In vitro probiotic characteristics of *Lactobacillus plantarum* ZDY 2013 and its modulatory effect on gut microbiota of mice

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ABSTRACT

Lactobacillus plantarum ZDY 2013, a novel strain isolated from Chinese traditional fermented acid beans, was systematically evaluated for its survival capacity under stress conditions (pH, bile salt, simulated gastrointestinal tract, and antibiotics), production of exopolysaccharide and antagonism against 8 pathogens. Its effect on mice gut microbiota was also investigated by quantitative PCR and PCR-denaturing gradient gel electrophoresis. The results showed that ZDY 2013 can grow at pH 3.5 and survive at pH 2.0 for 6 h and at 0.45% bile salt for 3 h. The exopolysaccharide yield was up to 204 ± 7.68 mg/L. The survival rate of ZDY 2013 in a simulated gastrointestinal tract was as high as 65.84%. Antagonism test with a supernatant of ZDY 2013 showed maximum halo of 28 mm against *Listeria monocytogenes*. The inhibition order was as follows: *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Enterobacter sakazakii*, and *Staphylococcus aureus*. *Lactobacillus plantarum* ZDY 2013 was sensitive to some antibiotics (e.g., macrolide, sulfonamides, aminoglycoside, tetracyclines and β -lactams), whereas it was resistant to glycopeptides, quinolones, and cephalosporins antibiotics. Denaturing gradient gel electrophoresis profile demonstrated that ZDY 2013 administration altered the composition of the microbiota at various intestinal loci of the mice. Moreover, the quantitative PCR test showed that the administration of ZDY 2013 enhanced the populations of *Bifidobacterium* and *Lactobacillus* in either the colon or cecum, and reduced the potential enteropathogenic bacteria (e.g., *Enterococcus*, *Enterobacterium*, and *Clostridium perfringens*). *Lactobacillus plantarum* ZDY 2013 exhibited high resistance against low pH, bile salt, and gastrointestinal fluid, and possessed antibacterial and gut microbiota modulation

properties with a potential application in the development of dairy food and nutraceuticals.

Key words: *Lactobacillus plantarum* ZDY 2013, stress resistance, quantitative PCR, antagonistics

INTRODUCTION

The beneficial effects of probiotics (e.g., *Lactobacillus* and *Bifidobacterium*) have been documented by numerous investigations over many years, and mainly included improvement in the health of intestinal tract, enhancement of immune system, reduction of intestinal disorders, and risk of certain cancers (Salminen et al., 1998; Parvez et al., 2006; Jankovic et al., 2010). Generally, it has been assumed that probiotics, being of intestinal origin, can be used for human or animal consumption for exerting beneficial effects on host health through the interaction with host and for providing positive influence in the alleviation or prevention of diverse intestinal disorders (Borriello et al., 2003). For example, *Lactobacillus rhamnosus* GG, one of the most successful commercial *Lactobacillus* strains isolated from human gastrointestinal tract, has been used for nutraceutical supplement in numerous microecological studies (García-Ródenas et al., 2006).

In addition to isolating probiotics organisms from human feces, traditional fermented foods have been another important source for isolating probiotic organisms in many countries (ben Omar and Ampe, 2000; Tamime, 2002; Cheng et al., 2014). Isolation of probiotic organisms and evaluation of their properties has attracted a lot of attention in recent decades. In this context, isolation of *Lactobacillus plantarum* is important, as it is found in traditional fermented food, especially in fermented foods from Korea, China, and India (Salminen et al., 1998; Jankovic et al., 2010). Kleerebezem et al. (2003) carried out the complete genome sequence *L. plantarum* WCFS1, which was isolated from kimchi, and annotated the interaction of this organism with its environment at molecular level. However, most of the work on *L. plantarum* focused on its antioxidant, antimutagen, modulation of immune system, lowering

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cholesterol level, and reducing lactose-intolerance capabilities (Naidu et al., 1999; Fooks and Gibson, 2002; Gill, 2004; Parracho et al., 2007). The behavior of *L. plantarum* is diverse depending on different sources of isolation. Some isolates developed resistance to extreme acidic conditions, whereas others can survive in high concentration of bile salt (Liong and Shah, 2005; Guidone et al., 2014). Kaushik et al. (2009) reported that *L. plantarum* LP9 survived at pH 1.5 to 2.0 or in 1.5 to 2.0% oxgall bile for 2 h. Similarly, Lee et al. (2011) reported that *L. plantarum* CJLP133, isolated from kimchi, could survive at pH 2.5 for 3 h and in 0.3% oxgall for 24 h. Therefore, characterization of isolates of *L. plantarum* from traditional fermented food is important to ascertain the mechanism of probiotic action for promoting human health. Very limited information exists on in vivo interaction of *L. plantarum* with intestinal microbiota. In our study, a novel strain, *L. plantarum* ZDY 2013, was isolated from Chinese home-made acid beans and was systematically assessed for its survival in stress conditions (pH, bile salt, mimic gastrointestinal tract, and antibiotics) and in vivo interaction with indigenous microbial biota using quantitative real-time PCR (qPCR) combined with denaturing gradient gel electrophoresis (DGGE).

