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ORIGINAL ARTICLE

Native chicken-derived *Lactobacillus* spp. strains with high probiotic, cholesterol-assimilation and aflatoxin-degradation capabilities

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ABSTRACT

Background and Objectives: Probiotics are added into the food or feed systems and provide beneficial effects to the human or animal health. This study aimed to isolate the gastrointestinal native *Lactobacillus* strains with high probiotic, cholesterol-assimilation and aflatoxin-degradation capabilities from native chickens.

Materials and Methods: About 70 *Lactobacillus* isolates were isolated from ileum of the Fars province native chickens and were investigated for their probiotic properties.

Results: Of 70 *Lactobacillus* isolates, 10 showed high probiotic capabilities, including survival at acidic conditions (pH up to 2.5), tolerance of 0.5% bile and 6-10% NaCl salts, growth in a wide range of temperature from 15 to 45°C, antagonistic effects against different important bacterial pathogens (*Pseudomonas aeruginosa, Escherichia coli, Streptococcus mutans, Clostridium defficile, Enterococcus hirae, Salmonella enterica* and *Staphylococcus aureus*) and sensitivity to some important antibiotics. The selected strains had an aggregation time less than 120 min. The 16S-rDNA sequencing showed that the selected strains were highly related to *Lactobacillus reuteri* and *L. casei*. Finally, the selected strains in this study along with 10 other probiotic strains isolated and characterized in our pervious study were used to evaluate their cholesterol assimilation and aflatoxin B1 degradation capabilities. The potentials of cholesterol assimilation of the selected strains were significantly different (P<0.05) and ranged from 2.3% to 99%. The highest content of cholesterol assimilation was obtained in isolates M20 and M4 with more than 98% absorption. Moreover, four strains 43, OR7, M21 and OR9 were able to absorb AFB1 with 58.6%, 52.33%, 47% and 31.6% efficiency respectively.

Conclusion: It could be concluded that the strains 43, M21 and OR7 showed high probiotic potentials for application in the poultry industry.

Keywords: Probiotics; Aflatoxin B1; Cholesterol; *Lactobacillus reuteri*; *Lactobacillus casei*; Poultry

INTRODUCTION

Probiotics are defined as live microorganisms commonly originated from gut of different organisms or from some traditional foods and dairy products, and confer beneficial effects to their hosts when administered in adequate amounts (1). These microorganisms are used in the food or feed systems and provide beneficial effects for human or animal health by improving the microbial flora balance of the digestive system (2). A wide range of microorganisms have been reported as probiotics, but most known

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types of them are lactic acid bacteria (LAB) and especially *Lactobacillus* species (3). The widespread LAB bacteria can be found in the fermented foods and the mucosal surfaces of humans, terrestrial and marine animals (4). LABs include a big group of different genera, such as *Lactobacillus, Lactococcus, Enterococcus, Oenococcus, Pediococcus, Streptococcus* and *Leuconostoc*. *Lactobacillus* bacteria are Gram positive and rod shape that comprise a large part of the LAB group. They are obligate and facultative anaerobes and predominantly found in the human and animal gastrointestinal and genitourinary tracts (5). Potentially probiotic strains should be evaluated based on certain desirable properties, such as acid and bile tolerance, production of antimicrobial compounds, adhesion to mucosal and epithelial surfaces, the ability to modulate immune responses and bile salt hydrolase activity (6). Probiotic microorganisms commonly have important beneficial effect for their hosts such as inhibiting the growth of pathogenic bacteria, protect the host against various toxins produced by various pathogens (7), decrease serum cholesterol levels (8), increase absorption of calcium and vitamins synthesis (9), modulating the immune system (10), reduction of cancer risk, especially colon cancer and increase nutritional value (11).

It is well confirmed that probiotics protect their hosts against toxic compounds, such as mycotoxins (12). Mycotoxins are toxic secondary metabolites of fungi that are capable of causing disease in both humans and animals. Aflatoxins is known as one of the most destructive fungal toxins that produced mainly by *Aspergillus* species, such as *A. flavus, A. nomius* and *A. parasiticus* that commonly contaminate a variety of animal foods (13). Among various type of aflatoxins (18 types), aflatoxin B1 (AFB1) is the most toxic aflatoxins that is associated with health complications in humans and animals and classified as Group I of carcinogen agents for humans by the International Agency for Research on Cancer (14). AFB1 can be directly affects DNA, RNA, and protein synthesis and alter nuclear morphology (15). Previously, different strategies have been developed for the elimination or inactivation of aflatoxins, including physical, chemical and biological methods. Among them, biological methods are more efficient than other methods due to their ease of application and affordable processes. It has been widely reported that some fungi and bacteria or their secondary metabolites or enzymes could be used for AFB1 biodegradation (16). Among them, LABs have high potential for biodegradation of AFB1 or protective effects against AFB1 (17).

