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PROBIOTIC PROPERTIES OF *ENTEROCOCCUS* STRAINS ISOLATED FROM THE SILAGE

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ABSTRACT

This study aimed to identify and evaluate the probiotic properties of five *Enterococcus* strains isolated from the silage using *in vitro* screening assays. Their metabolites, antibiotic susceptibility, antimicrobial activity, as well as their survival in the gastrointestinal tract (GIT) and adhesive ability to human intestinal cells were investigated. Among eight selected antibiotics, all the isolates showed sensitive traits to six particular antibiotics including tetracycline and vancomycin. L2–L5 showed inhibition zones against *Escherichia coli* and *Salmonella* except that L6 only inhibited the growth of *E. coli*. The GIT proved that all the strains were able to survive in low pH and bile salt conditions and they all can adhere to LS-174T intestinal cells. L2 and L5 were then chosen to examine their competition activity toward *E. coli* and inflammatory response. The results showed that both strains exhibited strong competition activities toward *E. coli.* The displacement ratios were 98.4 and 97.7%, respectively. Compared with *E. coli*, L2 showed lower stimulation of interleukin (IL)-8 and IL-1β, while L5 induced higher levels of cytokines to provoke an inflammatory response. Finally, the *in vivo* effect of L2 was investigated. The data showed that L2 was harmless. The results obtained suggest that L2 is potentially probiotic.

PRACTICAL APPLICATION

Enterococci are lactic acid bacteria that occur frequently in large numbers in various foods including vegetable, meat and dairy products. However, some of these bacteria are also recognized as nosocomial pathogens. Several properties may be evaluated to prove the potential of the bacteria before using them as probiotics. Results of this study indicated that five *Enterococcus* strains isolated from the mixed grass and corn stalk silage exhibited different capacties of antibiotic susceptibility, antimicrobial activity, tolerance to gastrointestinal conditions, adhesion to intestinal cells, and competitive exclusion of *E. coli* adhesion to epithelial cells and stimulation of immunity. Among these strains, L2 shows above-average results in all probiotic criteria observed *in vitro*. Additionally, L2 was evaluated to be safe in mice feeding. The findings of our study suggest that L2 isolated from the silage may be potentially used as probiotic in humans.

INTRODUCTION

The genus *Enterococcus* belongs to a group of microorganisms known as lactic acid bacteria (LAB) and forms an important part of environmental, food and clinical microbiology. It has a long history of safe use, especially in the dairy industry. Its normal habitat is the gastrointestinal tracts of humans and animals, a complex ecosystem in which a delicate balance exists between the intestinal microorganism and the host (Franz *et al.* 2003; Bhardwaj *et al.* 2008) In this system, *Enterococcus faecalis* and *Enterococcus faecium* are the most dominant species (Kayser 2003). *Enterococcus*

strains are also added to fermented foods on purpose, in which they contribute to the organism's properties (Giraffa 2002, 2003). Moreover, *Enterococcus* have been isolated from a wide variety of habitats, including human vaginal secretions (Martín *et al.* 2008), human milk (Martín *et al.* 2002), infant feces (Rodríguez *et al.* 2012), the preen gland secretion of birds (Martín-Platero *et al.* 2006; Soler *et al.* 2008) and a broad variety of fermented foods of both animal (milk, cheese, fermented sausages) and vegetable (fermented olives) origins, in which they may have beneficial effects (Foulquie Moreno *et al.* 2006).

Probiotics are defined as direct-feed microorganisms or microbial cell preparations with a beneficial effect on the health and well-being of the host (Nemcová 1997). According to the Food and Health Agricultural Organization of the United Nations/World Health Organization regulations (FAO/WHO 2002), probiotic microorganisms for use in health applications must be nonpathogenic, nontoxic and must survive the gastrointestinal transition to the target habitat and then settlement, serving to protect the host against infection by pathogenic microorganisms. Before using an organism as a probiotic, several properties may prove useful in evaluating the potential of the bacteria, e.g., strain origin, acid and bile tolerance, adhesion to the intestinal mucus, production of antimicrobial substances, and antibiotic resistance or sensitivity (Salminen *et al.* 1998). Recently, several strains of genus *Enterococcus* have been used as probiotics, which may improve the microbial balance of the intestine or can be used in the treatment of gastroenteritis in humans and animals (Giraffa 2003; Foulquie Moreno *et al.* 2006; Bhardwaj *et al.* 2008; Franz *et al.* 2011).

Silages have been used as diets of livestock for a long time all over the world. They contain a community consisting of different microorganisms, which usually include a mixed population of LAB, depending on the substrate and the site where they derive from (Pang *et al.* 2011). Therefore, there are different types of bacteria within different feeding silage-based diets. The present study aimed to search the potential probiotic *Enterococcus* isolated from the mixed grass and corn stalk silage. Five strains were evaluated for eligible probiotic traits such as antimicrobial activity, antibiotic resistance, tolerance to gastrointestinal conditions, adhesion to intestinal cells, competitive exclusion of *Escherichia coli* adhesion to epithelial cells and stimulation of immunity. Additionally, one of the strains L2 was selected to study its *in vivo* effect.

