Changes in gastric microbiota induced by *Helicobacter pylori* infection and preventive effects of *Lactobacillus plantarum* ZDY 2013 against such infection

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ABSTRACT

Helicobacter pylori is a gram-negative pathogen linked to gastric ulcers and stomach cancer. Gastric microbiota might play an essential role in the pathogenesis of these stomach diseases. In this study, we investigated the preventive effect of a probiotic candidate *Lactobacillus plantarum* ZDY 2013 as a protective agent against the gastric mucosal inflammation and alteration of gastric microbiota induced by *H. pylori* infection in a mouse model. Prior to infection, mice were pretreated with or without 400 μ L of *L. plantarum* ZDY 2013 at a concentration of 10^9 cfu/mL per mouse. At 6 wk postinfection, gastric mucosal immune response and alteration in gastric microbiota mice were examined by quantitative real-time PCR and high-throughput 16S rRNA gene amplicon sequencing, respectively. The results showed that *L. plantarum* ZDY 2013 pretreatment prevented increase in inflammatory cytokines (e.g., IL-1 β and IFN- γ) and inflammatory cell infiltration in gastric lamina propria induced by *H. pylori* infection. Weighted UniFrac principal coordinate analysis showed that *L. plantarum* ZDY 2013 pretreatment prevented the alteration in gastric microbiota post-*H. pylori* infection. Linear discriminant analysis coupled with effect size identified 22 bacterial taxa (e.g., *Pasteurellaceae*, *Erysipelotrichaceae*, *Halomonadaceae*, *Helicobacteraceae*, and *Spirochaetaceae*) that overgrew in the gastric microbiota of *H. pylori*-infected mice, and most of them belonged to the *Proteobacteria* phylum. *Lactobacillus plantarum* ZDY 2013 pretreatment prevented this alteration; only 6 taxa (e.g., *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae*), mainly from the taxa of *Firmicutes* and *Bacteroidetes*, were dominant in the gastric microbiota of the *L. plantarum* ZDY 2013 pretreated mice. Administration of *L. plantarum*

ZDY 2013 for 3 wk led to increase in several bacterial taxa (e.g., *Rikenella*, *Staphylococcus*, *Bifidobacterium*), although a nonsignificant alteration was found in the gastric microbiota. Overall, this study demonstrated that *L. plantarum* ZDY 2013 pretreatment played an important role in preventing gastric mucosal inflammation and gastric microbiota alteration induced by *H. pylori* infection, and the selective modulation in gastric microbiota posed by this intervention suggested that targeting gastric microbiota through oral administration of probiotics might be an alternative strategy to prevent *H. pylori* infection.

Key words: gastric microbiota, *Helicobacter pylori*, probiotic, *Lactobacillus plantarum* ZDY 2013, high-throughput

INTRODUCTION

Helicobacter pylori is a highly prevalent pathogen that colonizes approximately half of the world's population (Polk and Peek, 2010; Wroblewski et al., 2010). Although most subjects colonized by *H. pylori* are asymptomatic, this pathogen is strongly associated with gastritis, peptic ulcers, and gastric cancer in humans (Salama et al., 2013). A complex disease is rarely caused by a single factor. Previous studies have identified several factors that determine the disease outcome post-*H. pylori* infection (Martin and Solnick, 2014), whereas most of them have focused on the virulence factors carried by *H. pylori*, host genetics, and environment. Recently, with a rapid improvement and availability of sequencing-based analysis techniques, the role of host microbiota has attracted the interest of scientists to study disease associated with *H. pylori* infection (Sheh and Fox, 2013; Yang et al., 2013).