MATERIALS AND METHODS

Bacterial Strains Isolation and Identification

Traditional acid beans were collected from a village in Fujian province, China, and fermented under natural conditions without any additional bacteria. The bacterial strains were isolated from acid beans as described herein. Acid beans were collected from local village in Fujian province, China. Bacteria were isolated from acid beans according to a modified method described before (Wei et al., 2006; Wu et al., 2009). Briefly, 10 g of fermentation broth and beans were added to 90 mL of sterile PBS buffer, followed by homogenization. The suspension was serially diluted (10^{-1} , 10^{-2} , 10^{-3}) and 0.1 mL of each dilution was surface plated in duplicates on de Man, Rogosa, Sharpe (MRS) agar. The plates were incubated under anaerobic conditions at 37°C for 48 h. Colonies with distinct morphological differences, such as color, shape, and size, were selected and purified by streaking 3 times on MRS agar and were kept in MRS-agar stabs at 4°C, and frozen in 30% glycerol solution and stored at -80°C. A representative isolate was amplified by PCR method using the 16S rRNA universal primer. The amplicons were sequenced by Sangon Biotech, Ltd. (Shanghai, China) and the sequences were searched against NCBI database (<http://www.ncbi.nlm.nih.gov/index.html>) using the BLASTN

algorithm. Based on these, the organism was classified as *L. plantarum* and was given the strain no. ZDY 2013.

Acid and Bile Salt Tolerance Properties

The isolate *L. plantarum* ZDY 2013 was grown in sterile MRS broth at 37°C for 24 h, and centrifuged at $7,500 \times g$ for 10 min at 4°C. The cell pellet was diluted $100\times$ in sterile MRS broth and the pH was adjusted to 6.5, 5.0, 4.0, and 3.5 for acid adaptation; another set of media was adjusted to 3.0 and 2.0 for acid challenge studies. Bile salt tolerance was performed in the medium supplemented with 0, 0.15, 0.30, and 0.45% (wt/vol) bile salts (YuanYe Bio-Technology Ltd., Shanghai, China). Freshly prepared cultures were incubated at 37°C. The absorbance at 600 nm and pH values of fermentation cultures were measured immediately and at every 2 h. Viable counts were enumerated after acid challenge and bile salt tolerance test at 0, 3, 6, and 12 h.

Exopolysaccharide Yield

The MRS broth (100 mL) was inoculated with 1% ZDY 2013 and cultured at 37°C in 250-mL Erlenmeyer flasks. Aliquots (5 mL) were withdrawn at various time points to determine the viable cell counts and exopolysaccharide (EPS) yield. The EPS from cell-free culture supernatant was extracted and detected by the methods as described by Li et al. (2014a). Briefly, bacterial cells were removed by centrifugation at $4,000 \times g$ for 10 min at 4°C, and the cell-free culture supernatant was treated with ethanol overnight at 4°C followed by centrifugation ($8,000 \times g$, 20 min, 4°C). The precipitate was dissolved in 5 mL of distilled water. Proteins were removed using Sevig agent (chloroform: n-butanol; 4:1) followed by centrifugation ($8,000 \times g$, 10 min, 4°C). The supernatant was removed to another receptacle and precipitated with ethanol again. The precipitated EPS was dissolved with distilled water; EPS concentration was determined by the phenol-sulfuric acid assay method (Li et al., 2014a).

Antimicrobial Activities of ZDY 2013

Enterobacter sakazakii ATCC 29544, *Escherichia coli* O 157: H 7, *Salmonella typhimurium* ATCC 13311, *Shigella sonnei* ATCC 25931, *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* MCC 10104, *Listeria monocytogenes* CMCC 54007, and *Staphylococcus aureus* CMCC 26003 were selected to test the antibacterial activities of *L. plantarum* ZDY 2013. The antibacterial activity was investigated by agar diffusion assay as described previously by Chen et al. (2014b) with

a slight modification. Overnight-incubated culture of the indicator microorganisms was adjusted to 10^6 to 10^7 cfu/mL and 100- μ L aliquots of the organisms were spread on Luria-Bertani agar; then 200 μ L of cell-free supernatant of ZDY 2013 was added into the oxford cup (a stainless steel cylinder of outer diameter 7.8 ± 0.1 mm, inner diameter 6.0 ± 0.1 mm, and height 10.0 ± 0.1 mm) which was placed on the surface of the agar. The diameter of the inhibition zone around the cup (including that of the Oxford cup, 7.8 mm) was measured after 12 h of incubation. The experiment was performed in triplicate.

Antibiotic Sensitivity Test

The disk-diffusion method was used to determine the antibiotic susceptibility of ZDY 2013 according to Tan et al. (2013). Briefly, the organism was inoculated into MRS broth and incubated at 37°C overnight, 100 μ L of diluted culture (10^7 cfu/mL) was spread onto MRS agar, and the antibiotic discs were applied onto the surface. The concentrations of indicator antibiotics are shown in Table 1. Plates were incubated at 37°C and evaluated after 24 h. Inhibition zones around the discs were measured using a digital caliper.