MATERIALS AND METHODS

Bacterial Growth Conditions and Cell Cultures

In this study, we used five LAB isolates from the mixed grass and corn stalk silage. All strains were cultured in Man Ragosa Sharpe (MRS) broth (Ssincere, Shanghai, China) for 24 h at 37C in an incubator (HuaLida, Jiangsu, China) and were spread on the surface of MRS agar plates to check purity. A single colony was picked up to prepare new stocks (stored at −80C in MRS with 50% [v/v] glycerol). As standard procedure, LAB isolates from stocks were cultivated overnight at 37C, and used to inoculate in 2% fresh MRS media, which was cultivated for 24 h under the same conditions. Pathogenic bacteria were cultivated with Lysogeny Broth (LB) media under the same conditions.

The human colorectal cancer cell line LS-174T and the human macrophage cell line THP-1 were used. LS-174T was grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 ug/mL streptomycin. THP-1 was grown in suspension in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 ug/mL streptomycin, 2.5g/L glucose and 0.05 mM β-mercaptoethanol. All cell lines were cultured at 37C in an atmosphere of 5% CO_2 and 95% air in a humidified incubator (Heal Force151, Shanghai, China).

Identification of Isolates

In total, five isolates were identified by 16S r DNA sequence analysis. The 16S r RNA gene was amplified using universal primers. The polymerase chain reaction (PCR) primer sequences were: 5′-AGAGTTTGATCCTGGCTCAG-3′; 5′-GGTTACCTTTGTTACGACTT-3′ (Lee *et al.* 2011). The thermal cycling parameters were 30 cycles of denaturation at 94C for 30 s, annealing at 55C for 30 s, polymerization at 72C for 1 min. The sequences of the final products were analyzed by a Gel imager (Furi, Shanghai, China). Sequences were determined by Shanghai Sangni Company. Sequences homologies were examined by comparing the obtained sequences with those in the DNA Databases (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al.* 1990). The ability of lactic acid production was judged by soluble calcium ring experiment at the same time. Plates were prepared using MRS agar supplemented with 1% calcium carbonate. The overnight cultures of the isolates were spotted in duplicate on supplemented agar plates. Samples were incubated for 24 h and examined for bacterial growth and the plates change. The test was carried out in duplicate.

Genus-specific PCR was performed to identify the isolates at genus level. The PCR primer sequences were: 5′-TCAACCGGGGAGGGT-3′; 5′-ATTACTAGCGATTCCG G-3′ (Deasy *et al.* 2000). PCR conditions were the same as previously mentioned. Five isolates were spread on the surface of *Enterococcus* agar (Hope Bio-Technology, Qingdao, China) plates to check the genus level from physi-

ologic index (Deasy *et al.* 2000). The overnight cultures of the isolates were spotted in duplicate on *Enterococcus* agar plates. Samples were incubated for 24 h and examined for color change. The test was carried out in duplicate.

Metabolites Assay

The production of metabolites in the supernatants of 24 h grown LAB cultures was measured by high-performance liquid chromatography (HPLC) (Ayeni *et al.* 2011) using an LC-20AT system composed of an a SPD-20A detector and the LC solution software (Shimadzu, Kyoto, Japan) under conditions described as follows: column: inertsil ODS-4 (5 um, 250 × 4.6 mm1.D), eluent: 20 mM NH_4HPO_4 (pH2.0, H_3PO_4), flow rate: 0.8 mL/min, injection volum: 20 uL, col. temp: UV210nm.

Antibiotic Resistance Pattern

One hundred microliters of LAB fresh culture (A660 0.08–0.1) was spread on the surface of MRS agar plates for antibiotic disk diffusion tests. Four sterilized paper disks were placed aseptically on the agar surface after the agar plates had set and been surface dried. Agar plates with different concentration antibiotic (Sangon Biotech, Shanghai, China) disks were then incubated for 24 h (Nueno-Palop and Narbad 2011). The diameters of the inhibition zones were measured using a ruler under a colony counter apparatus. The results (average of five readings) were expressed as follows: − = no growth inhibition; + = inhibition zones of 1–11 mm in diameter; ++ = inhibition zones of 12–16 mm in diameter; +++ = inhibition zones of 17–20 mm in diameter; ++++ = inhibition zones larger than 21 mm relative to the reported standards (Acar and Goldstein 1991; Isenberg 1992; Woods and Washington 1995; Botes *et al.* 2008). The experiment was performed in triplicate.

Antimicrobial Activity

One hundred microliters of pathogenic bacteria fresh culture (A660 0.08–0.1) was spread on the surface of MRS agar plates for disk diffusion tests. LAB fresh cultures were centrifuged and the supernatants were obtained, and then 10 uL of supernatants was applied to each disk. Four sterilized paper disks were placed aseptically on the agar surface after the agar plates had set and been surface dried. Agar plates with disks were then incubated for 24 h. The diameters of the inhibition zones were measured using a ruler under a colony counter apparatus. The experiment was performed in triplicate.

Survival to the GIT

Isolates were inoculated in 2% of fresh broth with or without 25 ug/mL lysozyme (Sangon Biotech, Shanghai, China). The cultures were incubated at 37C for 5 min and plated on MRS agar (Rodríguez *et al.* 2012). Viable counts were determined after 24 h incubation under conditions in control and treated cultures. The test was carried out in duplicate.