Furthermore, decreased serum cholesterol levels is one of the important beneficial effect of probiotics bacteria. Heart or cardiovascular disease refers to conditions that involve the heart or blood vessels and could be led to a heart attack, chest pain (angina) or stroke (18). It is well confirmed that the significant increase of cardiovascular risk is directly related to elevated levels of serum cholesterol (19). According to prediction of World Health Organization (WHO), cardiovascular disease will be affecting about 23.6 million worldwide people through 2030. Moreover, this disease is a major cause of morbidity and mortality through the world. According to the previous studies, it is documented that LAB bacteria are potentially capable of reduction of serum cholesterol level through binding of cholesterol with the bacterial cells in the small intestine before cholesterol can be absorbed into the body (20). For instance, some *Lactobacillus* species, such as *L. reuteri* and *L. plantarum* presented high efficiency in reduction of serum cholesterol (21). So, finding probiotic bacteria with both aflatoxin and cholesterol reduction potentials is of importance. Therefore, the objectives of the present study were to isolate and identify *Lactobacillus* probiotic bacteria from digestive system of Iranian native chickens with high cholesterol and aflatoxin B1 reduction activities.

MATERIALS AND METHODS

Bacterial species and cultural conditions. Sampling was done from healthy household native chickens (*Gallus domesticus*) fed without antibiotics (under 1-year-old) from Fars province (Iran). The ileum contents of the chickens were diluted in peptone water (1/1), plated on MRS medium and then incubated in a modified atmosphere (5% CO_2 /95% air) at 37°C for 48 h. The purified isolates were stored deep-frozen with glycerol 15% (v/v) and skim milk 5% (v/v) at 80°C for future studies. Moreover, 10 strains of LAB probiotics bacteria, including *L. reuteri* (M20) *L. crispatus* (M21, OR7, OR9, OR10), *L. salivarius* (ES7), *L. oris* (M4, M9), *L. vaginalis* (M2) and *L. agilis* (M17) previously isolated from healthy Iranian household native chickens (22, 23).

Morphological and molecular identifications. At the first step, the isolates were evaluated by catalase test, Gram staining and bacterial morphology. Fermentation of carbohydrates assay were performed using D (+) galactose, lactose, sucrose, fructose, maltose l-hydrate, D (-) raffinose, D (+) trehalose, and D (+) xylose; glucose, arabinose, manose based on method that described in the pervious study (24).

At the next step, DNA from the selected isolates was extracted according to the protocol described by Pospiech and Neumann (1995) with minor modifications (25). The quantity and quality of the extracted genomic DNA were evaluated in 0.1% agarose gel electrophoresis. For molecular identification of the isolates, the polymerase chain reaction (PCR) was performed using the 27f (5′- AGAGTTTAGTCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) for amplification of 16S-rDNA region (35). The PCR reaction mixtures consisted of 1 µl template DNA (20 ng/µl), 0.75 U of Taq polymerase, 0.8 mM of dNTP mixture, 10 pmol of each primer, 1.5 mM $MgCl_2$ at 1/5 and 10× Taq polymerase in the supplied buffer in a final volume of 25 µl. Sequencing was carried out by Macrogen Company (Seoul, South Korea).

Phytogenic analyses were done using the Neighbor-Joining (NJ) algorithms and Maximum parsimony analyses (MP) in MEGA 6.0 software. MP analyses were performed using heuristic searches with 1000 random sequence additions and branch swapping with tree–bisection– reconnection (TBR) algorithm, gaps treated as missing data and the reliability of resultant trees were determined by bootstrap values in 1000 replicates. Topology of the phylogenetic tree was assessed by the neighbor-joining method and the reliability of generated trees was examined by bootstrap analysis with 1000 replicates. Bootstrap support values for NJ and MP higher than 50% are given above each branch (NJ/MP). All sequences were submitted to GenBank nucleotide database, and accession numbers have been recorded. Furthermore, the selected strains were deposited at the Agricultural Biotechnology Research Institute of Iran Culture Collection (ABRIICC) (Table 3).

Screening of *Lactobacillus* isolates for the probiotic properties. The isolated *Lactobacillus* bacteria were screened based on tolerance to acidic pH (at MRS medium adjusted at pH 2.5 and 5), tolerance to bile salt (MRS broth containing 0.3% oxgall; Ox-Bile LP0055; Oxoid), salt tolerance (MRS broth containing 6 and 10% (w/v) NaCl), temperature tolerance (15 and 45°C), antibiotic resistance assay and antagonistic activity based on method previously described by Aazami et al. (21, 22). Additionally, aggregation test was done according to the protocol of Reniero et al. (1992) in which strain was considered as aggregation positive when clearly visible sediment consist of aggregated cells were formed to the bottom of tubes within 2 h (lower aggregation time indicates greater aggregation of that strain) (26). The antibiotic resistance assay was performed using tylosin (10 µg/ml), ciprofloxacin (5 µg/ml), oxytetracycline (5 µg/ml), entrofloxacin (10 µg/ml) and chloramphenicol (3 µg/ml). Antagonistic activity of the isolates was assessed against important pathogens (prepared from Persian Type Culture Collection) including *Pseudomonas aeruginosa* (PTCC 1707), *Escherichia coli* (PTCC 1399), *Streptococcus mutans* (PTCC 1683), *Clostridium defficile* (PTCC 1765), *Enterococcus hirae* (PTCC 1239), *Salmonella enterica* (PTCC 1709), and *Staphylococcus aureus* (PTCC 1431) using agar diffusion method on solid medium (27).