Simulated Gastrointestinal Transit

The survival of ZDY 2013 in the simulated gastrointestinal tract (GIT) was studied in a simulation model with some modifications (Salazar et al., 2011, Tan et al., 2013). To simulate the GIT transit, the bacterial suspension was submitted to the gastric juice (GJ; con-

taining 3 g/L of pepsin dissolved with PBS, pH 3.0), duodenum juice [DJ; containing 1% (wt/vol) bile salt, pH 8.0], and intestinal juice [IJ; containing 0.3% (wt/vol) bile salt, 0.1% (wt/vol) pancreatin], sequentially. The bacterial cells were harvested by centrifugation ($7,500 \times g$, 5 min, 4°C) after 24 h of incubation and washed twice with PBS, and then a 1.0-mL suspension was centrifuged ($7,500 \times g$, 5 min, 4°C) and resuspended in 10 mL of GJ. Finally, bacterial suspension was incubated for 90 min at 37°C with shaking (100 rpm). Afterward, cells were harvested, resuspended in DJ, and incubated under static conditions for 10 min at 37°C in anaerobic condition. Finally, cells were harvested again, resuspended in IJ, and incubated for 120 min at 37°C in anaerobic condition. Viable counts were enumerated before each step and at the end of GIT transit.

Animal Model and Experiment Design

The protocol for carrying out animal experiments was approved by Nanchang University Animal Ethics Committee and all the ethical requirements to conduct the experiment were met. All procedures were conducted in compliance with protocols provided and approved by the Animal Care Review Committee (approval number 0064257) of Nanchang University (Jiangxi, China). Twenty 6- to 8-wk-old BALB/c mice obtained from Nanchang University Animal Science Department were used in the modulation of intestinal microbiota study after oral administration of ZDY 2013. The mice were acclimatized for 1 wk and assigned into their respective groups randomly (10 mice each group) based on baseline BW. Mice were gavaged twice a day (with an

Table 1. Antibiotic susceptibility of *Lactobacillus plantarum* ZDY 2013

Class	Antibiotic	Content (μg)	Diameter of inhibition zone (mm)	Sensitivity ¹
Macrolide	Erythromycin	15	33.13 ± 0.85	S
	Rifampicin	5	21.75 ± 0.29	S
Sulfonamides	Sulphamethoxazole	25	37.25 ± 0.96	S
	Aminoglycoside			
Aminoglycoside	Streptomycin	10	16.5 ± 0.58	S
	Gentamicin	10	21.50 ± 0.58	S
	Kanamycin	30	17.00 ± 0.82	I
	Nitrofurans			
Nitrofurans	Nitrofurantoin	300	32.63 ± 1.11	S
Tetracyclines	Tetracycline	30	25.88 ± 1.03	S
Amphenicols	Chloramphenicol	30	28.75 ± 0.96	S
Beta-lactams	Amoxicillin	10	28.25 ± 0.87	S
	Penicillin	10 U	28.88 ± 0.25	I
Glycopeptide	Bacitracin	0.04	6.17 ± 0.29	R
	Polymyxin B	30	7.25 ± 0.29	R
Quinolones	Ciprofloxacin	5	10.75 ± 0.96	R
	Nalidixic acid	30	6.63 ± 0.63	R
Cephalosporins	Cefoxitin	30	14.00 ± 0.82	R

¹S = susceptible; I = intermediate; R = resistance.

interval of 8 h) for 2 wk. Each mouse in the treatment group received 200 μ L of sterile PBS containing 10^7 to 10^8 cfu/mL of ZYD 2013 cells, whereas mice in the control group received an equal volume of sterile PBS. Activity, behavior, fur health, and live weight were observed twice daily during the experimental period. Twenty-four hours after the final administration, mice were euthanized by CO₂ inhalation followed by cervical dislocation. Blood samples were collected for chemical analysis, as discussed below. The kidneys, spleen, and thymus were weighed and their weights relative to the final BW (organ index) were calculated. Fecal pellets, cecum contents, and colon were also collected and stored at -20°C until analyzed.

Health Markers of Mice

Blood samples (approximately 0.8 mL) were taken from orbital sinus by quickly removing the eyeball the control and *L. plantarum* ZDY 2013 treatment groups (6 mice per group) after euthanizing them. Ten important hepatic markers were then measured, including alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, total protein, albumin, globulin, ratio of albumin to globulin, gamma glutamyl transaminase, alkaline phosphatase, creatinine, BUN, and urea. These various serum biochemical markers were indicators of liver and kidney functions. Blood samples were centrifuged (4,000 rpm, 10 min, 4°C) to obtain at least 0.25 mL of blood plasma for analysis of serum biochemistry using biochemical analyzer at the First Affiliated Hospital of Nanchang University, Nanchang, China (Yang et al., 2015).