MRS cultures grown for 24 h were washed twice in saline solution and resuspended in MRS at pH 2.5 for 1.5 h incubation at 37C. Then, bacterial suspensions were centrifuged, resuspended in MRS with 0.3% bile salts (Sangon Biotech, Shanghai, China) and incubated for 2 h. Experiments were carried out in triplicate and in all steps viable counts were obtained by plating in MRS agar.

Adhesion to Epithelial Intestinal Cell Lines

Epithelial intestinal cell line LS-174T was used to assess the adhesion ability of the five isolates. Briefly, a bacterial suspension (10^8 cfu/mL in RPMI1640 without antibiotics) was added to each monolayer of cells placed in 24-well tissue culture plates (10^5 cfu/mL, washed twice in phosphate buffered saline [PBS], to remove antibiotics) and incubated at 37C. Wells were gently washed three times with PBS to remove the nonadhered bacteria after 2 h of incubation. Then, monolayer was trypsinized with ethylenediaminetetraacetic acid (EDTA) trypsin solution (Sigma, St. Louis, MO, USA) and bacterial counts were carried out in MRS agar. Adhesion results were expressed as the percentage of bacteria adhered with respect to the amount of bacteria added (% CFU bacteria adhered/CFU bacteria added). Two replicated experiments were carried out for each isolate using two independent microplates (each isolate tested by duplicate in each plate).

Competitive Exclusion of *E. coli* Adhesion to Epithelial Cell Lines

The ability of the five isolates to compete with *E. coli* to adhere to intestinal epithelia, was tested using the cell lines LS-174T. Strains were grown for 24 h in MRS, as previously described, and *E. coli* was cultured overnight in LB medium at 37C under rigorous shaking. Bacterial cultures were washed with PBS and resuspended in the corresponding cell line media without antibiotic s at a concentration of about 10^8cfu/mL. Afterwards 1 mL of each LAB was added to cells placed in 24-well tissue culture plates (10^5 cfu/mL, washed twice in PBS, to remove antibiotics). Wells were gently washed three times with PBS to remove the nonadhered cells after 1 h of incubation. Then the same volume of *E. coli* (ratio of *Enterococcus*: *E. coli* about 1:1) was added to each

cell line type. Incubation took place for 1 h at 37C. Subsequently, *Enterococcus* and *E. coli* were counted using real-time PCR (each isolate tested by duplicate in each plate) in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). *E. coli* primer sequences were reported as: 5′-GACCTCGGTTTAGTTCACAGA-3′; 5′-CACACGCTG ACGCTGACCA-3′ (Candela *et al.* 2008).

Cytokines Modulation in Macrophage Cells

To analyze the modulation of cytokines (interleukin [IL]-8, IL-1β) in THP-1 cells, bacteria were inoculated into wells for 4 h. Ratio of bacteria: cell was about 1:1, 10:1,100:1. Then, RNA was extracted to real-time PCR. Primer sequences were: IL-8: 5′-ATGACTTCCAAGCTGGCCGT-3′; 5′-TCCTTGGCAAAACTGCACCT-3′. IL-1β:5′-GCAGTCT ACACAGCTTCGGG-3′; 5′-CCGCCTCAGCCTCCCAAAG-3′ (Cammarota *et al.* 2009). β -action: 5′-GCGAGAAGAT GACCCAGATC-3′; 5′-GGATAGCACAGCCTGGATAG-3′ (β-action was selected as internal controls).

The real-time PCR analysis was performed in triplicate assays using Super Real Premix Plus (SYBR Green) according to the manufacturer's instructions (TaKaRa, Tokyo, Japan) in a CFX96 Real-Time PCR Detection System (Bio-Rad). The reactions were as follows: activation of the Taq DNA polymerase at 95C for 15 min and 40 cycles of 95C for 10 s and 60C for 32 s. We quantified three genes based on cytokines IL-8 and IL-1β. The relative changes in gene expression were calculated using the equation: $2^{-\Delta\Delta CT}$, as Livak described (Livak and Schmittgen 2001).

Animal Feeding Procedure

Eighteen female Bagg albino/c mice aged 5 weeks were purchased from Slaccas Company (Shanghai, China). Animals were housed in plastic cages with free access to diet and water for 6 weeks. All animal experiments were approved and supervised by the local ethic committee at the East China University of Science and Technology. Mice were randomly assigned into three different groups. One group was used as a nonprobiotic treatment control, gavages 10% skim milk (BD) only. The other groups of mice were gavages administered with 10 uL/g of L2 suspensions daily at two different dose rates (low-dose 1×10^7 cfu/mL, high-dose 2×10^8 cfu/mL L2) in 10% skim milk. Mice received L2 suspensions by intragastric route with a stainless steel feeding needle (Medical Instruments, Shanghai, China) and a 1-mL syringe (Misawa Medical Industry, Shanghai, China). The treatments lasted for 30 days. Body weight was measured every 5 days.

Quantification of Bacteria in Feces

Aerobic bacteria in feces were quantified in day 30. Fresh feces were weighed, homogenized in sterile saline solution, and suitable dilutions of the homogenates were plated onto culture media agar plates and incubated at 37C for 2 days before colony enumeration. Tryptose Soya Agar (TSA) was used for aerobic bacteria CFU counted.

