. The simulated gastrointestinal juice was produced with following Shinde et al. (2019), and amylase, pepsin, bovine bile, and trypsin were purchased from Sigma. First, bacteria (1 ml, 1×10^9 CFU/ml) were suspended in 5 ml simulated salivary juice for 5 min. Then, the samples were resuspended to 10 ml gastric juice and incubated for 2 h. Subsequently, the samples were resuspended to 10 ml intestinal juice and incubated for 2 h. The entire digestion procedure was performed at 37°C, with stirring to simulate peristaltic contraction. After the simulated digestion process, the bacteria cell suspensions were diluted and plated onto MRS agar plates. The number of colonies was counted after 24 h of incubation at 37°C according to the formula:

$$\text{survival rate}(\%) = N1 / N0 \times 100\%$$

where N_1 was the total viable count of strains after treatment and N_0 was the total viable count of strains before treatment.

Adhesion to Caco-2 cell. Caco-2 cell cultures were determined by the method of Fonseca et al. (2021). After cultured in MRS broth for 24 h at 37°C and washed twice with phosphate-buffered solution, the bacteria were resuspended in DMEM approximately 10^9 CFU/ml. Then, 1 ml bacterial suspension was added to cells and incubated for 60 min at 37°C in a 5% CO_2 atmosphere. Subsequently, the cells were washed three times with 1 ml of PBS to remove non-adherent bacterial cells and lysed with 1 ml of Triton-X solution at 37°C for 5 min. After the above procedures, the solution was serial diluted and plated on MRS agar to determine the bacterial counts.

Animal, rearing and grouping. The mice (8-week-old male) used in the experiment were purchased from Vital River Laboratories Inc. (Beijing, China). Mice were singly caged under specific pathogen-free conditions at 20–22°C, and relative humidity of 40–60%. Before intragastric administration, the mice were weighed, and the feces were collected for 16S rRNA gene amplicon sequencing analysis. Then the mice were randomly allocated to four groups (each group n = 10):

control group (NC) and three treatment groups of *L. plantarum* BW2013: a low-dose (10^8 CFU/ml) group (LDG), a medium-dose (10^9 CFU/ml) group (MDG), and a high-dose group (10^{10} CFU/ml) (HDG). From 9 a.m. to 10 a.m. every day, the NC group was given sterile PBS (pH 7.4), and treatment groups were administered the corresponding of *L. plantarum* BW2013 suspension at 400 µl/d once daily over 28 days. All mice were weighed once a week. During the experiment, the mice were fed a normal diet.

16S rRNA gene amplicon sequencing. The fecal genomic DNA was extracted according to the manufacturer's guidelines of DP712-Magnetic Bead Soil and Fecal Genomic DNA Extraction Kit (Tiangen, China). For 16S rRNA gene amplicon sequencing, the DNA samples were amplified with primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3'), which targeted V3-V4 hypervariable regions of the bacterial 16S rRNA gene (Ranasinghe et al. 2012). PCR program was applied, as follows: the initial denaturation at 95°C for 15 min, the amplification of 34 cycles under various conditions (at 95°C for 30 s, 58°C for 30 s and 68°C for 1 min), and the final extension at 68°C for 5 min. Then the purified amplicons were sequenced with an Illumina Miseq sequencing platform at Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China).

The same volume of 1 × loading buffer (contained SYB green) was mixed with PCR products, and electrophoresis was operated on 2% agarose gel for detection. PCR products were mixed in equal density ratios. Then, the mixture of PCR products was purified with Gene JETTM Gel Extraction Kit (Thermo Scientific).

Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) following the manufacturer's recommendations. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific). At last, the library was sequenced on an Ion S5TM XL platform and 400 bp/600 bp single-end reads were generated.