qPCR Assays

The genomic DNA of intestinal contents and fecal samples were extracted using the bead-beating method (Li et al., 2014a). The qPCR amplification was performed using an ABI 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, CA) and the reaction of qPCR assays contained 10 μ L of SYBR Primer *EX Taq*II (Takara, Dalian, China), 0.4 μ L of ROX Reference Dye (50 $\times$; Takara), 1.0 μ L of DNA template, and 0.8 μ L of each of the primers (the final concentration was 0.4 μ M) with 7 μ L of milli-Q H₂O (Millipore, Billerica, MA). The amplification was programmed to start at 95°C for 30 s, followed by 40 cycles (degeneration at 95°C for 5 s, annealing at 60°C for 30 s; Wang et al., 2014). The relative population (fold change) of the target bacterial was analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method based on qPCR data according to Feng et al. (2010). Different groups and species of bacteria targeted by primers are listed in Table 2.

PCR-DGGE Analysis

The V3 region of the 16S rRNA gene was amplified using primers 338 (F) with GC clamp and 519 (R) followed by DGGE analysis, as described previously (Chen et al., 2014a; Li et al., 2014a). Briefly, the reaction mixture contained 25 μ L of 2 $\times$ Taq MasterMix (CWBIO, Beijing, China), 0.1 μ M of each primer, 1.0 μ L of DNA template, and milli-Q H₂O was added to obtain a final volume 50 μ L. The PCR reaction began with 1 cycle of 5 min at 94°C , followed by 30 cycles of 30 s at 94°C , 30 s at 56°C , and 45 s at 72°C , and a

Table 2. Primers (forward, F, and reverse R) used in this study¹

Target organism	Oligonucleotide sequence (5'—3')	Reference
V3 region	F: TACGGGAGGCAGCAG with GC clamp R: ATTACCGCGGCTGCTGG	Li et al., 2014a
Total bacterial	F: ACTCCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	Bartosch et al., 2004 and Li et al., 2014a
<i>Bifidobacterium</i> spp.	F: TCGCGTCCGGTGTGAAAG R: CCACATCCAGCATCCAC	Rinttilä et al., 2004
<i>Enterococcus</i> spp.	F: CCCTATTGTTAGTTGCCATCAT R: ACTCGTTGTACTTCCCATTTGT	Rinttilä et al., 2004
<i>Fusobacterium</i> spp.	F: CCCTTCAGTGCCGAGT R: GTCGCAGGATGTCAAGAC	Rinttilä et al., 2004
<i>Enterobacteriaceae</i>	F: CATTGACGTTACCCGCAGAAGAAG R: CTCTACGAGACTCAAGCTTGC	Bartosch et al., 2004
<i>Lactobacillus</i> spp.	F: CACCGCTACACATGGAG R: AGCAGTAGGGAATCTTCCA	Rinttilä et al., 2004
<i>Bacteroides</i> spp.	F: GGTGTCGGCTTAAGTGCCAT R: CGGATGTAAGGGCCGTGC	Rinttilä et al., 2004
<i>Clostridium perfringens</i>	F: CGCATAACGTTGAAAAGATGG R: CCTTGGTAGGCCGTTACCC	Wise and Siragusa, 2005

¹GC clamp = TACGGGAGGCAGCAG.

final extension at 72°C for 10 min. After amplification, the amplicons were separated by DGGE using the Dcode System (Bio-Rad, Hercules, CA; Chen et al., 2013) which was performed on 8% (wt/vol) polyacrylamide gels in running buffer containing 1× TAE [20 mmol/L Tris, 10 mmol/L acetate, 0.5 mmol/L EDTA (pH 8.0) and a denaturing gradient of 35–65% urea and formamide (Sigma-Aldrich, St. Louis, MO), where 100% is defined as 7 mol/L of urea and 40% (vol/vol) formamide]. Electrophoresis was conducted under 80 V at 60°C for 16 h. The DNA bands in gels were visualized by silver staining (Chen et al., 2012).

The DGGE patterns were subsequently normalized and analyzed using Bio-Rad Quantity One 4.4.0 software (Chen et al., 2011). The degree of similarity was described by a similarity coefficient. Digitalized DGGE patterns were analyzed by the principal component analysis (PCA) to change the microbiota into dots pattern. Richness indices were calculated according to equations previously described by Luo et al. (2004).

Statistical Analysis

Triplicate data were represented by mean ± standard deviation. We used SPSS 13.0 software to perform statistical analyses (SPSS Inc., Chicago, IL) by means of independent one-way ANOVA tests. The multiple comparison of each test was assessed by least significant difference test ($P < 0.05$).

