Screening Lactobacilli Isolates from Northern Iran Backyard Chickens as Bio-control Strategy Against Salmonella Enteritidis and Salmonella Typhimurium

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Abstract
In this study, Gram positive and catalase negative rod-shaped bacterial strains were isolated from Iran's backyard chicken intestines. After a preliminary screening for acid and bile tolerance, 14 Lactobacillus reuteri isolates were selected based on susceptibility to the antibiotics and the absence of beta haemolysis for antimicrobial activity against Salmonella Enteritidis and Salmonella Typhimurium. All 14 isolates were able to tolerate pH 3 for 3 h and their resistance to 0.3% bile salts was more than 50%. Among these 14 isolates, L. reuteri ABRIG22, L. reuteri ABRIG18, L. reuteri ABRIG17, L. reuteri ABRIG8 and L. reuteri ABRIG9 were able to withstand pH 2.3 for 2 h, and L. reuteri ABRIG17 was the only isolate with good survivability in pH 2.3 for 3 h. Five isolates were able to withstand stronger acidic conditions and inhibit Salmonella Enteritidis and Salmonella Typhimurium by more than 90% with less of their supernatants. The results of this study demonstrated that isolated L. reuteri from intestines of chickens native to Northern Iran could be introduced as potential antimicrobial probiotic strains to control S. Enteritidis and S. Typhimurium infection.

Keywords: Probiotic, Lactobacilli, Screening, Native chicken, Salmonella

INTRODUCTION
The appearance of drug resistance pathogens is one of the biggest health concerns. Multidrug-resistant Salmonella isolates have been recovered in patients [1,2] and retail chicken meat and giblets [3] in Iran. The emergence of
antibiotic resistant pathogens in chicken meat is the result of improper use of antibiotics by poultry farmers [4]. The probiotic bacteria are an important bio-control strategy against pathogens such as *Salmonella* in chickens [5]. Isolated *Lactobacillus* for use as an effective probiotic strain should be able to overcome the harsh conditions that they will be faced with after entering the gastrointestinal tract. Generally, in order to meet these conditions, new probiotic bacteria strains have been selected by passing experiments that simulate conditions, such as those of the gastrointestinal tract. Apart from ability to survive in the host gastrointestinal tract condition, novel strains should be identified properly using biochemical and molecular methods.

In order to select safe potential probiotic strains, the basic safety aspects such as absence of haemolytic activity and antibiotic susceptibility of novel probiotic bacteria strains must be evaluated. Eventually the novel probiotic bacteria strains must be able to inhibit the pathogens of interest in vitro [6,7]. The objective of this study was to test the anti-biotic sensitive *Lactobacillus reuteri* isolates (sensitive to ampicillin, clindamycin, gentamicin, streptomycin, tetracycline, erythromycin, kanamycin and chloramphenicol according to the EFSA (2012) method [7]; not published data), from the different parts of the gasterointestinal tract of backyard chickens from Northern Iran for their probiotic potential and inhibitory capacities as a bio-control strategy against *Salmonella enteritidis* and *Salmonella typhimurium*.

**MATERIAL and METHODS**

*Isolation*: Six healthy backyard chickens (about one year old) were collected from six different regions of Guilan province in Northern Iran. The selected chickens were kept in free range and were not fed antibiotics. The duodenum, jejunum, ileum and cecum contents of the chickens was removed aseptically and diluted in sterile phosphate buffered saline (PBS) (1/10). Then 10-fold serial dilutions of each sample were made in sterile PBS, 100 µL of the appropriate dilution was plated on the Man Rogosa and Sharpe (MRS) agar medium (Merk) and incubated anaerobically at 37°C for 24-48 h (5% CO₂). Strains without displaying blood lysis zones around the colonies were classified as non-haemolytic (without β-haemolysis) [6].