Haemolytic activity of the isolates was performed based on the method previously used by Maragkoudakis et al. (2006) onto blood agar base plates containing 5% (v/v) of sheep blood (28).

Cholesterol assimilation assay. The capability of the isolates for cholesterol assimilation was investigated in MRS broth medium. The amount of 100 mg/ml standard cholesterol powder was added in MRS broth medium as the cholesterol source (sterilized by 0.2 mL filter), then the isolates were inoculated with 1% concentrations (spectrophotometric optical density at 600 nm≈0.8) and incubated at 37°C for 24 hr. The overnight cultures were centrifuged at 10000 rpm for 5 min at 4°C and the supernatants containing non-assimilated cholesterol were collected. The measuring of cholesterol was performed according to the o-phthalaldehyde method described by Rudel and Morris (1973) (29). The amount of 1 ml of the supernatant were placed in a capped tube and 2 ml of 96% (wt/vol) ethanol, 1 ml of KOH (50% W/V) were added, mixed thoroughly, and heated in a 65°C water bath for 10 min. Tubes were placed at room temperature then, 3 ml hexane and 2 ml distilled water were added and mixed thoroughly (1 min). To permit phase separation (hexane and cholesterol), tubes were placed at room temperature for 15 min. Then, 1 ml of the hexane layer was transferred into duplicate test

tubes. The hexane was evaporated from each tube and 2 ml of o-phthalaldehyde reagent (contained 0.5 mg of o-phthalaldehyde per ml of glacial acetic acid) were added to each tube. Then remained at room temperature for 10 min and 500 µL of concentrated H_2SO_4 was added. The tubes were allowed to stand at room temperature for 20 min and then absorbance at 550 nm was measured with a spectrophotometer. The experiment was performed for each strain in three replications. The percentage of cholesterol assimilated was calculated as follows:

% cholesterol assimilated = 100 - (cholesterol in the supernatant containing the cells (mg/ml) /cholesterol in the supernatant control (no cell) × 100)

AFB1 binding assay. The selected probiotic isolates were used for aflatoxin assay, and cultured in MRS broth medium for 24 h at 37°C in a modified atmosphere (5% CO_2/95% air). The suspension was centrifuged (3000 rpm, 10 min, 4°C) and the bacterial pellets were washed three times with phosphate-buffered saline (PBS), and washed cells were dissolved in BPS buffer (with PH 6.5). Spectrophotometric optical density at 600 nm (OD) was set approximately 1 (10^{10} cfu/ml). To prepare standard stock AFB1 (100 µg/ml), 1 mg of solid aflatoxin B1 (Sigma Aldrich, Germany) was dissolved in 10 ml methanol (HPLC grade, Sigma 34860). Then, 10 ml of phosphate-buffered saline (BPS) was added and suspension was placed in 80°C water bath for 10 min (to evaporation of methanol) (30). In the next step, 950 µl of BPS was added to 50 µl of prepared standard stock (AFB1) for a final concentration of 5 µg/ml. Then, one ml of prepared cell suspension (OD=1, 10^{10} cfu/ml) centrifuged at 3000 rpm for 10 min and the supernatant contains BPS removed completely. Bacterial pellets were added to the 1 ml of AFB1 with 5 µg/ml concentration and mixed thoroughly. The tubes were incubated at 37°C in a modified atmosphere (5% CO_2/95% air). After incubation, the tubes were centrifuged at 10000 rpm for 10 min and the supernatant (containing unbound AFB1) was collected. A solution of mycotoxin in PBS was used as a positive control and bacterial suspension served as negative control.

AFB1 was collected for high-performance liquid chromatography (HPLC) analysis according to the method of El-Nezami et al. (1998) (17). The amount of 20 µl of the supernatant sample was injected directly into HPLC with 5 µm Supercosil TMLC-18 reverse phase column (250 × 4.6 mm, Supelco, Bellefonte, PA). The mobile phase was including water/methanol/acetonitrile (60:30:10, v/v/v) at the flow rate of 1 ml/min at 25°C. AFB1 detection was performed using fluorescence by excitation at 350 nm and emission at 460 nm. Aflatoxin B1 was quantified using the following formulae:

$$\text{Aflatoxin absorption \%} = 100 \times \left(1 - \frac{\text{AFB1 peak area in treated samples}}{\text{AFB1 peak area in control}}\right)$$

Statistical analysis. The statistical analysis of data was performed using a one-way analysis of variance (ANOVA), and the means were compared using Duncan's test by SPSS for Windows version 17 (Chicago, SPSS Inc.). P-value of ≤ 0.05 was considered as the statistical significance level.