Several studies have indicated that microbiota is involved in shaping host physiology and balance of immune system (Clemente et al., 2012), and altered microbiota is strongly associated with several diseases and conditions such as inflammatory bowel disease (Packey

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and Sartor, 2008), obesity (Ley et al., 2005), diabetes (Xu et al., 2015), and even liver cirrhosis (Qin et al., 2014). Previous studies have demonstrated that gastric microbiota played an important role in the development of gastric cancer (Wang et al., 2014). In particular, a recent study has demonstrated a gradual shift in the gastric microbiota profile from nonatrophic gastritis to intestinal metaplasia and then to intestinal-type gastric cancer (Aviles-Jimenez et al., 2014), indicating that an interaction took place between gastric microbiota and *H. pylori*. However, whether a specific pre-infection microbiota of the host would affect colonization of *H. pylori* and subsequent outcome after intervention with this microorganism for gastric disease is not yet known.

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, may have a positive effect to alter the microflora of host and exert a health benefit to the host” (Joint FAO/WHO Working Group, 2002). Oral administration of probiotic bacteria has been used as an alternative strategy to combat several gastrointestinal diseases (e.g., acute infectious diarrhea; Weichert et al., 2012). Currently, the role of probiotics in the treatment of *H. pylori*-related gastrointestinal diseases is increasingly being recognized as an alternate or complement to antibiotics, with a potential to decrease the use of antibiotics or to reduce their side effects (Patel et al., 2014). Several studies have shown that some probiotic strains (*Saccharomyces boulardii*, *Lactobacillus acidophilus*, or mixtures of strains) have specific mechanisms against *H. pylori* infection, including inhibiting *H. pylori* attachment to mucosal cells (Sakarya and Gunay, 2014), reducing *H. pylori*-associated gastric inflammation (Yang and Sheu, 2012), or other direct physiological effects (Gotteland et al., 2006). A recent meta-analysis found that adjunct use of a few probiotic strains improved *H. pylori* eradication rates and prevented the development of adverse events and antibiotic-associated diarrhea in those treated with standard eradication therapies (McFarland et al., 2015). Despite these advances, effects of probiotics on gastric microbiota, and a relationship between those effects and the disease outcome caused by *H. pylori* infection, are not yet examined.

In the present study, *L. plantarum* ZDY 2013, isolated from traditional Chinese fermented soybeans, was selected as probiotic as it exhibited antimicrobial properties in vitro (Pan et al., 2015) and showed modulatory effects on gut microbiota in mice (Huang et al., 2015). Pretreatment of C57BL/6J mice with this microorganism was carried out before *H. pylori* infection, and gastric mucosal immune response and changes in gastric microbiota post-*H. pylori* infection were examined by using quantitative real-time PCR (qPCR) assay and high-throughput 16S rRNA gene

amplicon sequencing, respectively. Our results showed that pretreatment with *L. plantarum* ZDY 2013 prevented gastric inflammation and changes in microbiota post-*H. pylori* infection. Moreover, 3 wk *L. plantarum* ZDY 2013 administration induced selective modulation in gastric microbiota, suggesting targeting the gastric microbiota through probiotics may be a new avenue for preventing *H. pylori* infection.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition

Lactobacillus plantarum ZDY 2013 was grown in de Man, Rogosa, Sharpe (MRS broth; Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) under anaerobic condition at 37°C overnight when the viable cell population reached at approximately 10⁹ cfu/mL for administration to mice.

The Sydney strain of *Helicobacter pylori* (SS1) was cultured on *Campylobacter* agar base (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (Solarbio, Beijing, China), 10 µg/mL vancomycin, 2.5 units/mL polymyxin B, 5 µg/mL amphotericin, and 5 µg/mL trimethoprim under micro-aerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C for 48 h. For mice infection, *H. pylori* was grown in Brain Heart Infusion (Oxoid) supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China) with gentle shaking at 37°C under micro-aerophilic conditions overnight.

