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Characterization of some potentially probiotic *Lactobacillus* strains isolated from Iranian native chickens

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(Received April 5, 2014; Accepted July 28, 2014)

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The objective of the present study was to isolate, identify and characterize new LAB strains with high probiotic potentials from Iranian (Isfahan) indigenous chickens. From 90 isolated LABs, 11 isolates had high growth rate under different stress conditions, including acid (pH 2.5), bile (0.5% ox-gall), salt (6–15%) and temperatures 15 and 45°C, and their aggregation time was less than 120 min. Based on the molecular identification using 16S rDNA sequencing and phylogenetic analysis, the isolates belonged to two *Lactobacillus salivarius* and *L. reuteri* species. The isolates showed different tolerance to 16 clinically and veterinary relevant antibiotics, and most of them were resistant to or semi-tolerant of 7–15 different studied antibiotics. The Es11, Es12, Es3 and Es13 strains with resistance to or semi-tolerance of 15, 14 and 13 different antibiotics, respectively, were the most tolerant strains. The selected isolates showed a wide range of antimicrobial activity against 7 different pathogenic strains. All the isolates exhibited antagonistic activity against *E. coli*, *Enterococcus hirae*, *Salmonella enterica* and *Staphylococcus aureus*. The isolates Es6 and Es11 with high antagonistic activity and resistance against 6 of the studied pathogens were the most powerful antagonistic isolates. The values and types of adhesion to the Caco-2 cell cultures were significantly different (0–40 bacteria/Caco-2 cell), and the maximum adhesion was observed for the isolates Es6 and Es13 with 35 and 40 bacteria adhe-

sion/cell, respectively. Finally, based on all the experiments, 7 strains, including Es1, Es6, Es7, Es11, Es12 and Es13, were selected for the further in vivo assays and possible use in the poultry industry.

Key words: adhesion; antagonistic activity; antibiotic tolerance; chicken; *Lactobacillus*; probiotic; 16S rDNA

Introduction

Pathogen resistance caused by wide application of various antibiotics in both the medical and veterinary fields has become a serious worldwide problem. Finding alternative methods, such as probiotics, to control and prevent pathogens is a challenge for animal nutrition in light of the ban of antibiotics as growth promoters (Damayanti et al., 2012; Telleza et al., 2012). These living microorganisms provide beneficial effects for human or animal health by improving the microbial flora balance of the digestive system and are used alone or incorporated into food or feed systems. This characteristic occurs via their antagonist action, production of different antimicrobial substances and supporting modulation of immune response (Ramirez-Chavarin et al., 2013).

Lactic acid bacteria (LAB), as common microorganisms in foods and the intestine of humans and most animals, are widely used as probiotics in humans and animals to restore the ecological balance of different mucosa (Chen et al., 2012; Musikasang et al., 2012). Some studies showed high effect of LAB on the economic yield properties (lay intensi-

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

ty, day egg weight and mean egg weight) indexes and resistance to pathogens in laying or meat chickens (Koudela et al., 2012; Pan et al., 2011; Yamazakia et al., 2012).

Recently, different groups have been focused on the isolation of new LAB strains with high probiotic potentials from industrial or indigenous laying or meat chickens for application in the poultry industry (Damayanti et al., 2012; Musikasang et al., 2012; Yamazakia et al., 2012). The presence of some *Lactobacillus* in the chicken gastrointestinal tract (GIT) has been described to be of great importance for regulating the composition of the intestinal microflora, developing immunity of the intestine, and promoting the health of chickens (Yamazakia et al., 2012; Muir et al., 2000). The results of these studies have demonstrated that some LABs of poultry origin were able to inhibit the growth of pathogens and survive through the gastrointestinal tract. So, the objective of the present study was to isolate and characterize new LAB strains with high probiotic potentials from Iranian indigenous chickens for application in the poultry nutrition industry.