RESULTS

Resistance to Acid and Bile Salts

Several strains of *Lactobacillus* were verified by PCR and sequenced from collections of isolates from Chinese traditional acid beans. Moreover, the first run evaluation of survival rate under extreme conditions (e.g., low pH, high concentration of bile salts) was performed and 1 strain with outstanding stress-resistant ability, *L. plantarum* ZDY 2013, was tested further for its sensitive to acid and bile salts. As shown in Figure 1, ZDY 2013 grew in a wide range of pH, from 6.5 to 3.5, although a minor difference in viability among groups was noted at pH 3, 3.5, and 4.0 (Figure 1A). To ascertain the survival of *L. plantarum* ZDY 2013 in extreme acidic conditions, a long-term resistant testing of viable cell counts (initial value of 2.24×10^7 cfu/mL) in low pH was performed. As shown in Figure 1B, the bacterial count did not change significantly at pH 3.0, or even after 6 h in pH 2.0, but decreased significantly after 12 h in pH 2.0. To observe the effect of the initial pH of

the original culture on *L. plantarum* ZDY 2013 fermentation, changes in pH values were tested for 36 h. As shown in Figure 1C, the pH of the culture at pH 6.5 and 5.0 decreased rapidly until 8 h and maintained this trend until 32 h, and pH 4.0 and 3.5 decreased slowly to a final pH of 3.2; however, only minor difference were noted among the various initial pH groups.

Considering common bile salt concentrations of human GIT range from 0.03 to 0.3%, we used 0.15, 0.30, and 0.45% as target challenge concentrations. As shown in Figure 1D, the survival rate of cells of *L. plantarum* ZDY 2013 was nearly stable in all test groups at 3 h; a reduction trend was observed in cell counts on increasing bile salt concentration. For example, the reduction in group of 0.45% was from 7.53×10^7 to 1.25×10^6 cfu/mL, whereas it was 1.78×10^7 and 5.01×10^6 cfu/mL, respectively, in 0.15 and 0.30% groups at 6 h; the cell counts maintained a high level at 0.3% bile salt even after 12 h (1.01×10^6 cfu/mL).

Growth and EPS Yield

Among metabolic substances produced from *L. plantarum* ZDY 2013, it is likely that EPS may have contributed to the resistance against extreme acid environment. In our preliminary study, this *Lactobacillus* strain produced high amount of EPS among our collection of *Lactobacillus* strains (data not shown). As shown in Figure 2, the yield of EPS was positively related to the biomass of cells of *L. plantarum* ZDY 2013; the highest yield of EPS (204 ± 7.87 mg/L) was achieved at 24 h, indicating that EPS may have been a primary metabolite of *L. plantarum* ZDY 2013. Interestingly, the amount of EPS decreased during the stationary phase.

Antagonistic Effect of Cell-Free Suspension of *L. plantarum* ZDY 2013 In Vitro

Antagonistic effect against pathogens was the main probiotic trait for the evaluation of the potential probiotic organism. As shown in Figure 3, 8 different food-borne pathogens were significantly inhibited by the cell-free culture supernatant of *L. plantarum* ZDY 2013. *Listeria monocytogenes* CMCC 54007 was the most sensitive organism, with a halo as high as 28.33 ± 0.76 mm, whereas *S. aureus* was the least sensitive, with a halo of 12.33 ± 0.29 mm.

Antibiotic Sensitivity of *L. plantarum* ZDY 2013

Considering potential application of *Lactobacillus* isolates together with a reasonable dose of antibiot-

ics as combination therapies for intestinal disorder, antibiotic sensitivity of *L. plantarum* ZDY 2013 was evaluated. As shown in Table 2, susceptibility of *L. plantarum* ZDY 2013 varied among classes of antibiotics, it exhibited a steady resistance to glycopeptide (bacitracin, polymyxin B), quinolones (ciprofloxacin, nalidixic acid), and cephalosporins (cefotaxime), whereas ZDY 2013 was sensitive to macrolide (erythromycin), sulfonamides (rifampicin, sulphamethoxazole), aminoglycoside (streptomycin, gentamicin), tetracyclines, nitrofurans (nitrofurantoin), amphenicols (chloramphenicol), and β -lactams (e.g., amoxicillin), but showed an intermediate sensitivity to penicillin and kanamycin.

Simulated Gastric-Duodenal-Intestinal Transit Testing

Based on the results of extreme acid and high concentration bile salt resistance of *L. plantarum* ZDY 2013, we further constructed a simulated gastric-duodenal-intestinal transit system, including mimic digestion enzyme (e.g., pepsin, pancreatin). As shown in Figure 4, a gradual loss of cell counts was observed in the 3 phases of mimic digestion (i.e., GJ, DJ, and IJ). The final survival of cells achieved was as high as 65.84%; the cell count was 6.31×10^7 cfu/mL after the complete gastric-duodenal-intestinal transit, with initial cell counts of 9.55×10^7 cfu/mL of *L. plantarum* ZDY 2013.

Figure 1. Sensitivity of *Lactobacillus plantarum* ZDY 2013 to acid and bile salts. Growth curve (A; optical density at 600 nm) and pH value of fermentation culture (C) of ZDY 2013 in de Man, Rogosa, Sharpe medium with different initial pH 6.5 (●), 5.0 (■), 4.0 (▲), 3.5 (▼), and 3.0 (◆); (B) viable counts of ZDY 2013 at extreme pH 2.0 (Δ) and 3.0 (○); and (D) bile salt resistance of ZDY 2013 in different sample points. Compared with initial value, * $P < 0.05$; ** $P < 0.01$.