Animal Experiments

The protocol for carrying out animal experiments was approved by Nanchang University Animal Ethics Committee. All the animal experiments were approved by the Animal Care Review Committee (approval number 0064257) of Nanchang University (Jiangxi, China). Female specific-pathogen-free C57BL/6J mice (SJA Laboratory Animal Co. Ltd., Hunan, China) were housed at the animal facility under standard conditions in sterile cages with sterile bedding and supplemented with sterile feed and water ad libitum. The timeline for the animal experiment is depicted in Figure 1A. Briefly, after 1 wk of acclimatization, 32 mice (~16 g, 6 wk old) were randomly divided into 4 groups receiving 4 different pretreatments as described below:

- Group NC (n = 8): normal chow diet with 400 µL of PBS (control),
- Group LP (n = 8): normal chow diet with 400 µL of viable cells of *L. plantarum* ZDY 2013 (~10⁹

cfu/mL) suspended in PBS and administered by oral gavages,

- Group **HP** (n = 8): normal chow diet with 400 μ L of PBS,
- Group **LP+HP** (n = 8): normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages.

After 3 wk of *L. plantarum* ZDY 2013 administration, mice in groups of HP and LP+HP were infected by oral gavages with 500 μ L of *H. pylori* ($\sim 10^8$ cfu/mL) per mouse twice at a 1-d interval. The mice were fasted overnight before gavages. The animals were killed via CO₂ narcosis at 6 wk postinfection; the stomachs were dissected and opened along the lesser curvature. After washing in cold sterile normal saline, the stomachs were divided into longitudinal strips and fixed in paraformaldehyde for hematoxylin-eosin staining or immediately

frozen in liquid nitrogen before stored in -80°C for DNA and RNA extraction.

Stomach Histology

Paraformaldehyde-fixed stomach tissues were embedded in paraffin, sectioned at 5 mm, and then stained with hematoxylin and eosin as previously described (Zhang et al., 2013).

qPCR Assay

Quantitative analysis of IL-1 β , IFN- γ , IL-17, and IL-10 in the mouse stomach tissues was performed by qPCR. Total RNA was isolated from the tissue samples of the mouse stomach using Takara MiniBEST Universal RNA Extraction Kit according to the manufacturer's protocol. The RNA (500 ng) was reverse transcribed into cDNA using the Takara PrimeScript RT reagent

Figure 1. (A) Timeline for animal experiment. (B) Body weight of mice in the 4 groups. NC = normal chow diet with 400 μ L of PBS (control); LP = normal chow diet with 400 μ L of viable cells of *Lactobacillus plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages; HP = normal chow diet with 400 μ L of PBS; LP+HP = normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages. Error bars represent mean $\pm$ standard deviation.

Table 1. Primer sequences for mouse inflammatory cytokine genes

Gene	Primers ¹	DNA sequence (5'–3')	PCR product size (bp)	Reference
<i>Gapdh</i>	Gapdh-F	ACCCAGAGGACTGTGGATGG	124	Duary et al. (2012)
	Gapdh-R	TCAGCTCTGGGATGACCTTG		
<i>IL-17</i>	IL-17-F	GAATGTGAAGGTCAACCTCAAAGTC	148	Shi et al. (2010)
	IL-17-R	TCCCAGATCACAGAGGGATATCT		
<i>IL-1β</i>	IL-1β-F	TCGCAGCAGCACATCAACAAG	114	Shi et al. (2010)
	IL-1β-R	CATGTCCTCATCCTGGAAG		
<i>IFN-γ</i>	IFN-γ-F	GATCCTTTGGACCCCTCTGACTT	148	Shi et al. (2010)
	IFN-γ-R	TGACTGTGCCGTGGCAGTAA		
<i>IL-10</i>	IL-10-F	ATGCAGGACTTTAAGGGTTACTTG	253	Duary et al. (2012)
	IL-10-R	AGACACCTTGGTCTTGAGCTTA		

¹F = forward; R = reverse.