Materials and Methods

Sampling and isolation of the bacteria. Eight healthy household Isfahan native chickens (*Gallus gallus domesticus*) fed without antibiotics (under 1 year old) were collected from Isfahan region. The ileum content of the chickens was removed, aseptically diluted in peptone water (1/1), plated on MRS medium and incubated anaerobically at 37°C for 48 h (Giraud et al., 1991). The cultures were stored deep-frozen with glycerol 20% (v/v) glycerol at -80°C. The isolates were evaluated by catalase test, Gram stain and bacterial morphology.

Tolerance to acidic pH and bile salt (Ox gall). The acid-tolerant LABs were selected at MRS medium adjusted at pH 2.5 according to the method of Erkkila and Petaja (2000). A bile tolerance test was performed in MRS broth containing 0.5% oxgall (Ox-Bile LP0055; Oxoid) as previously described by Arihara et al. (1998). The growth kinetics of the isolates was determined over 7 h at 1-h intervals at OD₆₀₀ with a Microplate Reader (Infinite M200 Pro, TECAN, Mannedorf, Switzerland). All tests were carried out in triplicate.

Aggregation test. The aggregation test was performed as previously described by Reniero et al. (1992). Aggregation was scored as positive when clearly visible sand-like particles were formed by the aggregated cells, gravitated to the bottom of the tubes, and left a clear supernatant fluid. The test was examined every 15 min for 2 h. The samples aggregated in less than 120 min were chosen for the next experiments.

Tolerance of NaCl and temperature. Viability of the LAB isolates was determined in 10 ml of MRS broth containing 6, 10 and 15% (w/v) NaCl. Samples were inoculated (1% v/v) with overnight cultures and incubated for 48–72 h at 30°C. Conventional MRS broth medium containing 0% NaCl was used as the control. To determine temperature tolerance of the isolates, samples were inoculated (1% v/v) with overnight cultures, and incubated for 48–72 h at 15, 37 (control) and 45°C. The experiments were carried out in three replications.

Antibiotic resistance test. The selected isolates were screened for resistance against 16 clinically and veterinary relevant antibiotics, namely ampicillin (10 µg/ml), vancomycin (30 µg/ml), rifampin (5 µg/ml), erythromycin (15 µg/ml), penicillin (10 µg/ml), ciprofloxacin (5 µg/ml), streptomycin (10 µg/ml), gentamycin (10 µg/ml), nitrofurantoin (30 µg/ml), co-trimoxazole (10 µg/ml), chloramphenicol (3 µg/ml), oxy-tetracycline (5 µg/ml), entrofloxacin (10 µg/ml), Linco-Spec (10 µg/ml), tylosin (10 µg/ml), and lincomycin (10 µg/ml), by the agar disk diffusion method as previously described by Bauer et al. (1966). Oxford *Staphylococcus aureus* used as a sensitive control. The strains *Lactobacillus helveticus* Lafti L10 and *Lactobacillus casei* ssp. *paracasei* L26 (LAFTI probiotics, Institut-Rosell-Lallemand, Quebec, Canada) were used as controls.

The antibiotic discs were supplied by PADTAN Teb (Tehran, Iran). The resistance to each antibiotic was measured based on the diameter of the inhibition zones, and graded according to the supplier's specifications as resistant (diameter of the inhibitory zones < 0–1 mm, R), intermediate (diameter of the inhibitory zones < 3 mm, I) or sensitive (diameter of the inhibitory zones > 3 mm, S). The assays were performed in three independent trials.

Antagonistic activity against pathogens. Antimicrobial activity of the *Lactobacillus* strains was assessed using the agar diffusion method on solid medium previously described by Liasi et al. (2009) and Tagg et al. (1976). The pathogen strains, namely *Pseudomonas aeruginosa* (PTCC 1707), *E. coli* (enterohemorrhagic PTCC 1399), *Streptococcus mutans* (PTCC 1683), *Clostridium defficile* (PTCC 1765), *Enterococcus hirae* (PTCC 1239), *Salmonella enterica* (PTCC 1709), and *Staphylococcus aureus* (PTCC 1431) were provided by Persian Type Culture Collection (PTCC). The pathogen growth-free zones of more than 6 mm was recorded as resistant, those less than 6 mm were reported as semi-resistant, and a free zone of less than 1 mm around a spot was scored as negative (sensitive).