RESULTS

About seventy bacilli form isolates were purified from ileum of the Fars province native chickens. Among them, 40 isolates were Gram-positive, catalase-negative and rod shape, identified as *Lactobacillus* sp. and selected for probiotic assays. Aggregation time, hemolytic activity, NaCl and temperature tolerance, acid and ox gall tolerance of selected strains are prepared in the Table 1. Ten isolates could have growth at temperatures 15-45°C, pH up to 2.5 and salinity up to 10%, showed aggregation time less than two hours, and were selected as potential probiotic strains. These strains also did not show any hemolytic activity (Table 1).

The isolate 48 and 67 showed the fastest (15 min) and slowest (120 min) aggregation time, respectively. Additionally, all the isolates could grow in the MRS containing 6 and 10% NaCl and their biomass was more than 90% compared to the control (100%). Moreover, the isolates tolerated the highest (15°C) and lowest (45°C) temperatures and their biomass was more than 90% compared to the control. The isolates 15P, 43, 2PH and 44 showed more than 80% growth percentage after 8 h in pH 2.5 compared to control. Subsequently, the isolates 48, 14P, 20P, 48, 67 and 52 had more than 70% compared to the control (normal condition). Maximum and minimum growth was related to the isolates 2PH (89.68%) and 45 (64.55%) compared to control. The isolates 48, 14p, 43, 2ph, 15p and 44 indicated more than 70% growth in the presence of 0.3% bile, while the isolates 20p, 45, 67 and 52 grew more than 60% compared to the control (Table 1).

Table 1. Acid and oxgall tolerance, NaCl tolerance and temperature resistance of the selected *Lactobacilli* strains.

Isolates	Collection number	NCBI accession number	Aggregation time (min)	Haemolytic activity	NaCl 6 & 10%	Temp. 15 & 45°C	Grow percentage after 8 h	
							pH 2.5	0.3% bile
2PH	ABRIICC 20082	KP757024	90	-	+	+	89.68 ± 0.16^{a}	79.36 ± 0.23^{c}
43	ABRIICC 20085	MN860067	70	-	+	+	80.94 ± 0.51^{b}	81.4 ± 0.13^{b}
14P	ABRIICC 20079	KP757023	45	-	+	+	79.58 ± 0.15^{bcd}	71.42 ± 0.22^{e}
48	ABRIICC 20083	MN860069	15	-	+	+	78.56 ± 0.38^{cd}	88.66 ± 0.14^{a}
20P	ABRIICC 20050	KP137677	105	-	+	+	74.43 ± 0.33^{e}	75.80 ± 0.12^{d}
45	ABRIICC 20084	MN860068	90	-	+	+	64.55 ± 0.41^{g}	70.40 ± 0.21^{e}
15P	ABRIICC 20080	KP137676	30	-	+	+	80.82 ± 0.26^{b}	74.75 ± 0.15^{d}
67	ABRIICC 20078	KP137678	120	-	+	+	77.95 ± 0.83^{d}	69.95 ± 0.21^{e}
44	ABRIICC 20077	KP757022	90	-	+	+	80.10 ± 0. 56^{bc}	75.98 ± 0.83^{d}
52	ABRIICC 20081	KP137675	45	-	+	+	72.31 ± 0.12^{f}	80.3 ± 0.52^{bc}

The pathogen inhibition and antibiotic resistance of the selected strains are shown in Tables 2 and 3, respectively. The pathogen inhibition zone for the selected strains varied from 0 to 14 mm (Table 2). Maximum antagonistic activity against *S. mutans, C. difficile, E. hirea, S. enterica, P. aeroginosa, S. aureus* and *E. coli* were related to the strains 43, 15p, 43, 48, 67, 43 and 20p, respectively. Moreover, maximum and minimum antagonistic activity was related to the strain 43 (average activity: 14.21 mm) and 2ph (average activity: 6 mm), respectively (Table 2).

The antibiotic susceptibility test was performed for all selected probiotic *Lactobacillus* spp. against 8 different widely used antibiotics in veterinary. Reactions of the studied isolates to antibiotics are shown in Table 3. The isolates indicated significantly different susceptibility or resistance to the studied antibiotics, however, a number of the isolates showed resistance to some antibiotics. Isolates 43 and 45 showed intermediate resistance to sensitive responses to all antibiotics. Moreover, other strains showed susceptibility to the majority of the studied antibiotics, however, they were resistant to at least one of the antibiotics. All the isolates showed sensitive or intermediate responses against rifampin and enrofloxacin, except isolates 67 and 14P, respectively. Carbohydrate fermentation profile of selected strains listed in Table 4. The selected isolates were able to ferment different carbohydrates. All isolates could ferment fructose and maltose as carbon source. Furthermore, the treated isolates could use lactose and mannose as carbon source except for 14P and 15P, respectively. The isolates 2PH and 43 showed similar carbohydrates fermentation profile by fermenting fructose, glucose, lactose, maltose, mannose and sucrose.