Kit with gDNA Erase. The qPCR was performed using SYBR Premix Ex Taq II. Primers were synthesized by Invitrogen China (Shanghai, China), and are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*Gadph*) was used as the housekeeping gene. The 20-μL reaction mix contained 0.8 μL (10 μM) of forward and reverse primers, 0.4 μL of ROX Reference Dye II (50×), 10 μL of SYBR Premix Ex Taq II (2×), 2 μL of cDNA samples, and the rest with nuclease-free water. The assays were performed on the 7900HT Fast Real-Time System (Applied Biosystems, Foster City, CA) with the following thermocycling program: pre-amplification stage 95°C for 1 min and then 40 cycles at 95°C for 5 s and 60°C for 1 min. All mRNA quantification data were normalized to the *Gadph* and are presented as fold change over control using the critical threshold (*Ct*) number and calculated using the $2^{-\Delta\Delta C_t}$ method (Yang et al., 2015).

Total DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Total genomic DNA was extracted from the gastric samples by using DNeasy Blood and Tissue Kit (Qiagen) combined with the bead-beater method (Bik et al., 2006). Briefly, gastric samples (~30 mg) were lysed in 180 μL of QIAamp ATL buffer and 20 μL of proteinase K for 1 h at 56°C. 0.2 g of zirconium beads (0.1 mm) were added, and samples were homogenized by bead beating twice for 45 s at a setting of 5.0 using a FastPrep-24 System (MP Biomedicals, Santa Ana, CA). An aliquot of 4 μL of RNaseA (100 mg/mL) was added and incubated for 2 min at room temperature (~22°C). Then DNA was recovered according to the manufacturer's protocol for tissue samples. The DNA concentrations and purity were examined by agarose gel electrophoresis and spectrophotometry using the NanoDrop (Thermo Scientific, Wilmington, DE). Purified DNA was stored at –80°C before 16S rRNA gene amplicon sequencing.

The 16S rRNA gene amplicon sequencing was performed on the Illumina MiSeq platform (BGI Tech Solutions Co. Ltd., Shenzhen, China) according to protocols described by Caporaso et al. (2012). The sequences of the primers targeting the V4 hyper-variable region of the bacterial 16S rRNA genes were as follows: 515F, 50-GTGCCAGCMGCCGCGGTAA-30; and 806R, 50-GGACTACHVGGGTWTCTAAT-30.

Bioinformatics Analysis

Bioinformatic analysis of sequencing data was conducted using the quantitative insights into microbial ecology (QIIME) pipeline (Caporaso et al., 2010). Briefly, raw data from all samples were filtered to eliminate the adapter pollution and low quality to obtain clean reads. High quality paired-end reads were combined to tags with an average read length of 252 bp using FLASH (fast length adjustment of short reads, v. 1.2.11). The tags were then clustered to OTU using USEARCH (v. 7.0.1090) at 97% sequence similarity and into phylotypes by clustering all the sequences belonging to the same genus. Taxonomic ranks were assigned to OTU representative sequence using Ribosomal Database Project Classifier v 2.2. Finally, an operational taxonomic units (OTU) table and a phylogenetic tree were produced for further α diversity (within a sample) and β diversity (between samples) analysis. Alpha diversity analysis included rarefaction curve, Shannon diversity index, and Simpson diversity. Principal coordinate analysis based on weighted or unweighted UniFrac was used for β diversity analysis. The statistical significance of the microbiota separation between groups was assessed by Adonis test utilizing the distance matrix between groups directly in R (version 2.15; The R Project for Statistical Computing) with Vegan packages (Oksanen et al., 2013). Differentially abundant bacterial taxa between groups were compared by using linear discriminant analysis effect size (LEfSe) method (Segata et al., 2011). A significant α

of 0.05 and an effect size threshold of 2 were used for all biomarkers discussed in this study.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation. Statistical significant difference among the groups was determined by one-way ANOVA followed by Tukey's post hoc test. An unpaired 2-tailed Student's *t*-test was used to analyze the significant differences between 2 groups. A *P*-value < 0.05 was considered statistically significant. GraphPad Prism version 5.01 for Windows (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis.