Detection of Spleen Weight Index

Mice euthanized at day 30. Spleen weight and live body weight in mice that were fed with different doses of *Enterococcus* L2 was measured. Spleen weight index (SWI) was expressed as the spleen weight (g) divided by the last measure of live body weight (g).

Accession Numbers

The 16S r DNA sequences of five isolates (L2, L3, L4, L5 and L6) were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov) under accession nos. KF670603, KF670604, KF670605, KF670606, KF670607, respectively.

Statistic Analysis

Results were expressed as mean ± standard deviation of triplicates for each sample. *P* values less than 0.05 were regarded as significant difference between means using a Student's *t*-test.

RESULTS AND DISCUSSION

Identification and Characterization of Five Isolates

The five isolates from the silage were identified at genus level by partially sequencing the 16S rDNA gene using genus-specific primers PCR and *Enterococcus* agar. The *Lactobacillus* primers gave a 1,500 base pair (bp) fragment when tested with *Lactobacillus* DNA (Lee *et al.* 2011). The size of 16S rDNA of five isolates is about 1,500 bp (Fig. 1A). The ability of lactic acid production was examined by soluble calcium ring experiment. All the isolates dissolved the calcium in the surrounding media after incubation for 24 h (Fig. 1C).

The *Enterococcus* primers gave a 733 bp fragment when tested with *Enterococcus* DNA (Deasy *et al.* 2000). Our data showed that the size of the five isolates is about 700 bp (Fig. 1B). These isolates also grew on *Enterococcus* agar, a medium generally considered to be selective for *Enterococcus* (Fig. 1D). *Enterococcus* grows as black colonies and produces a substance to blacken the surrounding media in this

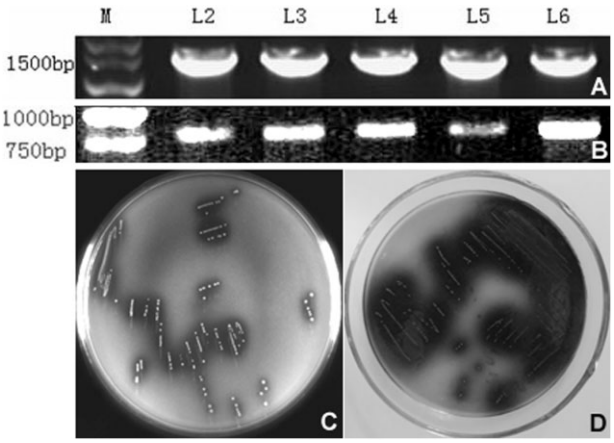

FIG. 1. THE IDENTIFICATION OF THE FIVE ISOLATES AT GENUS LEVEL BY PARTIALLY SEQUENCING THE 16S rRNA GENE (A) AND GENUS-SPECIFIC PRIMERS POLYMERASE CHAIN REACTION (B). THE FRAGMENTS WERE 1,500 AND 733 BP, RESPECTIVELY. THE OBSERVATIONS SHOW THAT L6 DISSOLVED THE CALCIUM OF THE SURROUNDING MEDIA AFTER INCUBATED FOR 24 H(C) AND L3 CHANGED THE *ENTEROCOCCUS* AGAR TO BLACK AFTER INCUBATED FOR 24 H (D)

agar. All the isolates changed the *Enterococcus* agar turned to black after incubation for 24 h (Fig. 1D). Afterward, the isolates and related reference strains were used to construct the phylogeny tree (Fig. 2), which is mainly composed of *Lactobacillus* and *Enterococcus* including the five isolates. Because of the limited selection of reference strains, L4 was far away from other four isolates in this tree. In short, the five isolates were identified as *Enterococcus*.

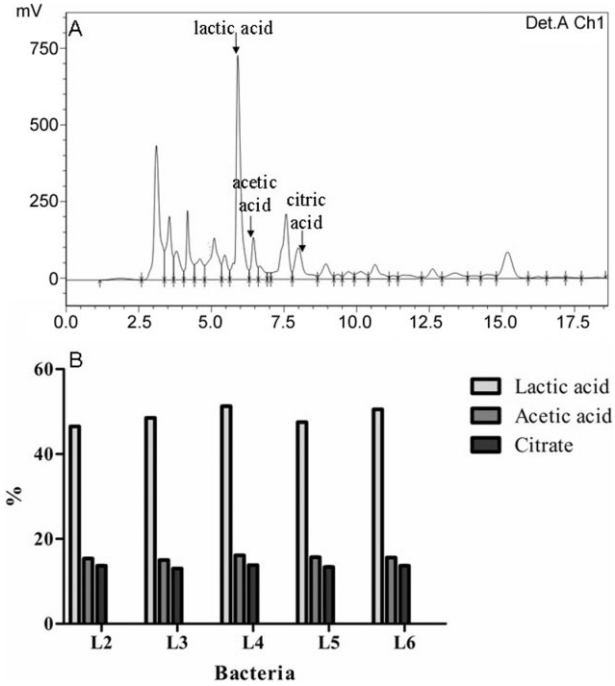

FIG. 3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-CHROMATOGRAM OF THE SUPERNATANT OF L2 IN MRS AFTER 24 H OF CULTIVATION (A). PERCENTAGES OF LACTIC ACID, ACETIC ACID AND CITRIC ACID IN THE SUPERNATANTS OF THE FIVE ISOLATES (B)