Molecular identification of the selected isolates was performed by amplification and sequencing of the 16S rRNA gene. The PCR amplification produced a band of approx 1500 bp (Fig. 1). Phylogenetic analysis was done based on maximum parsimony and neighbor-joining methods by using the 16S-rDNA sequences of 10 isolates together with the sequences of 16 isolates obtained from GenBank (NCBI). The *Enterococcus faecium* strain LGM 11423 was used as the outgroup taxon. The maximum parsimony tree was selected and is shown in Fig. 2. The isolates 52, 67, 45, 44, 48, 43 and 15P clustered with various strains of *L. reuteri* in a single clade (100% and 100% bootstrap

Table 2. Antimicrobial activities of the selected *Lactobacillus* isolates (growth inhabitation zone diameter (mm))

Strain	*S. mutans*	*C. difficile*	*E. hirea*	*S. enterica*	*P. aeroginosa*	*S. aureus*	*E. coli*	Mean
2PH	6	0	8	7	0	14	7	6
43	14.5	9	17	14	16	15	14	14.21
14P	0	14	6	13	8	13	0	7.71
48	8	10	12	19.5	11	13	13	12.35
20P	9	0	10	15.5	12	15	18.5	11.42
45	7	12	13	15	9	12	14.5	11.78
15P	7	18	7	15	8	0	13	9.71
67	8	16	11	13	18	10	13.5	12.78
44	0	7	11	14.5	6	13.5	12.5	9.21
52	8	11	11	11.5	7	7	16	10.21
Mean	6.75	9.7	10.6	13.8	9.5	11.25	12.2	

Table 3. Antibiotic resistance/susceptibility of the native *Lactobacilli* strains.

Strain	Chlo	Enr	Oxy	Cip	Str	Gm	RA	Tyl
2PH	R (0)	S (22)	S (20)	S (23)	R (0)	S (23)	S (25)	S (30)
43	I (16)	S (21)	S (17)	S (21)	S (25)	S (24)	S (25)	S (25)
14P	I (16)	R (0)	S (15)	S (24)	R (10)	S (23)	S (24)	R (12)
48	S (16)	S (22)	R (5)	S (22)	S (26)	S (25)	S (25)	R (0)
20P	S (15.5)	S (25)	S (24)	S (23)	S (21)	R (12)	S (23)	S (23)
15P	R (0)	R (4)	S (21)	S (22)	S (30)	R (0)	S (23)	R (0)
67	I (8)	S (16)	I (8)	R (11)	R (0)	S (23)	R (0)	S (27)
44	R (0)	I (15.5)	S (22)	S (25)	R (11)	R (0)	S (25)	R (10)
45	S (17)	S (22)	S (21)	S (25)	S (22)	S (22)	S (25)	S (25)
52	R (7)	S (21.5)	S (21)	S (23)	S (23)	S (23)	S (23)	R (0)

Inhibitory zone (mm) <15 (Resistant=R); 16≤ Inhibitory zone (mm) < 20 (Intermediate=I); 20≤ Inhibitory zone (mm) (Sensitive = S); Chlo = Chloramphenicol; Enr = Enrofloxacin; Oxy = Oxytetracycline; Cip = Ciprofloxacin; Tyl = TylosinK; Str = Streptomycin; Gm = Gentamycin; RA: rifampin.

Table 4. Carbohydrate fermentation profile of the selected probiotic isolates.

	Isolates									
	2PH	43	14P	48	20P	45	15P	67	44	52
Arabinose	-*	-	-	-	+	+	-	+	-	-
Fructose	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	+	-	-	-	+	-	+	-
Glucose	+	+	+	-	+	-	+	+	+	+
Lactose	+	+	-	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	-	+	+	+
Raffinose	-	-	+	-	-	-	+	-	+	+
Sucrose	+	+	-	+	-	-	+	-	-	-
Trehalose	-	-	+	-	-	+	-	-	-	+
Xylose	-	-	-	+	+	-	-	+	+	-

* (+): strain show capability of fermentation of the carbohydrate, (-): strain does not show capability of fermentation of the carbohydrate.

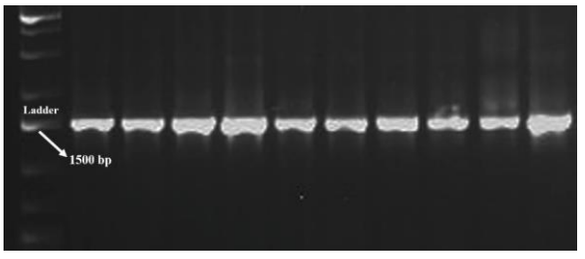

Fig. 1. The results of PCR amplifications of the bacterial isolates with 16S rDNA universal primers (27f & 1492r).

value for NJ and MP) and identified as *L. reuteri* (Fig. 2). Two other isolates 2PH and 14P were located together with *L. casei* strain DSM 20011 and identified as *L. casei* (Fig. 2).