**Identification of Lactobacillus Isolates with Ability to Survive and Growth in the Presence of Bile Salts and Low pH**: The DNA was extracted from the bacterial plates of the overnight culture (1.5 mL) of each of the 26 LAB isolate in MRS broth after centrifugation at 5,000 × g for 10 min at room temperature using a sinapure Gram positive bacteria DNA extraction kit (Sinagene, Iran). The 1.5 kb 16S rRNA genes of the isolates were amplified using universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT-3') [11,12].

The PCR amplification was performed in 25 µL reaction mixtures using a ASTEC Thermal Cycler (Fukuoka, Japan). The PCR condition was as follows: initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 1 min each, primer annealing at 55°C for 30 s and primer extension at 72°C for 2 min, and a final step of primer extension at 72°C for 5 min. After purification, PCR products were sequenced by the automated DNA sequencing system (Macrogen, Korea). Sequences were edited by Bioedit software version 7. Sequence similarity values were determined using the basic local alignment search tool (BLAST) of the National Centre of Biotechnology Information (NCBI).

The CLUSTAL W program of the Bioedit software version 7 was used for sequence alignment. A phylogenetic tree was constructed based on the 16S rRNA gene sequence analysis by the Neighbor-Joining tree method using MEGA6 software. In the construction of the phylogenetic tree, 34 nucleotide sequences comprised of 26 LAB isolates’ sequences derived from this study and 8 sequences belonging to *Lactobacillus* species that were obtained from GenBank, were involved. The *Lactococcus lactis* sequence (AB100803.1), was used as an outgroup.

**Antibiotic Susceptibility Test (Minimum Inhibitory Concentration)**: The assay for antibiotics susceptibility of the 26 isolated lactic acid bacteria to ampicillin, clindamycin, gentamicin, streptomycin, tetracycline, erythromycin, kanamycin, chloramphenicol and vancomycin was performed in 96-well plates using the broth microdilution method according to the EFSA (2012) [7] (not published data).

**Inhibition Assay I Against Salmonella Enteritidis and Salmonella Typhimurium**: The inhibitory activity of 14 LAB isolates against *S. enteritidis* and *S. typhimurium* was studied. First, to confirm the presence of lactate, pH 7.0, 100 µL of each sample was added to 900 µL of 100% TDS (95% toluene, 5% DMSO) solution and put into a test tube at 4°C. The tubes were incubated for 5 min at room temperature using a sinapure Gram positive bacteria DNA extraction kit (Sinagene, Iran). The 1.5 kb 16S rRNA genes of the isolates were amplified using universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT-3') [11,12].

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evaluated using the agar spot test as described by Toure et al.\cite{13}.

**Inhibition Assay II Against Salmonella Enteritidis and Salmonella Typhimurium:** The antimicrobial activities of different amounts of cell-free supernatants of the 14 susceptible isolated lactic acid bacteria to ampicillin, clindamycin, gentamicin, streptomycin, tetracycline, erythromycin, kanamycin, chloramphenicol and vancomycin without pH adjustment against \textit{S.} Enteritidis and \textit{S.} Typhimurium was assessed on 96-well microtitre plates. It was assessed according to a modified method described by Bian et al.\cite{14} with the absorbance reader (BIO TECK ELx 808) at wavelength 630 nm as the minimum inhibitory concentration on (MIC90). The isolated \textit{Lactobacillus} strains were cultured overnight in MRS broth medium at 37°C, then cell-free supernatants of each isolate were prepared by centrifuging the cultures at 650 g for 20 min at 4°C. The supernatant filter was sterilized (0.22 µm pore size, cellulose acetate filter) according to the method described by Jin et al.\cite{15} and used immediately after preparation. Ten µL of the activated culture of \textit{S.} Enteritidis and \textit{S.} Typhimurium (about 1.5X10^8 cfu/mL) were added to wells containing 10, 20, 30, 40, 50 and 60 µL of cell-free supernatant of each isolated bacteria. The total volume in each well was adjusted to 250 µL using tryptic soy broth (TSB). The pathogen inhibition was quantitatively determined on the basis of OD630 change in the microtitre plate, in comparison with untreated controls.