Production of Metabolites

The composition produced from the five isolates was analyzed by HPLC. Multiple metabolites were discovered in the

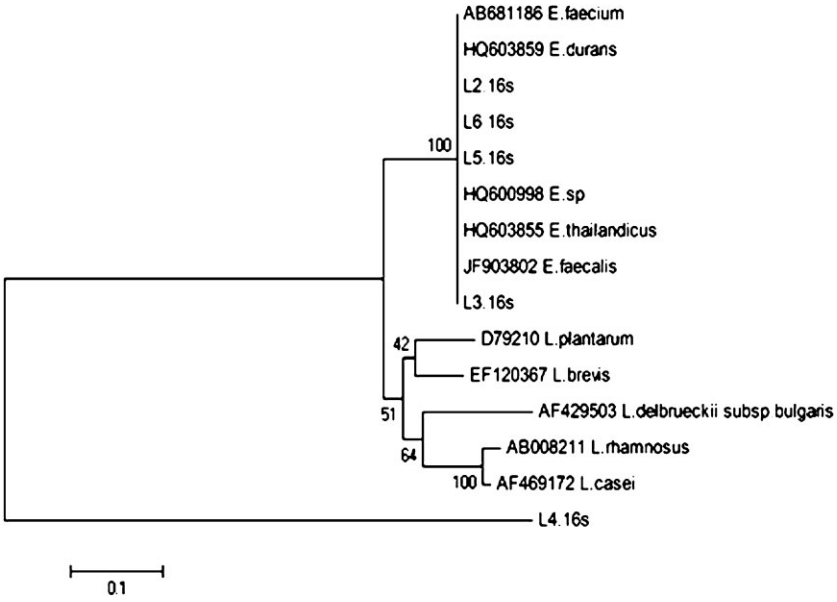

FIG. 2. PHYLOGENY TREE BASED ON 16S rRNA SEQUENCE ANALYSIS, SHOWING THE PHYLOGENY PLACEMENT OF THE FIVE ISOLATES

TABLE 1. THE ANTIBIOTIC SUSCEPTIBILITIES OF THE FIVE ISOLATES

	AMP*	KAN	TET	CHL	CIP	MET	VAN	ERY
Bacteria	10 μg/disk	30 μg/disk	30 μg/disk	30 μg/disk	5 μg/disk	5 μg/disk	30 μg/disk	15 μg/disk
L2	++++	_	++++	++++	+++	_	+++	++
L3	++++	_	++++	++++	+++	_	+++	++
L4	++++	_	++++	++++	+++	_	+++	++
L5	++++	_	++++	++++	+++	_	+++	++
L6	++++	_	++++	++++	+++	_	+++	++

–, no growth inhibition; +, inhibition zones of 1–11 mm in diameter; ++, inhibition zones of 12–16 mm in diameter; +++, inhibition zones of 17–20 mm in diameter; ++++, inhibition zones larger than 21 mm; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; KAN, kanamycin; MET, metronidazole; TET, tetracycline; VAN, vanomycin.

supernatant from L2 (Fig. 3A) and lactic acid was the major organic acid produced by these bacteria during growth (Fig. 3B). The data from other four isolates were similar to one of L2 (data not shown). In addition, the five isolates produced similar levels of acetic acid and citric acid, which were produced at low concentrations (Fig. 3B).

LAB can produce multiple metabolites including volatile acids (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids, *et al.*) and nonvolatile acids (lactic, succinic, oxalic, fumaric and malonic acids, *et al.*) (Urdaneta *et al.* 1995). It had been reported that acetic acid is a by-product of citrate metabolism in *Enterococcus* (Jensen *et al.* 1975; EI-Gendy *et al.* 1983). Thus, LAB can produce metabolites with different yields in different media.

Antibiotic Susceptibility

Table 1 shows the antibiotic susceptibility of the five isolates. All isolates were considered sensitive or moderately sensitive to ampicillin (β-lactam), tetracycline (tetracycline), chloramphenicol (chloramphenicol), ciprofloxacin (quinolones), vanomycin (glycopeptides) and erythromycin (macrolides), while they were resistant to kanamycin (aminoglycosides) and metronidazole (nitroimidazoles).

Native resistance is considered to present a minimal risk for spread while acquired resistance dominated by plasmids or transposons is considered to be of high risk (Ruiz-Moyano *et al.* 2009). For safety reasons, a critical criterion for *Enterococcus* to be used in foods is the lack of transferable antibiotic resistance (Cebrián *et al.* 2012). All the isolates in this study showed sensitive traits to six antibiotics, including the clinically relevant antibiotics tetracycline and vancomycin.