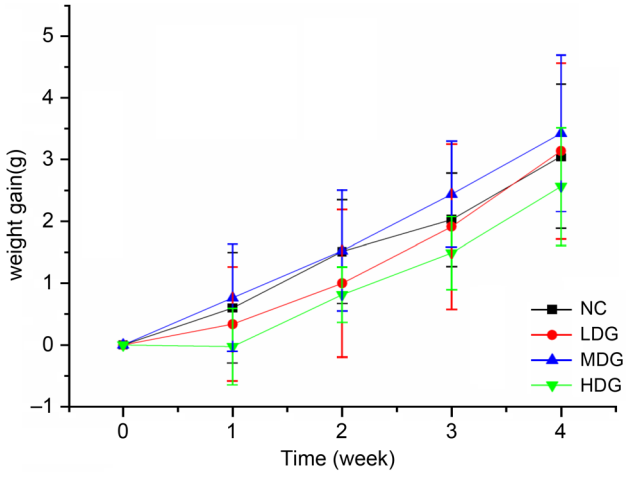

Fig. 1. Body weight variations of the mice after *L. plantarum* BW2013 gavage. The data are represented as mean ± SD.

NC – control group, LDG – low-dose group, MDG – medium-dose group, HDG – high-dose group.

Statistical analysis. The analysis of variance for multiple comparisons was performed in Prisma software (version 5). Statistical differences were evaluated by analysis of variance (ANOVA) and Dunnett-t pairwise comparisons. Cutadapt (V1.9.1) was used for quality control (Martin 2011). Uparse software (Uparse v7.0.1001, http://www.drive5.com/uparse/) was applied to cluster the clean reads to OTUs (Edgar 2013). Species annotation analysis was carried out using the Mothur method and SSSUrRNA database of SILVA132 (http://www.arb-silva.de/) to obtain taxonomic information at each taxonomic level (Quast et al. 2013).

Results

Tolerance to simulated digestion test and adhesion to Caco-2 cell. The survival rate of *L. plantarum* BW2013 after the simulated gastrointestinal digestion process was 2.90%, and the adhesion rates of *L. plantarum* BW2013 was 2.4%.

Effect of *L. plantarum* BW2013 on the body weight gain of mice. Before intragastric administration, the mice were weighed. Then body weight was recorded once a week. The weight changes of mice were shown in Fig. 1. There was no significant difference in body weight gain among the four groups.

Overall sequences and OTUs. An average of 85,386 reads was measured per sample by 16SrRNA amplicon sequencing, and an average of 80,298 clean reads was obtained after quality control. The clean reads of all samples were clustered by OTUs (operational taxonomic units) with 97% identity. A total of 1,120 OTUs were obtained, and 74 OTUs were annotated to the genus level.

Diversity indexes among the NC and treatment groups. Compared with NC group, the Chao 1 and ACE indexs (Table I) were slightly higher than those in the MDG group. But there were no significant differences for all the α-diversity indexes among the NC and treatment groups. Compared with the initial state, the Chao1 index of the NC and MDG groups increased significantly, respectively ($p = 0.0379$, $p = 0.0267$).

The changes in gut microbiota among groups were examined by using principal coordinate analysis (PCoA). Based on weighted unifrac distance, PCoA analysis was conducted to compare the microbial community composition of different samples (Fig. 2). On the weighted unifrac PCoA score plot, the NC group's symbols were separated from those of the treatment groups, which revealed that the microbiome