Figure 2. Bacteria cultivation and exopolysaccharide (EPS) production: Strain *Lactobacillus plantarum* ZDY 2013 was grown in de Man, Rogosa, Sharpe medium, and the cell number (line) and EPS production (column) were tested for a period of 36 h. Error bars represent SD.

Detection of the Major Species in the Intestine or Feces of Mice by qPCR

Principally, *L. plantarum* ZDY 2013 was a biota of the host. To investigate the exact effect of this organism on the major species of the intestinal- or fecal biota of the mice, we used qPCR for semiquantitative detection of intestinal biota constituents. After 2 wk of administration of *L. plantarum* ZDY 2013, the activity, behavior of mice, and the serum biochemical indexes as well as organ indices were normal (data not shown). As shown in Table 3, probiotics (i.e., *Lactobacillus* and *Bifidobacterium*) increased by at least 1.43 fold in the colon and cecum of mice; however, the former increased in feces by 1.91 fold and *Bifidobacterium* decreased by 0.9 fold. The potential enteropathogenic organisms (e.g., *Fusobacterium* and *Bacteroidetes*) decreased significantly in

Figure 4. Survival of cell counts and relative survival rate of *Lactobacillus plantarum* ZDY 2013 in transit under the simulated gastrointestinal tract. Viable counts (log cfu/mL) and the percentage of survival rate were obtained at each sampling point. GJ = gastric juice; DJ = duodenal juice; and IJ = intestinal juice. Symbols with different letters (a–c) are significantly different at $P < 0.05$. Error bars represent SD.

the colon; *Enterococcus* reduced at least by 3.35 fold both in intestinal tract and the feces, whereas both *Enterobacterium* and *Clostridium perfringens* decreased in the colon and cecum but increased in the feces.

General Health Status and Hepatic Indices of Mice

General health status of mice after oral administration with *L. plantarum* ZDY 2013 is shown in Supplementary Table S1 (<http://dx.doi.org/10.3168/jds.2014-9153>) and serum biochemical indices are shown in Supplementary Table S2 (<http://dx.doi.org/10.3168/jds.2014-9153>). The BW and organ indices were very similar (Supplementary Table S1). As shown in Supplementary Table S2, the 10 important hepatic indices (alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, total protein, albumin, globulin, ratio of albumin to globulin, gamma glutamyl transaminase, and alkaline phosphatase) were not significantly altered and were within a reasonable range independent of the oral administration of *L. plantarum* ZDY 2013 in comparison with the control. The indices of the kidney function monitored were creatinine, BUN, and urea, which exhibited similar levels as compared with the control group. A standard range of serum biochemical markers is reported in our previous study (Yang et al., 2015). Therefore, no hepatic and renal abnormality was observed in the animals after *L. plantarum* ZDY 2013 treatment, which suggested that mice were in a good health status with administration of probiotic, *L. plantarum* ZDY2013.

DGGE Analysis

To have a detailed overview of the individual biota in mice influenced by oral administration of *L. plantarum*

Figure 3. Antibacterial activities of the cultured supernatant of *Lactobacillus plantarum* ZDY 2013 against 8 kinds of food-borne pathogenic bacteria. Symbols with different letter (a–f) are significantly different at $P < 0.05$. Error bars represent SD.

Table 3. Fold change of internal microbiota population in colon, cecum, and feces as determined by quantitative PCR¹

Sample	ΔCt	$\Delta\Delta\text{Ct}$	$2^{-\Delta\Delta\text{Ct}}$
Colon			
<i>Enterococcus</i>	15.36 ± 1.94	3.88 ± 1.94	0.068 ± 0.14
<i>Lactobacillus</i>	4.35 ± 0.59	-0.63 ± 0.59	1.55 ± 0.75
<i>Fusobacterium</i>	8.29 ± 0.53	0.56 ± 0.53	0.68 ± 0.24
<i>Enterobacterium</i>	13.38 ± 0.57	0.98 ± 0.58	0.51 ± 0.24
<i>Bacteroidetes</i>	2.64 ± 0.36	0.56 ± 0.36	0.68 ± 0.18
<i>Bifidobacterium</i>	21.93 ± 0.38	-0.88 ± 0.37	1.84 ± 0.48
<i>Clostridium perfringens</i>	19.81 ± 0.39	0.99 ± 0.39	0.51 ± 0.12
Cecum			
<i>Enterococcus</i>	15.42 ± 0.41	3.77 ± 0.41	0.08 ± 0.02
<i>Lactobacillus</i>	4.71 ± 0.76	-0.65 ± 0.55	1.73 ± 0.82
<i>Fusobacterium</i>	7.53 ± 0.44	-0.67 ± 0.44	1.65 ± 0.50
<i>Enterobacterium</i>	10.40 ± 0.16	2.03 ± 0.16	0.25 ± 0.03
<i>Bacteroidetes</i>	2.54 ± 0.06	0.23 ± 0.06	1.17 ± 0.05
<i>Bifidobacterium</i>	18.26 ± 0.48	-0.45 ± 0.48	1.43 ± 0.46
<i>Clostridium perfringens</i>	17.16 ± 0.56	0.49 ± 0.56	0.75 ± 0.28
Feces			
<i>Enterococcus</i>	13.27 ± 1.08	2.11 ± 1.08	0.23 ± 0.21
<i>Lactobacillus</i>	5.40 ± 0.71	-0.93 ± 0.71	1.91 ± 0.19
<i>Fusobacterium</i>	8.96 ± 0.15	-0.74 ± 0.15	1.67 ± 0.18
<i>Enterobacterium</i>	9.81 ± 0.18	-0.89 ± 0.18	1.85 ± 0.23
<i>Bacteroidetes</i>	2.72 ± 0.24	-1.52 ± 0.24	2.86 ± 0.48
<i>Bifidobacterium</i>	21.04 ± 0.43	0.99 ± 0.43	0.50 ± 0.15
<i>Clostridium perfringens</i>	19.48 ± 0.30	-1.61 ± 0.30	3.05 ± 0.60