The percentage of pathogen inhibition was calculated as:

\[
\% \text{ inhibition} = \frac{\text{OD untreated control} - \text{OD treatment}}{\text{OD untreated control}} \times 100 \quad [14].
\]

**RESULTS**

**Identification and Phylogenetic Analysis Using 16S rRNA Gene Sequences:** The results of the 16S rRNA gene sequence showed that all 26 LAB isolates belonged to the \textit{Lactobacillus} genus (Table 1). Twenty two isolated isolates from 26 LAB isolates were 99% similar to \textit{L. reuteri} and 4 of them showed 99% similarity to \textit{L. johnsonii}. The 16S rRNA gene sequences of the 26 \textit{Lactobacillus} strains

<table>
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<tr>
<th>Isolate</th>
<th>Source of Isolate</th>
<th>Accession Number</th>
<th>The Nearest Matched Species from GenBank</th>
<th>Similarity (%)</th>
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were deposited in the GenBank database under accession numbers MF686461 to MF686486 for ABRIG1 to ABRIG26 isolates. Fig. 1 shows the phylogenetic tree based on the 16S rRNA gene sequence analysis of 26 Lactobacillus strains obtained in this study (ABRIG1 to ABRIG26) and 8 Lactobacillus strains obtained from GenBank. Lactococcus lactis (NR 040955.1) was used as the outgroup.

The 26 isolates were divided into two main groups, one group was similar to the L. reuteri species and the second group was similar to the L. johnsonii species. The strains ABRIG5, ABRIG7, ABRIG14 and ABRIG24 isolated from chicken ileum were clustered together and grouped with L. johnsonii NR_117574.1 (bootstrap value of 99%). The other 22 Lactobacillus strains isolated from quaternary areas of the native chicken intestine were grouped together and formed a monophyletic clade with previously found L. reuteri strains from the Gene bank NR_075036.1 and NR_113820.1 with a bootstrap value of 96%. In the L. reuterie group ABRIG3 and ABRIG12 clustered together with a bootstrap value of 88% and ABRIG2, ABRIG9, ABRIG13, ABRIG15 and ABRIG19 clustered together with a bootstrap value of 57%.

In vitro Assessment of Characteristics of the L. reuteri Isolates for Survival in the Gastrointestinal Tract Bile Salt and Acid Tolerance: Fig. 2 shows the bile salt resistance of the 14 isolated strains of L. reuteri in this study. 0.3% oxgall exerted an inhibitory effect on all of the isolates in comparison with the control treatment (MRS without bile salt). All of the 14 isolated L. reuteri strains exhibited more than 50% tolerance to 0.3% (w/v) bile salt. Maximum

![Phylogenetic tree based on the neighbor-joining method of 16S rRNA gene sequences. The analysis involved 26 sequences of Lactobacillus strains obtained in this study, 8 sequences of Lactobacillus species obtained from the GenBank, and the out group was Lactococcus lactis NR_040955.1. Bootstrap values above 50% are indicated at the nodes of the tree. The scale bar represents 0.02-nucleotide substitutes per position.](image)
resistance was observed with *L. reuteri ABRIG3* at 70.52% resistance. Then, the resistance percentage for *L. reuteri ABRIG23, L. reuteri ABRIG17* and *L. reuteri ABRIG25* was 67.87%, 66.78%, and 66.41%, respectively. *L. reuteri ABRIG15* showed the lowest resistance at 52.96%. The viability of the 14 *L. reuteri* isolates in this study to acidic conditions of pH 2.3, and 3 for 3 h and pH 2.3 for 2 h is shown in Table 2. The survival rate of LAB in low pH is important for understanding their ability to withstand the initial acid stress on the upper parts of the intestine.