Antimicrobial Activity

We studied the antimicrobial activities of the five isolates. None of the supernatant from the five isolates inhibited the growth of the pathogenic *Shigella*, *Listeria monocytogenes*, *Staphylococcus aureus* strains. L2–L5 showed inhibition zones against *E. coli* and *Salmonella*, while one of L6 only inhibited the growth of *E. coli* (Table 2). Ehrmann *et al.* (2002) reported acid as the major factor of the antimicrobial multivariate mechanism of LAB against harmful intestinal bacteria. In addition, the antimicrobial activity of several recognized probiotic *Lactobacillus* strains was mainly attributed to a pH-lowering effect (Fayol-Messaoudi *et al.* 2005). Therefore, inhibition effects can be explained by bacteriocin action or the production of organic acids along with the low pH.

Survival to the GIT

Microorganisms interact with the lysozyme-containing saliva in the mouth, and resistance to lysozyme is recommended as a criterion for strains suitable for use in the milk industry (Guglielmotti *et al.* 2007). Survival rates to lysozyme at 25 ug/mL were observed in all isolates tested. Fig. 4A showed that L3 is resistant to lysozyme. The results are similar in other four isolates (data not shown). For bacteria to be effective as a probiotic, their ability to survive the passage through the upper digestive tract to reach the intestine where its beneficial action is expected is an essential requirement (Marteau *et al.* 1997; Charteris *et al.* 1998; Tuomola *et al.* 2001). The pH in the human stomach ranges from 1 (during fasting) to 4.5 (after a meal), and food ingestion can take up to 3 h (Maragkoudakis *et al.* 2006). The five isolates showed the ability to resist pH 2.5 treatment

TABLE 2. THE ANTIMICROBIAL ACTIVITIES OF THE FIVE ISOLATES

Bacteria	*Escherichia coli*	SAL	SHI	LIS	STA
L2	7.87 ± 0.15*	6.43 ± 0.25	0	0	0
L3	7.83 ± 0.25	6.3 ± 0.2	0	0	0
L4	7.7 ± 0.2	6.27 ± 0.06	0	0	0
L5	7.97 ± 0.15	6.43 ± 0.15	0	0	0
L6	7.87 ± 0.21	0	0	0	0

* The diameters of the inhibition zones were measured (mm).
SAL, *Salmonella*; SHI, *Shigella*; LIS, *Listeria monocytogenes*; STA, *Staphylococcus aureus*.

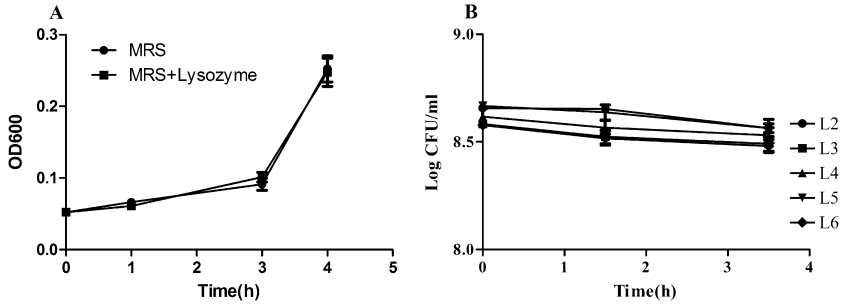

FIG. 4. SURVIVAL RATES TO 25 MG/ML LYSOZYME IN L3 (A). COUNTS (LOG CFU/ML) OF THE FIVE ISOLATES AFTER THE CHEMICALLY SIMULATED GIT (B). 0 H: MRS, PH6.8; 1.5 H: MRS, pH2.5; 3.5 H: MRS+0.3% BILE SALTS, PH6.8

(Fig. 4B). These results are in agreement with those obtained from previous similar studies, where *Lactobacillus* strains were able to retain their viability when exposed to pH values of 2.5–4.0, but displayed loss of viability at lower pH values (Jacobsen *et al.* 1999; Dunne and Mahony 2001). Moreover, all strains examined in this study could survive well in the presence of bile salts (0.3%, w/v) (Fig. 4B). So far, studies have shown that the majority of the strains survived well under such extreme conditions, suggesting a potential recuperation of the initial levels during the passage of the small intestine (Charteris *et al.* 1998; Jacobsen *et al.* 1999; Fernandez *et al.* 2003).

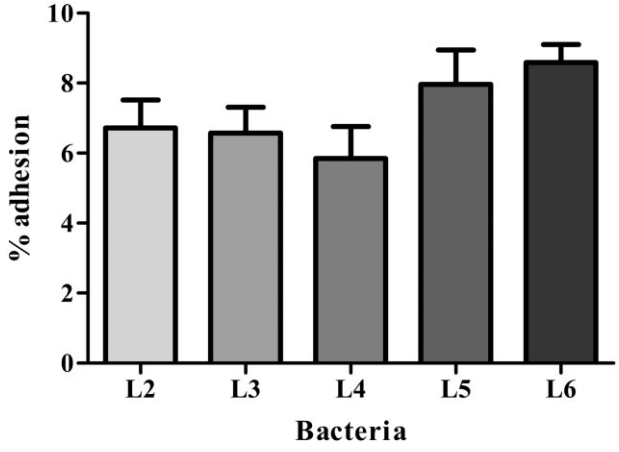

FIG. 5. PERCENTAGES OF ADHESION (CFU/ML OF ADHERED BACTERIA WITH RESPECT TO CFU/ML OF ADDED BACTERIA) OF THE FIVE ISOLATES TO THE EPITHELIAL INTESTINAL LS-174T CELLS