¹Samples were run in triplicate and represented by mean ± SD. ΔCt represents the difference between the cycle threshold (Ct) value with the primers to each bacterium and the Ct value with the primers to total bacteria. $\Delta\Delta\text{Ct}$ represents the difference between the ΔCt value of *Lactobacillus plantarum* ZDY 2013 group and the ΔCt value of the control. $2^{-\Delta\Delta\text{Ct}}$ are fold changes to represent relative abundance (reference samples of control group = 1.0) of the population.

ZDY 2013, 6 samples were collected randomly from colon, cecum, and feces of control and ZDY 2013-challenged groups and examined by PCR-DGGE followed by PCA. The bacterial profiles, PCA plots (Figure 5-A, B, C), and the richness (Figure 5-D) of different parts in mice intestine of the control and ZDY 2013-challenged mice are shown in Figure 5. All the PCA dots in the control group gather together, except for the separation of the number 6 mice in colon and cecum, which illustrates the stability of gut microbiota in mice. However, the PCA plots exhibited the tendency of separation in the profiles after the interference of ZDY 2013 for 14 d, especially in cecum and fecal parts in mice intestine. In addition, the richness which represented the band numbers reduced significantly in the cecal ($P < 0.05$) DGGE profiles, whereas they increased in fecal ($P < 0.05$) but not in colon profiles.

DISCUSSION

The beneficial effects of probiotics, including modulation of biota balance in GIT, and enhancement of the host immunity have attracted attention of many researchers for novel and potential commercial strains of lactic acid bacteria and bifidobacteria from traditional fermented food. We isolated a novel *L. plantarum*

ZDY 2013 from Chinese traditional acid beans and systematically evaluated its potential as a probiotic by testing its resistance in extreme acid condition, bile salt concentration, antibiotic sensitivity, and its effects on intestinal biota constituents.

Lactobacillus plantarum, already used in starter culture of yogurt and other products, has been known for resistance to extreme acid and bile salt and could be a potential candidate for use as a probiotic. Lee et al. (2011) reported that several isolates, identified as *L. plantarum* from Korean kimchi, could survive at pH 2.5 for 2 h. Some *L. plantarum* maintained 1.0×10^5 cfu/mL after a treatment at pH 1.5 for 4 h (Chen et al., 2014b). Generally, the reduction of cell counts of *L. plantarum* in various studies was at least one order of magnitude (Anderson et al., 2010; Zago et al., 2011; Guidone et al., 2014). However, we found that *L. plantarum* ZDY 2013 can survive for 6 h at pH 2.0 with loss of less than one order of magnitude. Therefore, *L. plantarum* ZDY 2013 demonstrated better survival in extreme acid environment as compared with other reported strains.

Transit ability during passage through the GIT was the main focus of evaluation of *L. plantarum* ZDY 2013 for its potential use as a probiotic. In addition to facing challenges of extreme acidity and high concentrations

Figure 5. The DGGE fingerprint and principal component analysis of the colon contents (A), cecum contents (B), and feces (C) for the microbial community of mice. 1–6 = 6 mice for control group; 7–12 = 6 mice for experimental group of *Lactobacillus plantarum* ZDY 2013. (D) Richness of intestinal tract and feces microbiota. Compared with control group, * $P < 0.05$. Error bars represent SD.

of bile salt, the survival of probiotics is also affected by the digestive enzymes (pepsin, pancreatin). As demonstrated in our study, as high as 65.84% of the *L. plantarum* ZDY 2013 cells could pass through the subsequent challenge of simulated GJ, DJ, and IJ. In contrast, Zago et al. (2011) reported that the reduction in the 9 isolates of *L. plantarum* from an initial 10^9 cfu/mL to a final 10^6 cfu/mL in GIT transit. A high level of survival indicated that *L. plantarum* ZDY 2013 can potentially be orally administrated as a probiotic.