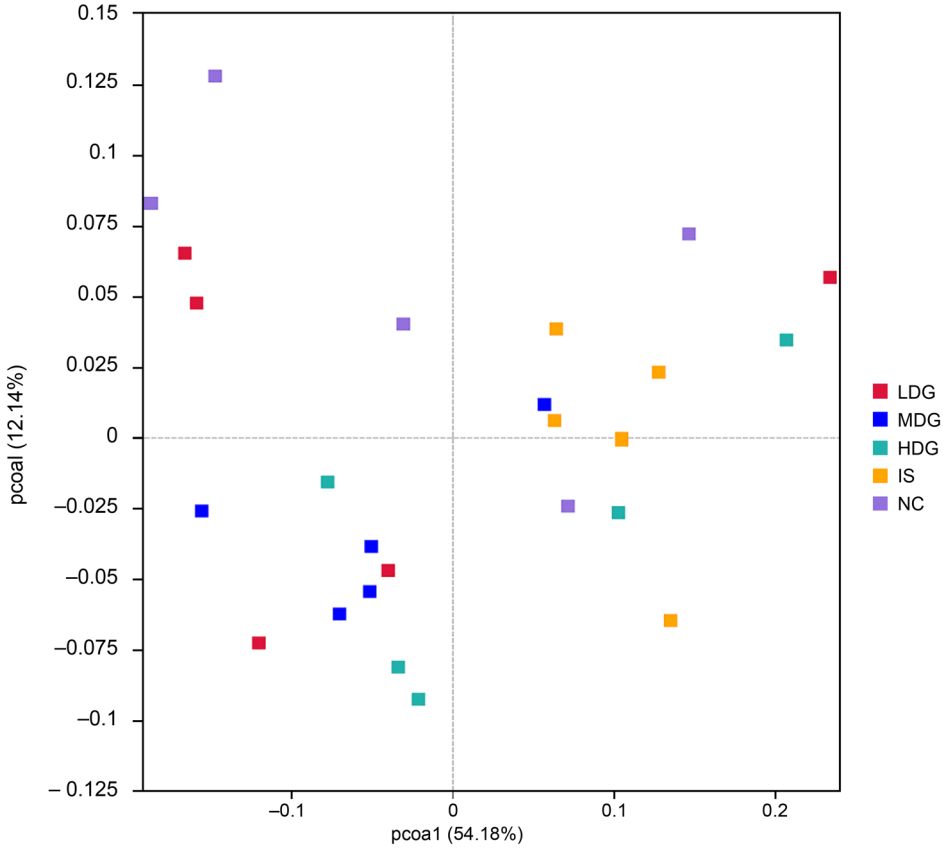

Fig. 2. Principal co-ordinates analysis (PCoA) of the microbial communities of different groups. Initial state (IS) stands for mice before intragastric administration.
NC – control group, LDG – low-dose group, MDG – medium-dose group, HDG – high-dose group.