In vitro adherence assays. The adherence of bacterial strains to Caco-2 intestinal cells was tested as described previously by Jacobsen et al. (1999) and Hsieh et al. (2013). Adherent *Lactobacillus* cells in 100 random Caco-2 cells were counted. *Lactobacillus* strains were scored as non-adhesive when an average of fewer than 5 bacteria was present in each Caco-2 cell, weak when there were 1–20 bacteria per cell, intermediate (adhesive) when there were 20 to 35 bacteria per cell, and strongly adhesive when there were more than 35 bacteria per cell. The strains *L. helveticus* Lafti L10 and *L. casei* ssp. *paracasei* L26 (LAFTI probiotics, Institut-Rosell-Lallemand, Quebec, Canada) were used as controls.

Statistical analysis. All the experiments were carried out in three replications. Analysis of variance, average comparing and treatment groups score were obtained using SAS (version 9.1) and the Duncan Multiple test ($p < 0.01$).

Molecular identification. The 16S rRNA genes of the isolates were amplified using universal primers 16sF: (5'-AGAGTTTGATCCTGGCTCAG-3') and 16sR: (5'-AAGGAGGTGATCCAGCCGCA-3') (Michlmayr et al., 2010). The PCR was performed according to Lawalata et al. (2011). Sequences were edited and analyzed with the BioEdit program and basic local alignment search tool (BLAST)

program in the Gene Bank (NCBI). Phylogenetic analysis was performed by the Neighbor-Joining tree method using the Clustal W and MEGA 4 programs. Grouping stability was calculated using 1,000 bootstrap values.

Results

A total of 120 *Lactobacillus* colonies were isolated from ileum of the Isfahan native chickens. Ninety isolated Gram-positive bacteria with catalase-negative reaction and bacillus shape were subjected to further analysis. Forty *Lactobacillus* isolates were considered to be intrinsically acid-resistant since their growth rate was above 75% compared to the control after 7 h of incubation in MRS broth adjusted to pH 2.5. The acidic condition had no significant negative effect on the growth of 19 isolates ($p < 0.05$), and showed maximum growth (100%) in comparison to the normal condition (Table 1).

Among the 90 isolates, 38 isolates showed high or moderate tolerance to oxgall, and showed more than 70% of the growth rate after 7 h in comparison to the control. Among 38 tolerant isolates, 10 isolates, such as Es2, Es6 and Es11, showed high resistance, and their growth was not significantly reduced compared to the control (Table 1). Thirty-

seven isolates showed a lower tolerance, and their growth rate was below 60% after 7 h compared to the control. In total, 35 isolates had high growth rates in both pH 2.5 and the presence of 0.5% oxgall, and were selected for the next experiments.

The aggregation experiment results showed that 11 isolates from 35 selected isolates had an aggregation time less than 120 min. The time needed for significant aggregation of these isolates was between 10 and 120 min. The isolate Es1 showed the fastest aggregation time (less than 15 min), and four isolates, including Es 7, Es 8 and Es 12, had aggregation times of less than 30 min (Table 1). All the eleven selected *Lactobacillus* isolates were able to grow at both 15 and 45°C, and their biomass was more than 90% compared to the control. Moreover, the isolates could grow in the MRS containing 6 and 10% NaCl, and their biomass was 100% and more than 90% compared to the control, respectively. Eight of the isolates could grow with more than 50% biomass efficiency in the MRS containing 15% NaCl, but 3 isolates, namely Es 4, Es 6 and Es 13, could not tolerate 15% NaCl, and did not show any growth.