RESULTS

Effects of *L. plantarum* ZDY 2013 Pretreatment on Gastric Mucosal Inflammation

Compared with the normal group, mice in the HP group showed a slight reduction in BW (Figure 1B) after wk 5 (1 wk postinfection) but exhibited a significantly elevated level ($P < 0.01$) of inflammatory cytokine mRNA for IL-1 β (Figure 2A), IFN- γ (Figure 2B), and IL-17 (Figure 2C) in gastric mucosa at 6 wk postinfection. *Lactobacillus plantarum* ZDY 2013 pretreatment for 3 wk prevented the increase in mRNA for IL-1 β (Figure 2A) and IFN- γ (Figure 2B), and significantly reduced ($P < 0.05$) the mRNA level for IL-17 (Figure 2C). The mRNA level of IL-10 (Figure 2D) showed no significant changes among all the groups. Moreover, hematoxylin-eosin staining of the gastric mucosal tissue showed severe inflammatory cell infiltration in the gastric lamina propria of mice from HP group, whereas mice from LP+HP showed no inflammation (Figure 2E).

Effects of *L. plantarum* ZDY 2013 Pretreatment on Gastric Bacterial Diversity and Bacterial Community Alteration

Rarefaction curve (Figure 3A), Shannon's diversity index, and Simpson's diversity index (Figure 3B) were calculated to analyze the α diversity changes in gastric microbiota of the 4 groups (Figure 3A). In comparison, *H. pylori* infection led to a decrease in α diversity of gastric microbiota at 6 wk postinfection. Pretreatment with *L. plantarum* ZDY 2013 prevented the decrease in Shannon's diversity index and Simpson's diversity index (Figure 3B).

The changes in gastric microbiota across groups were examined by using principal coordinate analysis (PCoA) based on the unweighted and weighted Uni-

frac distance matrixes. The microbiota in each group showed an apparent clustering based on unweighted distance matrixes (Figure 3C), but robust clustering based on weighted Unifrac distance matrixes (Figure 3D). While using the Adonis test, both of them yielded a *P*-value of 0.001, indicating at least one group being separated from the others. To further analyze the effect of *H. pylori* infection and *L. plantarum* ZDY 2013 pretreatment on the gastric microbiota, a similar PCoA based on weighted Unifrac distance was carried out to compare the NC versus HP or LP+HP, and the Adonis test showed a significant difference in microbiota between NC and HP ($P = 0.024$), but not between NC and LP+HP ($P = 0.084$). Additionally, a nonsignificant microbiota difference was observed between NC and LP ($P = 0.105$, Adonis test).

The Bacterial Taxa Response to *H. pylori* Infection and *L. plantarum* ZDY 2013 Pretreatment

The gastric bacterial community composition of the 4 groups is shown in Figure 4. It was demonstrated that 4 phyla (in descending order: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia*) were the most dominant in 13 bacterial phyla; *Firmicutes* and *Bacteroidetes* represented on an average as much as 75.6% of each sample's OTU.

To identify the specific bacterial taxa associated with *H. pylori* infection and *L. plantarum* ZDY 2013 pretreatment, the gastric microbiota in the group of HP was compared with that of LP+HP using LEfSe. The greatest difference in taxa between the 2 communities is displayed in Figure 5A. Twenty-two bacterial taxa (e.g., *Pasteurellaceae*, *Erysipelotrichaceae*, *Halo-monadaceae*, *Helicobacteraceae*, and *Spirochaetaceae*) were significantly ($P < 0.05$) much more abundant in HP group, whereas only 6 taxa were overgrown ($P < 0.05$) in the group of LP+HP (e.g., *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae*). Particularly, the average relative abundance of bacterial genus of *Helicobacter* in HP group was 0.0281, whereas it decreased to 0.006 in the LP+HP group (Figure 5B).