Twenty strains including the selected strains in this study along with 10 strains from our pervious study were used to evaluate their effect on cholesterol assimilation *in vitro*. The levels of cholesterol assimilation by the strains are shown in Fig. 3. The content of cholesterol removed varied (P<0.05) and ranged from 2.3% to 99%. The maximum cholesterol assimilation was observed for the strains M20 and M4 with more than 98% absorption. The strains 52 and 43 showed 80-90% cholesterol assimilation, while it was 60-80% for the strains OR9, 67, M21, ES7 and M9. Other strains assimilated less than 60% cholesterol. The strain M17 with 2.3% showed lowest absorption capacity (Fig. 3). Twenty strains including the selected strains in this study along with 10 strains from our pervious study were used to evaluate their AFB1 binding *in vitro*. AFB1 was calculated in three times, including 30 minutes (T0), 4 h (T1) and 12 h (T2) after adding bacteria and incubation at 37°C. The results showed that four strains, including *L.reuteri* strain 43, *L. crispatus* strain OR7, *L. crispatus* strain M21 and *L. crispatus* strain OR9 were able to absorb AFB1 (Fig. 4). The highest reduction of AFB1 observed 12 h after inoculation. The strains 43 and OR7 showed 58.6% and 52.33% removal of AFB1 compared to the initial mycotoxin concentration after 12 h. The amount of mycotoxin reduction in the samples including M21 and OR9 was 47% and 31.6% after 12 h, respectively.

DISCUSSION

The application of probiotics in the poultry industry has attracted much attention in recent years. Recently, some studies have been dedicated on evaluation of probiotic potentials of LAB bacteria derived from the digestive systems of indigenous layers or broilers. These microorganisms are able to protect their hosts against different biotic and abiotic stresses. Additionally, LABs not only have high potentials for removing and degradation of mycotoxins especially various known types of aflatoxins, but also, are potentially capable of reduction of serum cholesterol level. The present study was dedicated to isolation and characterization of some native strains of *Lactobacillus* bacteria with high probiotic potentials from healthy Iranian household native chickens. The selected strains showed growth and tolerance at harsh conditions, including acidic pH (pH 2.5), bile (0.3% oxgall), salt (6-10%) and temperature range from 15 and 45°C. Bile tolerance is known as one of the key factors for candidate probiotics strains which shows survival and growth rate of the probiotic in the gastrointestinal tract (GIT). The concentration of bile salt in the chicken GIT is about 0.175 that probiotics bacteria must be tolerate it. In this study, we assayed tolerance of LABs at 0.3% bile salt, which is more than that of recommended in other studies. All the strains showed high tolerance to 0.3% bile. These results are similar to the reports of Reuben et al. (2019) (31), in which LABs could tolerate more than 0.3% bile salts. Acidic pH tolerance also is known as one of the important factors for probiotic bacteria, as survival in the acidic pH of host gut during passage through the gastrointestinal tract is of importance for efficient durability and functionality of probiotics bacteria (32). In the present study, candidate probiotic strains of both *L. casei* and *L. reuteri* showed moderate to high tolerance to acidic pH and showed 60-86% growth at pH 2.5 compared to the control (pH: 5.6). The *L. casei* strain 2PH showed maximum tolerance to the acidic pH and had 86% growth compared to the normal conditions. Previous studies showed that LABs isolated from chickens have moderate to high tolerance to acidic conditions (31).

The strains were identified as *L. reuteri* (strains 52, 67, 45, 44, 48, 43 and 15P) and *L. casei* (strains 2PH and 14P) based on 16S rRNA gene sequencing. The aggregation time of the selected strains was between 15 to 120 min. All the selected strains showed a wide range of antagonistic activity against various Gram-negative and positive pathogens that are commonly cause different diseases in poultry and humans (33). Beneficial effects of lactobacilli on inhibition of