The resistance or sensitivity of the 11 studied *Lactobacillus* isolates to the selected antibiotics is shown in Table 2. The isolates showed different responses to the antibiotics,

Table 1. Acid and oxgall tolerance and aggregation times of the selected *Lactobacilli* strains.

Strains	MRS pH 2.5	MRS oxgall (0.5%)	Aggregation time (min) ^b
	Growth rate after 7 h ^a	Growth rate after 7 h ^a	
Es1	100	68	30
Es2	100	100	105
Es3	100	80	90
Es4	100	79	90
Es6	100	100	15
Es7	100	83	30
Es8	100	82	30
Es9	83	84	105
Es11	75	100	60
Es12	100	71	30
Es13	100	91	15

^aGrowth rate compared to the control (100% growth).

^bTime needed to give a clear supernatant fluid; lower aggregation time indicates greater aggregation of that strain.

Table 2. Antibiotic resistance of the *Lactobacilli* strains.

Bacteria	Antibiotics															
	Ot ^b	Enf	Ls	Tyl	Lin	Sxt	Chl	Str	Gm	Pen	Ery	Van	RA	Am	Cp	FM
L26	>5 ^a (S)	>5 (S ^c)	>5 (S)	>5 (S)	>5 (S)	3 (S)	4.8 (S)	0 (R)	0.8 (R)	4 (S)	3.9 (S)	0 (R)	2.7 (I)	3.1 (S)	3 (S)	>5 (S)
L10	4 (S)	3.8 (S)	2 (I)	3.1 (S)	4 (S)	1.5 (I)	3 (S)	1 (R)	1 (R)	4.2 (S)	3 (S)	2.6 (I)	2.8 (I)	4 (S)	1.7 (I)	1.7 (I)
Es 1	1.2 (I)	2.2 (I)	1.5 (I)	2.4 (I)	4.4 (S)	2.5 (I)	1.1 (I)	0 (R)	0 (R)	3.1 (S)	1.2 (I)	1 (R)	2.4 (I)	2.7 (I)	1.6 (I)	2 (I)
Es 2	3.9 (S)	3.2 (S)	2.5 (I)	2.6 (I)	4.4 (S)	0 (R)	3.4 (S)	1.9 (I)	2.3 (I)	3.2 (S)	2.8 (I)	2.5 (I)	3 (S)	3.4 (S)	0 (R)	2 (I)
Es 3	0 (R)	2.4 (I)	1.2 (I)	1.8 (I)	0 (R)	2.4 (I)	2.6 (I)	0 (R)	0 (R)	3 (S)	0 (R)	0 (R)	4.8 (S)	4.8 (S)	0 (R)	1.5 (I)
Es 4	4.2 (S)	4.2 (S)	2.8 (I)	3.8 (S)	5 (S)	1.2 (I)	3 (S)	0 (R)	1 (R)	3 (S)	3.7 (S)	1.5 (I)	2.4 (I)	3.5 (S)	1 (R)	3 (S)
Es 6	3 (S)	2.8 (I)	1.7 (I)	2.6 (I)	3.8 (S)	2.4 (I)	3.2 (S)	0 (R)	0 (R)	3.2 (S)	2.4 (I)	0 (R)	2.3 (I)	2.3 (I)	0 (R)	2.8 (I)
Es 7	2.7 (I)	3.2 (S)	2 (I)	2.8 (I)	2.6 (I)	1.5 (I)	1 (R)	0.9 (R)	1.1 (I)	2.1 (I)	3.1 (S)	0.9 (R)	2.8 (I)	2.4 (I)	2.6 (I)	3.8 (S)
Es 8	1.6 (I)	2.3 (I)	2.3 (I)	2.3 (I)	2.7 (I)	1.6 (I)	1.2 (I)	0 (R)	1.2 (I)	2.5 (I)	2.5 (I)	0 (R)	3 (S)	2.7 (I)	0 (R)	3 (S)
Es 9	3.8 (S)	2.6 (I)	2.4 (I)	2.2 (I)	3.8 (S)	0 (R)	1.5 (I)	0 (R)	0 (R)	3.6 (S)	3.8 (S)	0 (R)	3.8 (S)	3.5 (S)	0 (R)	2.6 (I)
Es 11	2.4 (I)	2.2 (I)	1.8 (I)	2.5 (I)	3.1 (S)	0 (R)	2.7 (I)	0 (R)	0 (R)	2.6 (I)	2.7 (I)	0 (R)	2.6 (I)	2.7 (I)	1.6 (I)	2.4 (I)
Es 12	2.9 (I)	2.8 (I)	1.8 (I)	2 (I)	3.2 (S)	0.9 (R)	1.4 (I)	1 (R)	1 (R)	3 (S)	2.4 (I)	0 (R)	1.6 (I)	2 (I)	0.9 (R)	2.5 (I)
Es 13	3.4 (S)	3 (S)	1.8 (I)	2.2 (I)	5 (S)	2 (I)	2.4 (I)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	2.5 (I)	3.2 (S)	0 (R)	4 (S)