Bacterial Taxa Associated with Administration for 3 wk of *L. plantarum* ZDY 2013

The hypothesis that *L. plantarum* ZDY 2013 administration may change the gastric microbiota, which can pose a preventive effect against *H. pylori* infection, was interesting. To explore the bacteria taxa associated with *L. plantarum* ZDY 2013 administration, a LEfSe comparison of the gastric microbiota between the group NC and LP was performed. As shown in Figure 6, at family level, 5 bacterial families (i.e., *Rikenellaceae*,

Figure 2. The LP pretreatment suppresses gastric mucosal inflammation induced by *Helicobacter pylori* infection. Gastric mucosal inflammatory cytokine mRNA for IL-1 β (A), IFN- γ (B), and IL-17 (C) were significantly suppressed by LP pretreatment. Cytokine mRNA for IL-10 (D) showed no change. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by ANOVA followed by Tukey post hoc test. (E) Gastric histopathology from C57BL/6J mouse inoculated with *H. pylori* showed moderate inflammatory cell infiltration of the lamina propria, whereas 3 wk pretreatment of LP prevented inflammatory cell infiltration in lamina propria. Hematoxylin-eosin stain; original magnification $\times 150$. NC = normal chow diet with 400 μ L of PBS (control); LP = normal chow diet with 400 μ L of viable cells of *Lactobacillus plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages; HP = normal chow diet with 400 μ L of PBS; LP+HP = normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages. Error bars represent mean $\pm$ standard deviation. Color version available online.

Figure 3. Bacterial community diversities and structures of the mice gastric microbiota in different treatments. (A) Rarefaction curves of operational taxonomic units (OTU) reached saturation, reflecting that samples had the most bacterial diversity within a sample. (B) *Helicobacter pylori* infection induced a decline in Shannon and Simpson index, and pretreatment with LP prevented this decline. * $P < 0.05$ by ANOVA followed by Tukey post hoc test. Error bars represent mean $\pm$ standard deviation. (C) Principal coordinate analysis (PCoA) showed an apparent clustering of the gastric microbiota of 4 groups based on unweighted Unifrac distance. (D) Principal coordinate analysis based on weighted Unifrac distance showed a robust clustering of the gastric microbiota of the 4 groups. Blue triangles represent groups of LP, green circles represent groups of NC, pink triangles represent groups of LP+HP, and red squares represent groups of HP. NC = normal chow diet with 400 μ L of PBS (control); LP = normal chow diet with 400 μ L of viable cells of *Lactobacillus plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages; HP = normal chow diet with 400 μ L of PBS; LP+HP = normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages. Color version available online.

Ruminococcaceae, *Coriobacteriaceae*, *Staphylococcaceae*, and *Bifidobacteriaceae*) were much more abundant in the gastric microbiota of LP group, whereas at the

genus level, 4 bacterial genera, including *Rikenella*, *Staphylococcus*, *Bifidobacterium*, and *Corynebacterium*, were observed.

DISCUSSION

Lactobacillus plantarum ZDY 2013 was previously proven to be an ideal probiotic organism due to its high survival under acidic conditions and exhibition of acid tolerance response (Huang et al., 2015). In this study, *L. plantarum* ZDY 2013 was administered for its preventive effect against *H. pylori* infection. Our results demonstrated that pretreatment with *L. plantarum* ZDY 2013 can prevent gastric mucosal inflammation and gastric microbiota alteration induced by *H. pylori* infection.