Adhesion to Epithelial Intestinal Cell Lines

Once the probiotic microorganisms have reached the intestine, adhesion to the intestinal mucosa and epithelial cells is a prerequisite for colonization and they must adhere to the mucus layer to avoid being removed from the colon by peristalsis (Saarela *et al.* 2000). Adhesion of LAB has been claimed to be essential for the exertion of a beneficial (probiotic) effect in the intestine. In our case, the adhesion of the five isolates to the epithelial intestinal cell line LS-174T was evaluated by a real-time PCR. As shown in Fig. 5, adhesion percentages were 6.7, 6.5, 5.7, 7.9 and 9.1% in the five isolates (L2–L6), respectively.

Competitive Exclusion of *E. coli* Adhesion to Epithelial Cell Line

Although the capability of adhesion to epithelial cells is a criterion for defining a new probiotic strain (Saarela *et al.* 2000), current research also focuses on its interference with pathogen adhesion rather than merely on the simple adhesion of putative probiotics. In competition experiments, L2 and L5 were chosen because of their high adhesive abilities and antimicrobial activities. The adhesive inhibition of *E. coli* to LS-174T by L2 and L5 were estimated using real-time PCR. The ability of the strains to competitively exclude

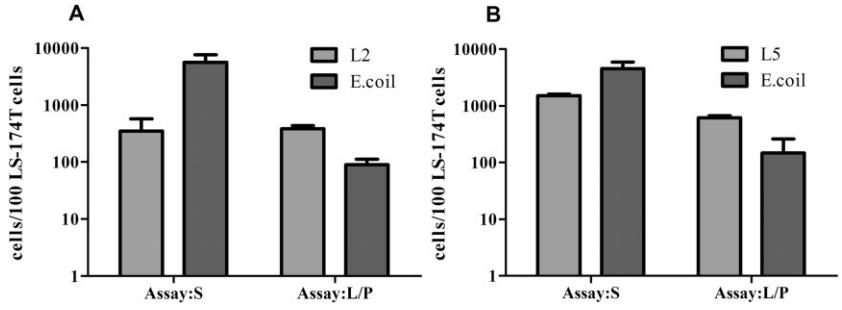

FIG. 6. COMPETITIVE ACTIVITIES OF L2 AND L5 AGAINST *ESCHERICHIA COLI* ADHERE TO LS-174T. THE LOG VALUES OF BACTERIA BOUND TO 100 LS-174T CELLS, AS OBTAINED IN ASSAYS CONTAINING THE SINGLE STRAINS (ASSAYS S) AND THE DISPLACEMENT ASSAYS (ASSAY L/P) ARE GIVEN

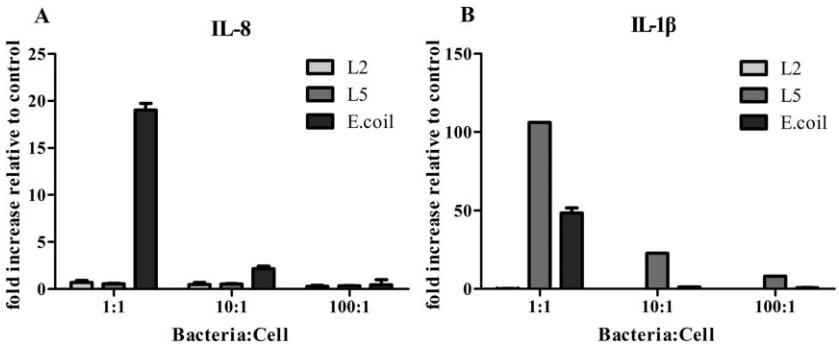

FIG. 7. THE mRNA LEVELS OF CYTOKINES IN THP-1 CELLS TREATED WITH L2, L5 AND *ESCHERICHIA COLI*. RESULTS ARE SHOWN AS THE FOLD CHANGE IN EXPRESSION RELATIVE TO THE CONTROL

the *E. coli* adhesion to LS-174 T-cell monolayer was evaluated by calculating the percentage of the reduction of the adhered *E. coli* cells. The results showed that L2 and L5 had the strongest competition activities toward *E. coli.* The displacement ratios were 98.4 and 97.7% for L2 and L5, respectively (Fig. 6).

Cytokines Modulation in Macrophage Cells

Immune modulation by probiotics is presumed to be one of the main mechanisms of probiotic action in human health, explaining reported effects on inflammatory bowel diseases and allergies (Cross 2002; Guarner and Malagelada 2003; Mecennier *et al.* 2003). For instance, in response to enteropathogens infection, the intestinal epithelium releases the cytokines IL-8, IL-1β and other pro-inflammatory molecules that provoke an acute inflammatory response. Herein, the expression levels of IL-8, IL-1β were evaluated in THP-1 cells treated with L2, L5 and the pathogen *E. coli*, respectively. As shown in Fig. 7, the two isolates induced very low levels of IL-8 compared with the pathogen *E. coli* at any ratio of bacteria to cell. Meanwhile, L2 induced very low levels of IL-1β compared with the *E. coli*, whereas L5 resulted in a significant increase of IL-1β production, which was even higher than the *E. coli*. These pro-inflammatory cytokines contribute to the host's defense mechanisms in response to external invasion, but in some cases, over-mass lymphocytes may perpetuate inflammation and ultimately lead to cell damage, epithelial barrier dysfunction and host tissue injury (Liu *et al.* 2011). Thus, L2 was safer, and has potential to be probiotic.