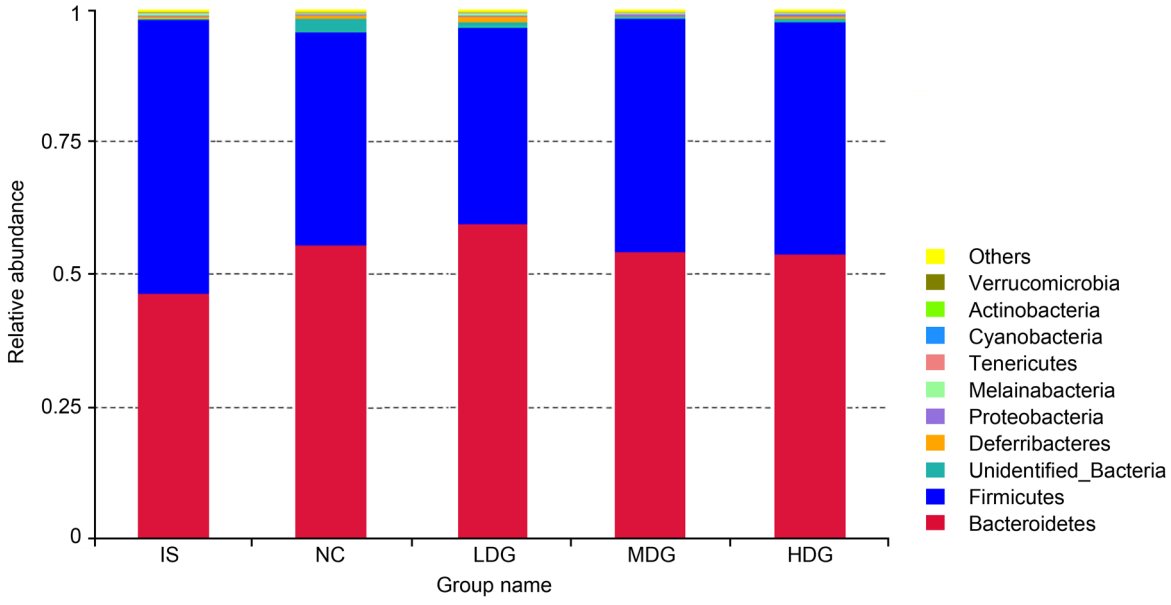

Fig. 3. Microbial community bar plot at the phylum level. Initial state (IS) stands for mice before intragastric administration.
NC – control group, LDG – low-dose group, MDG – medium-dose group, HDG – high-dose group.

composition of treatment groups was different from those of the NC group, but there was no significant difference. Additionally, there was no significant difference between initial state and NC group.

Relative abundance of gut microbiota at phylum and genus levels. The relative abundance of gut microbiota was measured at the phylum (Fig. 3) and genus (Fig. 4) levels.

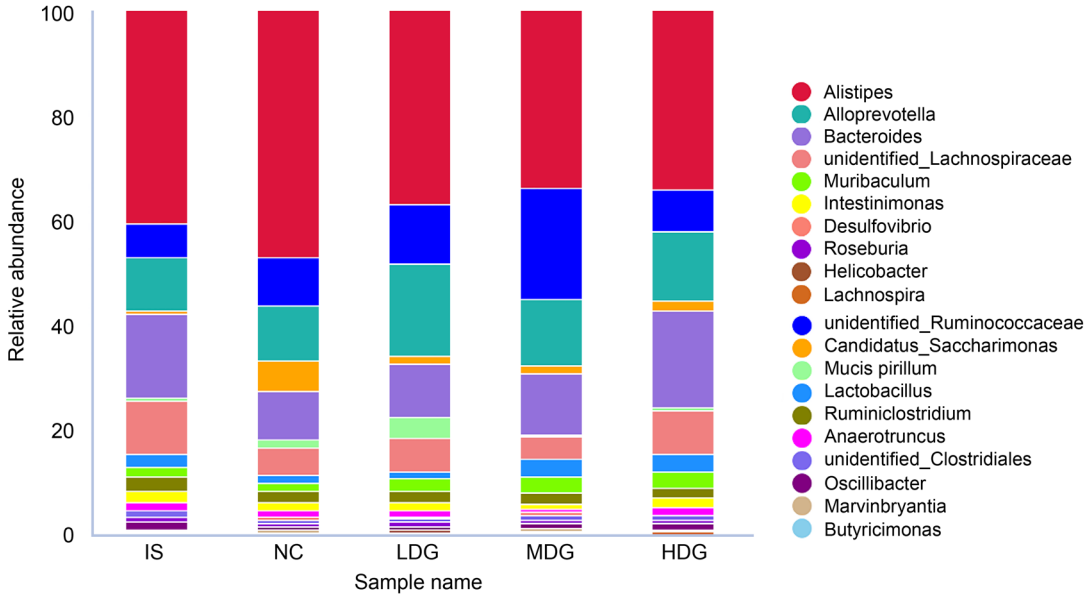

Fig. 4. Microbial community bar plot at the genus level. Initial state (IS) stands for mice before intragastric administration. NC – control group, LDG – low-dose group, MDG – medium-dose group, HDG – high-dose group.

Examining the changes in the gut microbiota, the top ten bacterial phyla of the NC and treatment groups were evaluated. The results showed that Bacteroidetes was the most abundant phylum, followed by Firmicutes, Deferribacteres, and Proteobacteria in the NC and treatment groups. *L. plantarum* BW2013 mainly affected the abundance of Bacteroidetes and Firmicutes, which accounted for 97% of the total bacteria (Fig. 3). Compared with the NC group, the abundance of Bacteroidetes increased significantly in the LDG group ($p = 0.04$). Additionally, the relative abundance of Firmicutes increased significantly in the MDG group compared to the NC group ($p = 0.01$).