For host immunity stimulation, *H. pylori* has been implicated in triggering the Th1/Th17 cell response in the gastric mucosa (Shi et al., 2010). In agreement with that, in this study we found an elevated level of inflammatory cytokine RNA for IL-1 β , IFN- γ , and IL-17 at 6

wk postinfection, whereas in the LP+HP group, an elevated mRNA level for IL-1 β and IFN- γ was inhibited, indicating that pretreatment with *L. plantarum* ZDY 2013 could prevent the Th1 cell response triggered by *H. pylori* infection. Numerous reports have mentioned the immune-modulatory properties of probiotics. However, pro- or anti-inflammation for the host immune system might occur on a case by case basis upon use of different probiotic strains (Turroni et al., 2014). Previous studies have reported an anti-inflammatory property of *Lactobacillus plantarum* in several animal disease models (Liu et al., 2011; Thiraworawong et al., 2014). Studies in the mouse colitis model demonstrated that an anti-inflammatory cytokine of IL-10 was up-regulated by *Lactobacillus plantarum* supplementation (Duany et al., 2012); however, no significant increase in IL-10 was found in *L. plantarum* ZDY 2013 pretreated

Figure 4. Composition of mice gastric bacterial community in different treatments. Each bar represents the relative abundance of operational taxonomic units (OTU) at the phylum level in the gastric microbiota within a sample. *Firmicutes* and *Bacteroidetes* represented on an average of as much as 75.6% of each sample's OTU. NC = normal chow diet with 400 μ L of PBS (control); LP = normal chow diet with 400 μ L of viable cells of *Lactobacillus plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages; HP = normal chow diet with 400 μ L of PBS; LP+HP = normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages.

mice, suggesting that other factors rather than IL-10 were involved in the anti-inflammatory activity of *L. plantarum* ZDY 2013.

Probiotics can affect the immune homeostasis by altering the microbial balance in the gastrointestinal tract and thus enhancing the immune system of the

Figure 5. Difference in bacterial taxa between group of HP and LP+HP. (A) Linear discriminant analysis (LDA) coupled with effect size (LEfSe) showed that the bacterial taxa significantly differentiated between HP and LP+HP group. The HP group-enriched bacterial taxa are indicated with negative LDA score (red), and bacterial taxa enriched in LP+HP group are indicated with a positive LDA score (green). $P < 0.05$, LDA significant threshold > 2 . (B) Average relative abundances of *Helicobacter* in HP and LP+HP. *** $P < 0.001$ by analysis of unpaired 2-tailed Student's t -test. Error bars represent mean $\pm$ standard deviation. NC = normal chow diet with 400 μ L of PBS (control); LP = normal chow diet with 400 μ L of viable cells of *Lactobacillus plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages; HP = normal chow diet with 400 μ L of PBS; LP+HP = normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages. Color version available online.

host (Clemente et al., 2012; Turrone et al., 2014). Due to the extreme acidic environment of the stomach, few probiotics can resist such conditions and remain alive. Upon the extreme gastric environment resistance of *L. plantarum* ZDY 2013, we examined the effect of its pretreatment on gastric microbiota post-*H. pylori* infection. In our study, *H. pylori* infection resulted in the decrease of α diversity accessed by Shannon index and Simpson index, *L. plantarum* ZDY 2013 pretreatment prevented that tendency to decrease. Furthermore, a significant gastric microbiota separation between NC and HP (Adonis test < 0.05) was found according to PcoA analysis based on Unifrac distance, whereas nonsignificant gastric microbiota separation was found between NC and LP+HP (Adonis test > 0.05), indicating *L. plantarum* ZDY 2013 pretreatment corrected the alteration of microbiota induced by *H. pylori*. Selective modulation of the microbiota by using probiotics is a developing strategy for many diseases such as obesity (Delzenne et al., 2011), inflammatory bowel disease (Jones and Foxx-Orenstein, 2007), and liver disease (Xu et al., 2012). However, with regard to the beneficial effects of probiotics against *H. pylori* infection, the pro-

posed mechanisms were limited to the biological properties of the strain itself, such as immune-modulatory (Yang and Sheu, 2012) and antimicrobial effects (Chen et al., 2012), due to previous bias that the normal stomach is sterile. Up to date, little was known about the niche of gastric flora, regardless of the change in gastric microbiota by intervention with lactic acid bacteria. Thus, to our knowledge, this is the first study to examine the effect of probiotics on gastric microbiota against *H. pylori* infection.