^aInhibitory zone (mm), ^bOT: oxytetracycline, ENF: enrofloxacin, LS: Linco-Spec, Tyl: tylosin, 5: lincomycin, AM: ampicillin, Van: vancomycin, FM: nitrofurantoin, Ery: erythromycin, Pen: penicillin, CP: ciprofloxacin, Str: streptomycin, GM: gentamycin, SXT: co-trimoxazole, Chl: chloramphenicol, RA: rifampin, ^cR=resistant, I=intermediate, S=sensitive.

and most of them were resistant to or semi-tolerant of 7–15 different studied antibiotics. The isolates Es3 and Es13 showed the maximum resistance to 7 and 6 different antibiotics, respectively. The isolates Es11, Es12, Es3 and Es13 showed resistance to or semi-tolerance of 15 (4+11), 14 (5+9) and 13 (7+6) different antibiotics, respectively, were the most tolerant isolates. All the studied isolates and commercial strains were sensitive to or semi-tolerant of entrofloxacin, linco-spec, tylosin, rifampin, ampicillin, nitrofurantoin, but were resistant to or semi-tolerant of streptomycin, vancomycin, gentamycin and ciprofloxacin (Table 2).

Antimicrobial activity of the *Lactobacillus* strains was assessed using the agar diffusion method on solid medium. The selected isolates showed a wide range of antimicrobial activity, and differently were able to inhibit growth of all the pathogenic strains (*P. aeruginosa*, *E. coli*, *S. mutans*, *C. difficile*, *E. hirae*, *S. enterica*, and *S. aureus*). Although the halo zones of growth inhibition varied in diameter among the isolates (from 0 to 21 cm), all the isolates (exception for Es8) were resistant or semi-resistant to *E. coli*, *E. hirae*, *S. enterica* and *S. aureus*. The maximum growth inhibition for all the strains was observed against *S. enterica* (Table 3). The isolates Es6 and Es11 with high antagonistic activity against 6 of the studied pathogens showed the maximum range of antagonistic activities. The isolates Es1, Es7 and Es13 showed resistance to or semi-tolerance of all the pathogens (Table 3).

The relative adhesion of the native strains to the intestinal epithelial cells was between 0–40 bacteria/ Caco-2 cell (Fig. 1, Table 4). The maximum adhesion was observed for the isolates Es6 and Es13 with 35 and 40 bacteria adhesion/cell, respectively. The isolates Es2, Es4, Es 7 and Es12 showed intermediate adhesion capacity, but the isolates Es1, Es8 and Es9 showed a weak adhesion to the Caco-2 cells. Different types of adhesion, including aggregative, disperse, and local, were observed for the studied isolates.