The four most prevalent bacterial genera in the guts of the NC and treatment groups were *Alistipes*, *Alloprevotella*, *unidentified_Ruminococcaceae,* and *Bacteroides* (Fig. 4). Compared to the NC group, *Alistipes* exhibited significantly decreased proportions in the HDG group ($p = 0.038$). In addition, the abundance of *Candidatus_Saccharimonas* decreased significantly in the MDG and HDG groups ($p = 0.01$, $p = 0.007$). By contrast, *Alloprevotella*, *unidentified_Ruminococcaceae,* and *Bacteroides* showed an upward trend. The abundance of *Alloprevotella* in the LDG group was significantly higher than that in the NC group ($p = 0.001$). Moreover, compared with the NC group, the abundance of *unidentified_Ruminococcaceae* in the MDG group increased significantly ($p = 0.014$), while the abundance of *Lactobacillus* increased slightly, but there was no significant difference. In addition, the proportion of *Bacteroides* increased significantly in the HDG group compared to the NC group ($p = 0.038$).

Clustering analysis of species abundance. The heat map showed the relative abundance of the main identified bacteria at the genus level. As shown in Fig. 5, the clustering of gut microbiota was different in the groups. In the initial state, gut microbiota was mainly clustered in Firmicutes. In the NC group, Bacteroidetes were concentrated in the genus of *Desulfovibrio*. Both *Parabacteroides* and *Alloprevotella* from Bacteroidetes dominated the LDG group. In the MDG group, there were large quantities of *Ruminococcaceae* and *Lachnospira* in Firmicutes. In the HDG group, Bacteroidetes were concentrated in the genus of *Bacteroides.*

Table I
The α diversity index of gut microbiota in each group.

Group	Observed species	Shannon	Simpson	Chao1	ACE	Goods coverage
IS	532	6.566	0.971	534.371	540.911	0.999
NC	535.6	6.433	0.968	538.985	545.437	0.999
LDG	559	6.622	0.976	563.100	570.366	0.999
MDG	580	6.728	0.978	582.440	588.671	0.999
HDG	584.8	6.640	0.971	616.129	619.041	0.999