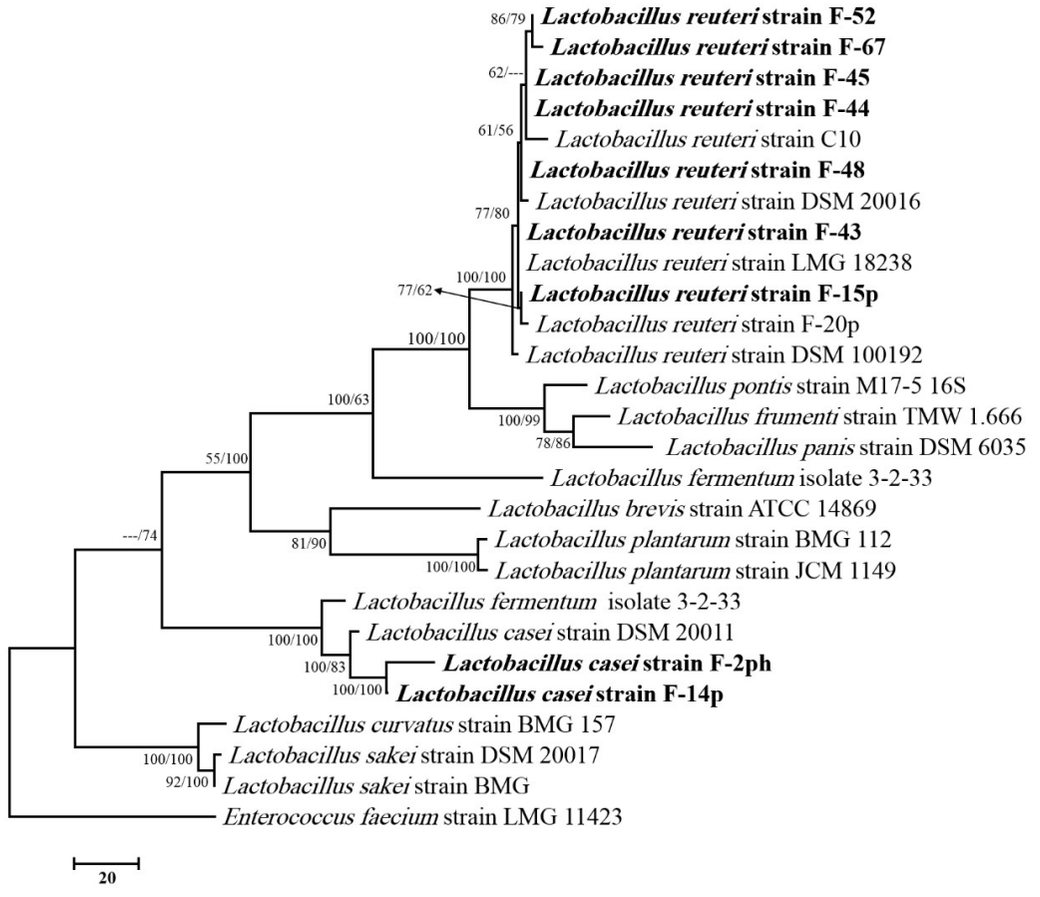

Fig. 2. The phylogenetic tree constructed using maximum parsimony method based on the nucleotide sequences of 16SrDNA of the studied strains. Bootstrap support values for maximum Parsimony and neighbor-joining higher than 50% are given above each branch (MP/NJ). The *Enterococcus faecium* LMG 11423 is the outgroup taxon.

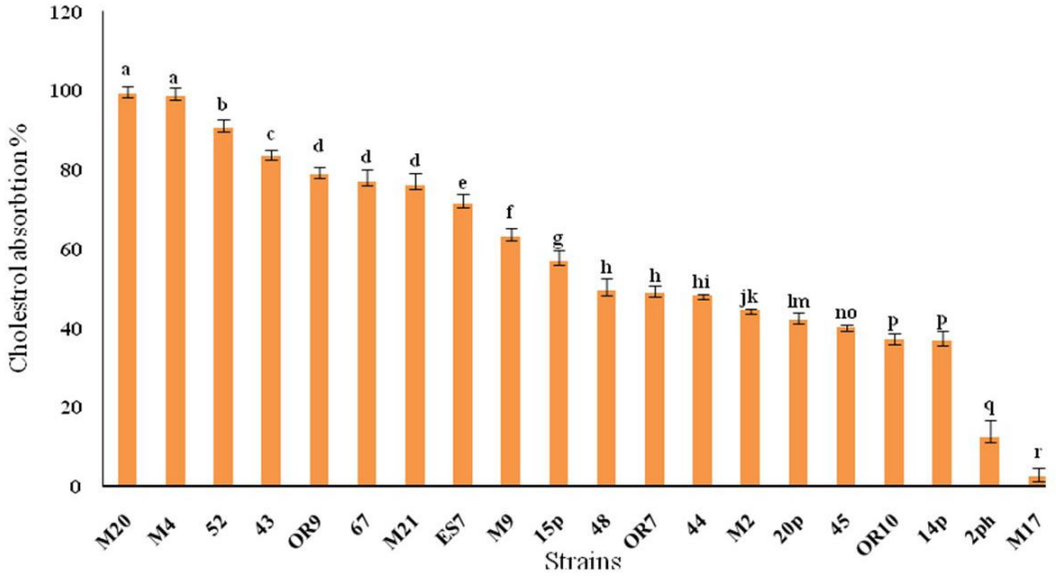

Fig. 3. The results of *in vitro* cholesterol absorption by of the native *Lactobacillus* strains. Error bars refer to standard deviation. The letters above bars indicate treatments with significant difference ($P < 0.05$).

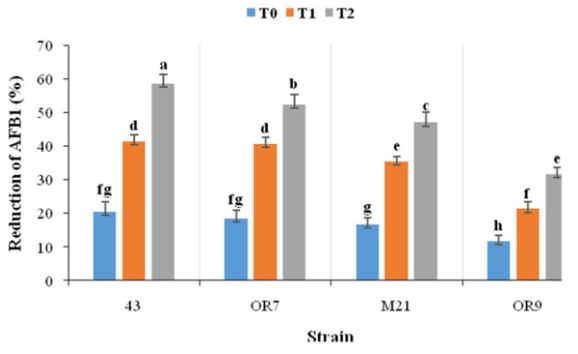

Fig. 4. Aflatoxin reduction in different incubation time intervals (T0, T1 and T2 = 30 min, 4 h and 12h after inoculation, respectively). Error bars refer to standard deviation. The letters above bars indicate treatments with significant difference ($P < 0.05$).