The 16S rRNA gene of the selected *Lactobacillus* isolates was amplified and observed on agarose gel. For each strain a PCR product about 1,500 bp in size was achieved (Fig. 2).

Fig. 1. The adhesion capacity of the studied *Lactobacillus* strains to Caco-2 cells (Magnification 1,000 ×).

(a) Strongly adhesive (adhesion type: local), (b) intermediately adhesive (adhesion type: disperse).

Table 3. Antimicrobial activities of the selected *Lactobacillus* isolates.

Bacteria	Pathogens							
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>C. difficile</i>	<i>E. hirae</i>	<i>S. aureus</i>	<i>S. mutans</i>	Mean
Es 1	16 ^a (R)	7 (R)	18 (R)	3 (SR)	8 (R)	3 (SR)	4 (SR)	8.4 (R)
Es 2	3 (SR)	0 (S)	5 (SR)	0 (S)	4 (SR)	3 (SR)	0 (S)	2.1 (SR)
Es 3	2 (SR)	2 (SR)	5 (SR)	6 (R)	2 (SR)	5 (SR)	0 (S)	3.1 (SR)
Es 4	2 (SR)	3 (SR)	5 (SR)	3 (SR)	3 (SR)	3 (SR)	0 (S)	2.7 (SR)
Es 6	12 (R)	15 (R)	15 (R)	8 (R)	7 (R)	9 (R)	4 (SR)	9.5 (R)
Es 7	7 (R)	7 (R)	8 (R)	10 (R)	10 (R)	5 (SR)	3 (SR)	7.1 (R)
Es 8	2 (SR)	0 (S)	10 (R)	0 (S)	6 (R)	0 (S)	0 (S)	2.5 (SR)
Es 9	3 (SR)	5 (SR)	6 (R)	4 (SR)	5 (SR)	5 (SR)	0 (S)	4 (SR)
Es 11	10 (R)	5 (SR)	12 (R)	6 (R)	6 (R)	6 (R)	2 (SR)	6.7 (R)
Es 12	8 (R)	3 (SR)	10 (R)	3 (SR)	3 (SR)	3 (SR)	0 (S)	4.2 (SR)
Es 13	6 (R)	7 (R)	10 (R)	9 (R)	6 (R)	6 (R)	2 (SR)	6.2 (R)
L26	8 (R)	6 (R)	21 (R)	3 (SR)	4.5 (SR)	3 (SR)	2 (SR)	6.9 (R)
L10	10 (R)	5 (SR)	10 (R)	5 (SR)	5 (SR)	4 (SR)	3 (SR)	6 (R)

^aGrowth free zones (in mm), S: sensitive, SR: semi-resistant, R: resistant.

Table 4. The adhesion capacity of the studied *Lactobacilli* strains to Caco-2 cells.

Bacteria	Number of Bacteria/cell	Place of adhesion	Type of adhesion	Adhesion rate
Es 1	15 ± 2	S and A ^a	Local	Weak
Es 2	25 ± 4	A	Aggregative	Intermediate
Es 3	0	—	—	Non-adherent
Es 4	20 ± 3	S and A	Dispersive	Intermediate
Es 6	35 ± 5	S and A	Local and dispersive	Strongly adherent
Es 7	20 ± 3	A	Dispersive	Intermediate
Es 8	10 ± 2	S	Dispersive	Weak
Es 9	15 ± 3	S and A	Dispersive	Weak
Es 11	3 ± 1	—	—	Non-adherent
Es 12	25 ± 3	A	Local	Intermediate
Es 13	40 ± 5	S and A	Local	Strongly adherent

^aS: surface of Caco-2 cells, A: around Caco-2 cells.

Fig. 2. Amplification of the 16S rDNA gene of the selected strains using specific primers.

M: size marker, C: control, 1–11: Es1–Es13, respectively.

Fig. 3. The phylogenetic characteristics of the selected *Lactobacillus* strains.