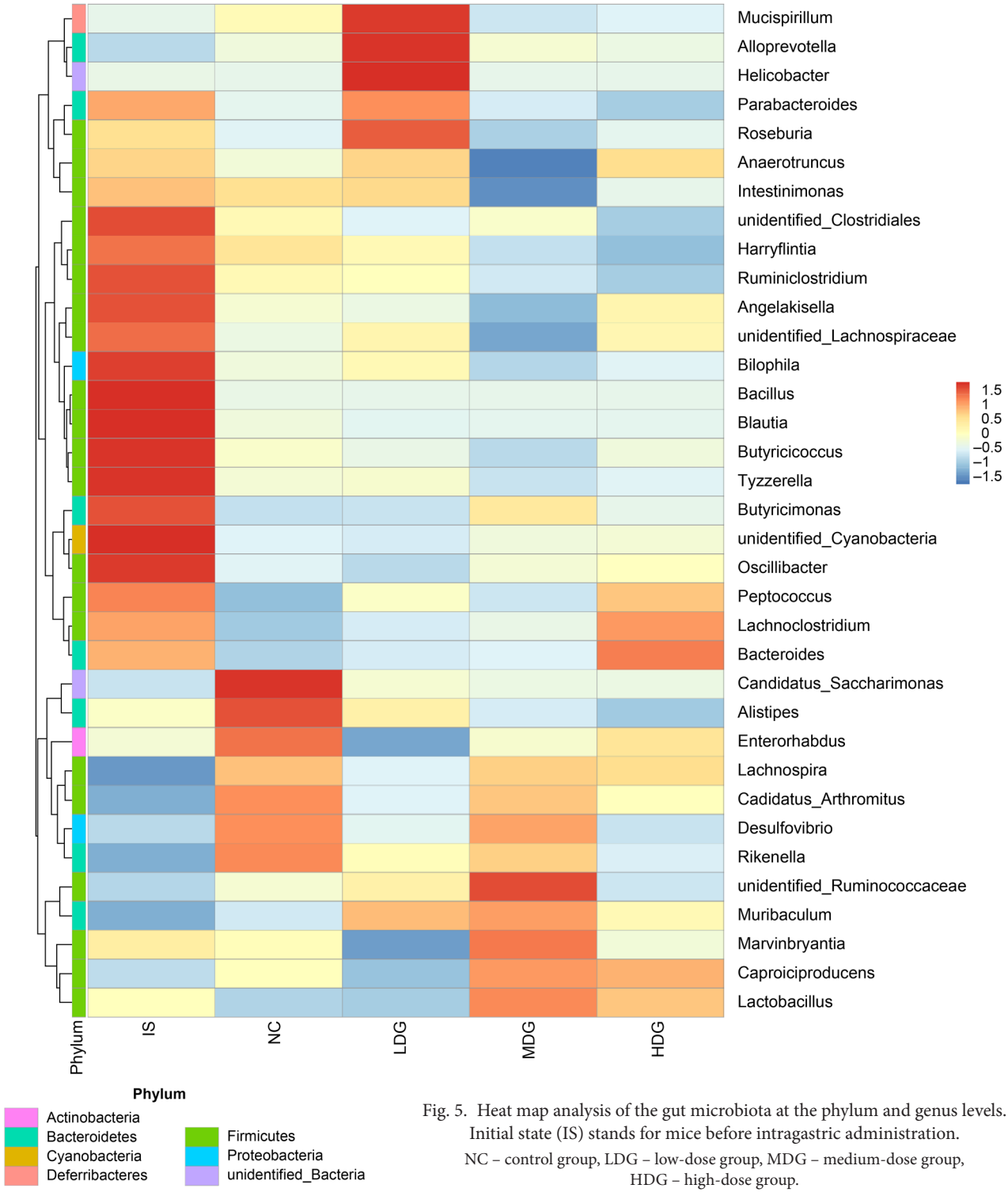

Fig. 5. Heat map analysis of the gut microbiota at the phylum and genus levels. Initial state (IS) stands for mice before intragastric administration.
NC – control group, LDG – low-dose group, MDG – medium-dose group, HDG – high-dose group.

Discussion

The consumption of probiotics has been reported to modulate the composition and structure of the gut microbiome and treat multiple diseases, but some functional activities are strain-specific (Biagioli et al. 2019; Hsu et al. 2019). There are also various methods for detecting gut microbiota, among which 16S rRNA amplicon sequencing is a faster and cheaper way to study the gut microbiome (Goldfeder et al. 2017). In this study, the effect of *L. plantarum* BW2013 on gut microbiota composition in BALB/c mice was investigated by 16S rRNA amplicon sequencing.

16S rRNA amplicon sequencing could be used to determine all microorganisms' genetic composition and community function in environmental samples (Qi et al. 2019). In our study, the results of 16S rRNA amplicon sequencing showed that the ACE index and Chao1

index slightly increased in the MGD group compared to the NC group, but there was no significant difference. A previous study also showed that the microbial richness (Chao1 and Shannon) was not significantly improved after the *L. plantarum* LIP-1 treatment (Song et al. 2017), which is consistent with our results. Based on PCoA, there was no significant difference in β diversity in the treatment groups compared to the NC group. There was also no significant difference between the initial state and NC group.

At the phylum level, the abundance of Bacteroidetes and Firmicutes accounted for 97% of the total bacteria. Bacteroidetes can regulate the chaotic state of intestinal microorganisms to a balanced state (Wang et al. 2020a). Firmicutes may play important roles in gastrointestinal health, and affect the metabolism and function of gut microbes (Zhao et al. 2018). In this study, the relative abundance of Bacteroidetes in the LDG group was significantly higher than that in the NC group. Li et al. (2017) found that *L. casei* CCFM419 increased the abundance of Bacteroidetes, which is similar to our result. Our study showed that the abundance of Firmicutes increased significantly in the MDG group compared with the NC group. *L. plantarum* 12 increased the relative abundance of Firmicutes (Sun et al. 2020), which is similar to our result. Contrary to our results, a strain of *L. plantarum* decreased the abundance of Firmicutes (Zhang et al. 2019).