Potential of ZDY 2013 as a probiotic was based not only on survival ability during passage through the GIT, but also by the antagonistic effect against pathogens and in vivo microbial interference. Our data showed that ZDY 2013 inhibited 8 common food-borne pathogens in this order: *L. monocytogenes*, *P. aeruginosa*, *S. typhimurium*, *E. coli*, *B. cereus*, *S. sonnei*, *E. sakazakii*, and *S. aureus*. In a similar study, Kaushik et al. (2009) reported that the inhibition order was *B. cereus*, *L. monocytogenes*, and *E. coli*. Among pathogens, *L. monocytogenes* was the most sensitive and was inhibited by *L. plantarum* either in diffusion test or coinoculation test, as we achieved a halo as large as 28 mm in diameter against *L. monocytogenes*. Antagonistic bacterial substances from *L. plantarum* may include bacteriocin (e.g., plantaricin Y) and organic acid (e.g., lactic acid and acetic acid), or even EPS (Crowley et al., 2013; Anas et al., 2014; Chen et al., 2014c; Li et al., 2014b). In our study, we found that the acidification with ZDY 2013 took place even at pH 3.5; with the growth of cells and accumulation of EPS, those organic acids contributed to the antagonistic effect, which was also documented by Wilson et al. (2005). In our previous study with *L. plantarum*, we reported that *L. monocytogenes*, *S. aureus*, and *B. cereus* were significantly inhibited by EPS produced by *L. plantarum* R315 (Li et al., 2014b).

High transit efficiency of ZDY 2013 provided a precondition for colonization of intestinal tract, and thereafter resulted in specific host-microbial interactions. In our study, qPCR in vivo analysis of mice showed that the administration of ZDY 2013 had successfully enhanced the native bacterial population (e.g., *Bifidobacterium* and *Lactobacillus* groups in colon and cecum) and reduced the potential enteropathogenic bacteria (e.g., *Enterococcus*, *Clostridium perfringens*). Its antagonistic effect might affect the binding of pathogenic organisms to the GIT lining and thus help in expediting their clearance from the host. Some studies have also demonstrated that feeding rats with probiotics, prebiotics, or synbiotics promoted increases in *Bifidobacterium* and *Lactobacillus* population at different locations in the rat GIT (Tuohy et al., 2003; Quigley, 2010). The DGGE profile demonstrated that the probiotic properties of *L.*

plantarum ZDY 2013 may partially depend on its effect on the microbial diversities at various intestinal loci.

Moreover, for therapeutic or prophylactic applications of ZDY 2013, the antibiotic sensitivity of this organism was also investigated and demonstrated that ZDY 2013 was sensitive to amoxicillin, tetracycline, and rifampicin and resistant to bacitracin, ciprofloxacin, and cefoxitin. Whereas *L. plantarum* ZDY 2013 showed high resistance to bacitracin and intermediate resistance to penicillin, Charteris et al. (1998) reported that 46 *Lactobacillus* strains from human and dairy sources were assayed for susceptibility to 44 antibiotics; however, only 4 human strains and 1 dairy strain exhibited resistance to bacitracin. Temmerman et al. (2003) reported that antibiotic resistance among 187 isolates was detected, but only 23% of strains were resistant to penicillin. *Lactobacillus plantarum* ZDY 2013 originated from natural fermented product and presented specific antibiotic resistance to bacitracin and penicillin, which might be an advantage considering a joint use for probiotic therapy in the future. The information in our study provides a reasonable basis for the application of this probiotic along with certain antibiotics for improving the balance of the host GIT biota.

CONCLUSIONS

Lactobacillus plantarum ZDY 2013 was systematically evaluated for its survival ability under an extreme acid environment, a high concentration of bile salt, and a simulated GIT in vitro. The interference of ZDY 2013 to the diversity and constituents of biota in mice was monitored by qPCR and PCR-DGGE. The antagonistic effect on pathogens and antibiotic sensitivity as well as EPS yields were also tested in this study. Our results showed that ZDY 2013 was a potential probiotic candidate for its outstanding stress-resistant ability (e.g., survival at pH 2.0 for 6 h and at 0.45% bile salt concentration for 3 h, maintaining 65.84% survival rate after GIT transit, and producing as high as 204 mg/L of EPS). *Lactobacillus plantarum* ZDY 2013 showed high resistance to bacitracin and intermediate resistance to penicillin, providing a possibility for using this organism along with certain antibiotics in a reasonable dosage for treating GIT disorders, but this claim needs further study. The results also showed an antagonistic effect on 8 common food-borne pathogens; the effect on the microbial diversities at various intestinal loci was shown in the results of PCR-DGGE and the reduction of potential enteropathogenic bacteria while enhancing *Bifidobacterium* and *Lactobacillus* group was confirmed by qPCR. The results suggested that ZDY 2013 could be a probiotic candidate, which might have potential applications in nutraceutical and dairy products.

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