Nine isolates showed maximum similarity to *L. salivarius* and two strains showed maximum similarity to *L. reuteri*. The sequences were submitted to the GeneBank (the accession numbers KC561104 to KC561133). After blasting and alignments, two phylogenetic trees were constructed for two species (Fig. 3). The isolates with similarity to *L. salivarius* and *L. reuteri* were divided in two separate main groups. The isolates from the *L. salivarius* group were divided in two sub groups. The first group included Es1, Es2, Es6, Es7, Es8, Es9, Es11 and Es13. The second group included only one isolate, Es12.

Discussion

It is well known that resistance of the indigenous laying or meat chickens (especially those produced in village societies) to different biotic and abiotic stresses, is related to presence of LAB strains with high probiotic potentials in the digestive systems of such chickens (Damayanti et al., 2012; Musikasang et al., 2012; Yamazakia et al., 2012). The presence of some lactobacilli bacteria in the chicken GIT has been described to be of great importance for regulating the

composition of the intestinal microflora, developing immunity of the intestine, and promoting the health of chickens (Yamazakia et al., 2012 ; Muir et al., 2000). So the objective of the present study was to isolate and characterize new LAB strains with high probiotic potentials from Iranian indigenous chickens for application in the poultry nutrition industry. It has been proved that those orally delivered probiotic bacteria are effective which survive during the passage through the gastrointestinal tract to their site of function. The first main hurdle to be overcome during this transport is survival in the low pH environment of the proventriculus and gizzard (Yamazakia et al., 2012; Ehrmann et al., 2002; Van Coillie et al., 2007). In the present study, 19 *Lactobacilli* isolates were considered to be intrinsically acid-resistant (pH 2.5) since their growth rate was similar to that of the control. Acidic environment tolerance by LABs has been reported previously by many researchers (Yamazakia et al., 2012; Ehrmann et al., 2002; Van Coillie et al., 2007, e.g.), but no one reported growth at such low pH (2.5). If probiotic bacteria survive through the acidic environment, the next major challenge is to withstand the presence of bile acids, a major hurdle to bacterial survival and growth in the small intestine. In total, 35 isolates had high growth rates in both pH 2.5 and the presence of 0.5% oxgall, and were selected for the next experiments. It was previously confirmed that these kinds of strains could survive better in the gastrointestinal tract than other strains with low acid and bile tolerance in in vivo studies (Jacobsen et al., 1999).

The aggregation experiment results showed that 11 isolates from the 35 selected isolates were able to grow at both 15 and 45°C, and in 6, 10 and 15% NaCl, and had an aggregation time of less than 120 minutes (Table 1). Many authors have reported that the aggregation test is appropriate for screening because it is a simple method applicable to a large number of test strains and this ability of *Lactobacilli* species might enable them to produce a barrier that prevents colonization by pathogenic bacteria (Ehrmann et al., 2002; Zhang et al., 2011; Taheri et al., 2009).

All the studied isolates and commercial strains were sensitive to or semi-tolerant of entrofloxacin, Linco-Spec, tylosin, rifampin, ampicillin, and nitrofurantoin, but were resistant to or semi-tolerant of streptomycin, vancomycin, gentamycin and ciprofloxacin (Table 2). These results were in accordance with previous works reporting different resistance of isolated *Lactobacillus* strains to some antibiotics, such as gentamicin, ciprofloxacin, clindamycin, sulfonamides, glycopeptides and tetracycline (Liasi et al., 2009; Klein., 2011).