At the genus level, *L. plantarum* BW2013 significantly increased the abundance of *Alloprevotella* and *Ruminococcaceae*, and significantly decreased the abundance of both *Alistipes* and *Candidatus_Saccharimonas*. *Alistipes* is pathogenic in colorectal cancer and is associated with mental signs of depression (Parker et al. 2020). *Candidatu_Saccharimonas* has been associated with inflammatory diseases, such as gingivitis and other periodontal dysfunctions (Cruz et al. 2020). *Alloprevotella* can produce vitamin B1 and folic acid, and an increase in the abundance of *Alloprevotella* was associated with the improvement of intestinal disorders Seddik et al. 2017; Qi et al. 2019). Based on our results, the abundance of *Alloprevotella* in the LDG group was significantly higher than in the NC group. Kong et al. (2018) found that the abundance of *Alloprevotella* increased significantly after probiotics treatment. This finding is similar to our result. *Ruminococcaceae* can produce butyrate, which can provide energy for intestinal epithelial cells (LeBlanc et al. 2017). In our study, compared with the NC group, the abundance of *Ruminococcaceae* in the MDG group increased significantly. Wang et al. (2018) found that the abundance of *Ruminococcaceae* showed a decreasing trend in *L. plantarum* ZDY2013 group, which is different from our result. It may be due to the different strains used in the experiment. Biagioli et al. (2019) mentioned that some functional activities are strain-specific. Our study showed that compared with the NC group, the abundance of *Bacteroides* in the HDG group increased significantly. Li et al. (2017) found that *L. casei* CCFM419 increased the abundance of *Bacteroides*, which is consistent with our result. *Bacteroides* are producers of short-chain fatty acids (SCFAs) (Du et al. 2020). SCFAs are produced by the fermentation of microorganisms in the gut and help regulate host energy homeostasis and physiological processes (Horiuchi et al. 2020). This correlation means that the presence of *L. plantarum* BW2013 can provide a positive impact on host health. In our study, *Desulfovibrio* was enriched in the NC group in the heat map, while *Parabacteroides*, *Alloprevotella*, *Ruminococcaceae*, *Lachnospira*, and *Bacteroides* were concentrated in the treatment groups. Our results collectively suggested that *L. plantarum* BW2013 the effect of ameliorating gut microbiota composition, but its effects vary with the dose.

Conclusion

In this study, our results showed that treatment with *L. plantarum* BW2013 exerted an effect on the gut microbiota composition in mice. At the phylum level, the abundance of Bacteroidetes increased in the LDG group compared with the NC group, while the abundance of Firmicutes increased in the MDG group. At the genus level, the abundance of *Alloprevotella* was higher in the LDG group compared with the NC group. By contrast, the abundance of *Ruminococcaceae* increased in the MDG group, but *Candidatus_Saccharimonas* decreased. In addition, *Bacteroides* abundance increased in the HDG group, but *Alistipes* and *Candidatus_Saccharimonas* decreased. These results indicated that *L. plantarum* BW2013 had the effect of ameliorating the composition of gut mice, but its effect varies with dosing.

Ethical approval

This project was approved by the Ethics Committee of Functional Inspection Center of Health Food of Applied Science and Humanities in Beijing Union University (No. 2019-04).

Authors' contributions

Zuming Li, Jia Liu and Zhihui Bai conceived and designed the experiments; Tong Tong, Qian Li and Yuxi Ling performed the experiments; Tong Tong, Qian Li, Xiaohui Niu and Michael Zhang analyzed the data; Tong Tong and Xiaohui Niu wrote the paper; Ran Xia, Zhichao Wu and Xiu Liu gave important suggestions; Zuming Li, Michael Zhang,Tong Tong and Xiaohui Niu revised the manuscript.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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