Gram-positive and Gram-negative pathogenic have been reported by many researchers (33, 28). All the strains belonging to *L. reuteri* in the present study indicated higher antimicrobial activity than those of *L. casei* strains (2PH and 14P). Most strains showed high antagonistic effects against *E. coli, S. enterica* and *S. aureus.* The highest inhibition was observed against *S. entrica* (average activity: 13.8 mm), *E. coli* (average activity: 12.2 mm) and *S. aureus* (average activity: 11.25 mm). The lowest inhibition was observed against *S. mutans* (average activity: 6.75). These results agree with those reported by other studies about inhibitory activity lactobacilli probiotic bacteria against pathogenic bacteria *Salmonella* and *E. coli* (34). Among the Gram-negative pathogens, all the isolates displayed weak activity against *P. aeruginosa* that is similar to the results of Sharma et al. (2017) (33). Commonly, it has been well known that *Lactobacillus* species show less antagonistic properties against Gram-positive pathogens compared to Gram-negative pathogens such as *E. coli* and *Salmonella* (35). However, strains of the present study showed high antimicrobial activity against Gram-positive bacteria such as *C. difficile* that was not consistent with previous studies.

Safety of probiotic strains is another key factor which should be taken into account during the process of strain screening. Absence of genes responsible for antibiotic resistance is important option for safety of probiotics. Antibiotic susceptibility of novel probiotic strains was assessed for 10 various antibiotics. Fortunately, the majority of strains showed susceptibility to most of the antibiotics, however, a number of strains showed resistance to a few studied antibiotics, and therefore, should be considered if the strains will be commercially used for poultry (Table 2). Furthermore, all the LAB strains did not show any hemolytic activity. Similar to the present study, Reuben et al. (2019) reported that the majority of LAB strains isolated from gut of chickens and broilers were non-haemolytic (36).

Aflatoxin B1 (AFB1) is one of the most toxic fungal toxins that produced mainly by *Aspergillus* species (12). The AFB1 can be entered into the body through foods and can cause tissue necrosis, hepatic cirrhosis and liver cancer. The presence of AFB1 in poultry resulting in significant economic costs by reduction in growth, decreased quality of egg and meat production, and compromised immune functions. In addition, annually approximately 25% of the world's food supply are contaminated with mycotoxin. Moreover, contaminated poultry products can be transferred to human body that causes various destructive disease. LABs have also high potentials for removing or degradation of AFB1. So, application of probiotics with capability of AFB1 degradation to the poultry diet, can guarantee poultry health and subsequently human health. In this study, the number of 20 strains were evaluated for their AFB1 degradation activities. Four strains, including *L. reuteri* strain 43, *L. crispatus* strain OR7, *L. crispatus* strain M21 and *L. crispatus* strain OR9 were able to bind to AFB1. The highest amount of AFB1 was bound by the strain 43 after 12 h inoculation. These results were in accordance with those of Peltonen et al. (2000) (37), which assessed various strains of *Lactobacillus* probiotic bacteria including *L. crispatus* strains for their binding ability to aflatoxin B1. They showed that *L. crispatus* has high potential for binding to AFB1. However, there are many studies on various species of *Lactobacillus* on binding ability to AFB1 that show their high potentials for aflatoxin B1 reduction (17).

Cardiovascular disease is a major cause of mortality in many countries worldwide. Epidemiological studies have been demonstrated increasing of cardiovascular risk is directly related to elevated levels of serum cholesterol (19). Recent studies have been dedicated to evaluation of the effect of prebiotic bacteria (especially LAB Bacteria) on reduction of serum cholesterol level (7, 37). We investigated cholesterol-lowering effect of the selected native probiotic bacteria *in vitro.* The efficiency of the strains for cholesterol assimilation was between 2.3-98%. The highest content of cholesterol assimilation was ob-

tained for *L. reuteri* strain M20, *L. oris* M4, *L. reuteri* strain 52 and *L. reuteri* strain 43 with 99.4%, 98.6%, 90.6% and 83.43% absorption, respectively. However, other tested strains have between 60-80% cholesterol assimilation. The cholesterol assimilation in the other studies have been 40-67%, which are less than the results of the present study (29). Recently, some studies have showed that use of probiotic bacteria in the poultry diet can be significantly result in reduction of serum, egg yolk and meat cholesterol (38, 39). So, it is expected that commercial application of the selected strains in the present study in the poultry diet will reduce serum, egg yolk and meat cholesterol and increase product quality in the layers or broilers. Finally, it could be concluded that the strains *L. reuteri* strain 43, *L. crispatus* strains M21 and OR7 have sufficient potentials to be evaluated in the farm trials for commercial use in the poultry industry.

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