The demonstration of antimicrobial activity towards pathogenic species in vitro may be considered a desirable attribute of some probiotic bacteria. The pathogens studied in the present work commonly cause different diseases in poultry and humans, so they are used as standards in antimicrobial activity tests of potentially probiotic microorganisms (Ramirez-Chavarin et al., 2013; Yamazakia et al., 2012; Ramos et al., 2012; Tsai et al., 2008; Valdéz et al., 2005). The selected *Lactobacillus* isolates showed a wide range of antimicrobial activity, and differently were able to inhibit growth of all the pathogenic strains, and interestingly, all the isolates exhibited antagonistic activity against *E. coli*, *E. hirae*, *S. enterica* and *S. aureus* (except for Es8). The isolates

showed the maximum growth inhibition against *S. enterica* (Table 4). This result agrees with the report of Taheri et al. (2009), who reported that all 62 isolates showed higher inhibition activity against *Salmonella* than *E. coli*. Interestingly, the *L. salivarius* strains were more effective against the pathogens than the *L. reuteri* strains. These results are in agreement with those reported by Yamazaki et al. (2012) showing that some of the studied *L. salivarius* strains isolated from chickens and hens of egg-laying strains inhibited the growth of *S. enteritidis* and *Typhimurium* more than other Lactobacilli, including *L. reuteri*. Casey et al. (2004) also reported that *L. salivarius* strains demonstrated the most antimicrobial activity against *Salmonella*. Gilliland and Speck (1977) and Kizerwetter-sweda and Bineck (2005) reported that Lactobacilli have lower activity against Gram-positive pathogenic bacteria such as *S. aureus* and *C. perfringens* than Gram-negatives such as *E. coli* and *Salmonella*. These reports were not in agreement with our results, because in the present study it was shown that most of the Lactobacilli isolates had antibacterial effects against both the Gram-negative and Gram-positive pathogens, but some of them were resistant or sensitive to both Gram-positive and Gram-negative pathogens (Table 3).

Adherence to the intestinal epithelium is considered to be the most important physiological function of probiotic bacteria. Caco-2 cells are the most generally used cell line for adherence assays. In this work 9 strains were able to adhere to Caco-2 cells; most of them adhered both to the surface of Caco-2 cells and around Caco-2 cells with three patterns of adherence (Table 4). Adhesion to the cell line was previously reported by some authors (Chauvière et al., 1992; Perea Vélez et al., 2007) but none of them described a characteristic pattern of adherence or place of adhesion. Chauvière et al. (1992) reported that adherence to cell lines is a strain-specific characteristic, and some strains may adhere to the cell line whereas some of them may not. Strains Es 6 and Es 13, with high aggregation rates, showed strong adhesion to Caco-2 cells. Similar results have previously been reported by Garriga et al. (1998) in which the strains with high aggregation rates had better attachment to the epithelial cells.

The phylogenetic studies of 16S rRNA gene sequences showed that the selected *Lactobacillus* isolates belonged to *L. salivarius* and *L. reuteri*. *L. salivarius* is one of the few species that can achieve numerical dominance among the intestinal *Lactobacillus* populations of certain individuals. The ability of specific *L. salivarius* strains to remain viable and adhere to the host's intestine is potentially desirable as a means to maximize probiotic-derived host benefits at the target site (Pineiro and Stanton, 2007). *L. reuteri* is another dominant Lactobacilli found in the gastrointestinal tract of various animals (Ehrmann et al., 2002; Van Coillie et al., 2007).

Based on the results of the present study, it can be concluded that household native chickens could be used as source of powerful probiotic LABs. The seven selected strains showed high growth rate under different stress conditions, including acid (pH 2.5), bile (0.5% oxgall), salt (up to 15%) and temperature (15 and 45°C), and their aggregation time was less than 120 min. These strains also showed high tolerance of the majority of 16 clinically and veterinary relevant

antibiotics, high antimicrobial activity against 7 different pathogenic strains, and maximum adhesion to the Caco-2 cell cultures. The strains will be used in further in vivo assays at farm level, and the most effective combinations will be used in the poultry industry after bioprocess engineering optimizations.

Acknowledgments

This project was financially supported by Deputy Minister of Agriculture in livestock production and Agricultural Biotechnology Research Institute of Iran (ABRII).

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