To further ascertain the bacterial taxa associated with *H. pylori* infection and *L. plantarum* ZDY 2013 pretreatment, LEfSe analysis was performed. We found that 22 bacterial taxa were more dominant in the HP group; most of them belonged to the *Proteobacteria* phylum, whereas in the LP group, 6 dominant phyla were present, mainly from *Firmicutes* and *Bacteroidetes*. The expansion of the *Proteobacteria* was strongly associated with several diseases because this phylum includes gram-negative bacteria, which includes a wide variety of pathogenic species, such as *Escherichia*, *Salmonella*, and *Vibrio* (Mukhopadhyay et al., 2012). In this study, the inhibition of *Proteobacteria* in LP+HP

Figure 6. Difference in bacterial taxa between group of NC and LP. Linear discriminant analysis (LDA) coupled with effect size showed the significantly different bacterial taxa between NC and LP groups. The LP group-enriched bacterial taxa are indicated with negative LDA score (red), and NC group-enriched bacterial taxa with a positive LDA score (green). $P < 0.05$, LDA significant threshold >2. NC = normal chow diet with 400 μ L of PBS (control); LP = normal chow diet with 400 μ L of viable cells of *Lactobacillus plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages; HP = normal chow diet with 400 μ L of PBS; LP+HP = normal chow diet with 400 μ L of viable cells of *L. plantarum* ZDY 2013 ($\sim 10^9$ cfu/mL) suspended in PBS and administered by oral gavages. Color version available online.

indicates that *L. plantarum* ZDY 2013 pretreatment successfully prevented the gastric microbiota alteration induced by *H. pylori* infection. Particularly, analysis of the abundance of bacterial genus of *Helicobacter* alone found a significant decrease in *Helicobacter* (from 0.0281 to 0.006), suggesting that colonization of *Helicobacter* was not totally prevented but suppressed by *L. plantarum* ZDY 2013 pretreatment.

For examination of the beneficial mechanism of probiotics on the host, ample evidence suggests that probiotics can inhibit potential pathogens directly through production of antimicrobial substance (Gotteland et al., 2006). Currently, other indirect benefits of probiotics administration including increasing the diversity or number of “good” bacteria, such as *Bifidobacterium*, *Fecalibacterium*, and *Clostridium*, and so on, have also been reported (Zhang et al., 2014). Thus, we are interested in the hypothesis that the pretreatment with *L. plantarum* ZDY 2013 might change the gastric microbiota, and in turn provide a preventive effect against *H. pylori* infection. According to the Adonis test, however, no significant microbiota alteration was found after 3 wk of *L. plantarum* ZDY 2013 administration ($P > 0.05$). But through LEfSe analysis, we still found several bacterial taxa (e.g., *Rikenella*, *Staphylococcus*, *Bifidobacterium*) more abundant in *L. plantarum* ZDY 2013 treated mice when compared with the control group. These data suggest that the preventive effect of *L. plantarum* ZDY 2013 administration might occur through selective modulation of specific bacterial taxa in gastric microbiota rather than by changing the overall gastric microbiota.

In addition, except for its effect on stomach microbiota, the administration of *L. plantarum* ZDY 2013 and its effect on constitution of intestinal microbiota might be helpful against the infection of HP as well; therefore, as another task, a systemic analysis of the gastrointestinal microbiota is worthy for exploring the mechanism of probiotic intervention against infection of *H. pylori*.

CONCLUSIONS

In summary, our results show that *L. plantarum* ZDY 2013 pretreatment posed a selective modulation in gastric microbiota, which may have contributed to an important preventive effect on *H. pylori* infection, including prevention in gastric mucosal inflammation and gastric microbiota alteration post-*H. pylori* infection. Our work suggests that targeting gastric microbiota through oral administration of probiotics might be an alternative strategy to prevent *H. pylori* infection.

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