Quantification of Bacteria in Feces

There was no significant body weight gain of the mice after the treatments of different doses of L2 (Fig. 8). We counted bacteria in feces used TSA agar and found that total aerobic microbiota was not quantitatively changed by the administration of L2 (Fig. 9). Therefore, the presence of L2 in the gut had no effect on the indigenous intestinal microbial populations. These intestinal microbial usually are beneficial to the host. If the balance is destroyed, it will be harmful to the body. L2 did not cause change of intestinal microbial population, which indicated that L2 is harmless in mice feeding.

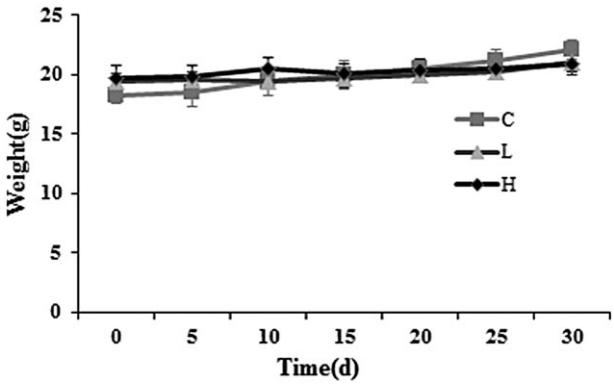

FIG. 8. THE BODY WEIGHT OF MICE DURING ADMINISTRATION OF L2. C, CONTROL; L, LOW DOSE OF L2; H, HIGH DOSE OF L2

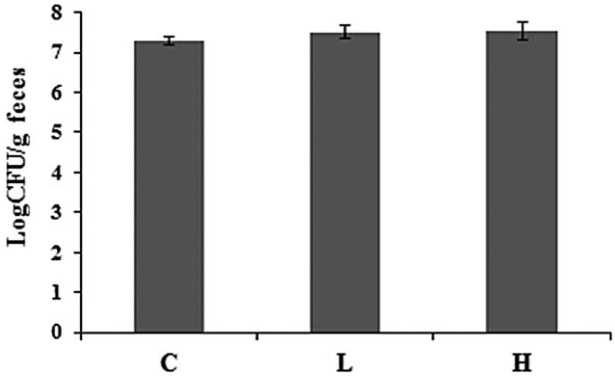

FIG. 9. THE BACTERIA IN FECES WERE COUNTED USED TSA AGAR AFTER THE ADMINISTRATION OF L2 TO MICE FOR 30 DAYS. RESULTS ARE SHOWN AS THE LOG CFU IN 1 G FECES. C, CONTROL; L, LOW DOSE OF L2; H, HIGH DOSE OF L2

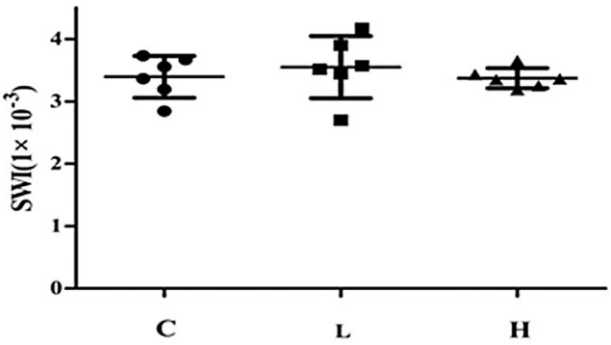

FIG. 10. THE SPLEEN WEIGHT INDEX (SWI) OF MICE ORALLY INOCULATED WITH L2 FOR 30 DAYS. SWI = SPLEEN WEIGHT (G)/BODY WEIGHT (G). C, CONTROL; L, LOW DOSE OF L2; H, HIGH DOSE OF L2

Detection of SWI

No obvious difference in the size and appearance of visceral organs between each group was observed, including splenomegaly As shown in Fig. 10, there was no significant difference in the SWI among the different groups of mice. All animals were healthy and survived the inoculation after 30 days. L2 did not exhibit gross acute oral toxicity effects on the experimental animals' general health status, growth and development.

CONCLUSION

In conclusion, the five isolates from the silage were identified to be strains belonging to *Enterococcus* at the genus level by partially sequencing the 16S rRNA gene, soluble calcium ring experiment, genus-specific primers PCR and *Enterococcus* agar. Among the five strains, L2 shows above-average results in all criteria observed *in vitro* including antibiotic susceptibility, antimicrobial activity, tolerance to gastrointestinal conditions, adhesion to intestinal cells and competitive exclusion of *E. coli* adhesion to epithelial cells and stimulation of immunity. Additionally, the *in vivo* data showed that L2 is safe in mice feeding. These findings demonstrate that L2 could be a promising candidate for future use as probiotic in humans. Further investigations need be conducted to assess the *in vivo* efficacy of the strain in animal experiments.

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