The ability to survive in the acidic condition (pH 2.3 and 3) was assessed by measuring reduction in cell viability (log units) after the 2 (pH 2.3) and 3 h (pH 2.3 and 3) incubation time. The results of the acid tolerance of the isolated *L. reuteri* isolates at pH 3 showed that all strains were tolerant to these acidic conditions. But reduction in viable cell number increased with pH reduction. The reduction in cell viability (log units) in pH 3 was less than one unit and was between 0.04 (*L. reuteri ABRIG13, L. reuteri ABRIG22* and *L. reuteri ABRIG17*) and 0.38 (*L. reuteri ABRIG4, L. reuteri ABRIG20*). But isolate viability reduction in pH 2.3 was more than the bacterial cell viability reduction in pH 3 and only 6 isolates could survive in pH 2.3 for 2 h. *L. reuteri ABRIG22, L. reuteri ABRIG3, L. reuteri ABRIG18, L. reuteri ABRIG17, L. reuteri ABRIG8* and *L. reuteri ABRIG9* showed good vitality in pH 2.3 for 2 h. However, among the 14 isolated bacteria, only *L. reuteri ABRIG17* was able to survive in pH 2.3 for 3 h.

**Antimicrobial Activity:** All the isolates inhibited the growth of *S. Enteritidis* and *S. Typhimurium* in different inhibition zone diameters (*Table 3*). The inhibitory zone against *S. Enteritidis* was between 3.66±0.33 mm and 13.33±1.25 mm for *L. reuteri ABRIG22* and *L. reuteri ABRIG19*, respectively. In the case of the *S. Typhimurium* the mean inhibition zone was between 4.7±0.34 mm to 12.67±1.70 mm for *L. reuteri ABRIG9* and *L. reuteri ABRIG20*, respectively. The second experiment was conducted to measure the anti-microbial activity of different amounts of 20-h pHunadjusted cell-free supernatants of the 14 antibiotic-susceptible isolated *L. reuteri* against two food-borne pathogens. The results showed that the *L. reuteri* had different levels of inhibition against *S. Enteritidis* (*Fig. 3*) and *S. Typhimurium* (*Fig. 4*).

The results of the co-incubation assay show that isolated *Lactobacillus* cell-free supernatants better inhibited *S. Enteritidis*. 30 μL of the 5 isolated *L. reuteri (ABRIG3, ABRIG4, ABRIG17, ABRIG18 and ABRIG22)* were able to inhibit this pathogen growth more than 90%. In the case of the *S. Typhimurium*, among the 14 isolated *L. reuteri*, *L. reuteri ABRIG4* and *L. reuteri ABRIG22* best inhibited this pathogenic bacteria. Thirty μL of their supernatant was able to inhibit *S. Typhimurium* more than 90%. However, the isolates *L. reuteri ABRIG9, L. reuteri ABRIG4*

![Fig 2. Growth of Lactobacillus reuteri isolates in MRS broth (control) and MRS broth containing 0.3% bile salt](image1)

![Fig 3. % Salmonella Enteritidis inhibition by different amounts of the Lactobacillus reuteri cell free supernatants after 24 h](image2)
Probiotic Isolation from Avian Gut

and *L. reuteri* ABRIG25 showed a weaker performance in this regard, 50 μL of cell-free supernatant ABRIG25 could inhibit the growth of the *S. Typhimurium* more than 90%. Sixty μL of *L. reuteri* ABRIG9 and *L. reuteri* ABRIG13 cell-free supernatant inhibited growth of *S. Typhimurium* the least (Fig. 4).

### DISCUSSION

From a probiotic point of view, a bacterium with a suitable probiotic potential should be able to survive in a bile-rich condition and the acidic environment of the upper parts of the gastrointestinal tract in the host’s...
The vitality of the isolated L. reuteri of this study in acidic conditions is in accordance with the reported literature. In our study, isolated Lactobacillus showed better stability at pH 3 rather than pH 2.3 and reduction in cell viability was less than 1 log unit in pH 3 for all isolates. Only 6 Lactobacillus reuteri isolates were able to tolerate pH 2.3 for 2 h with a reduction in cell viability between 0.17 to 1.56 log units for L. reuteri ABRIG17 and L. reuteri ABRIG9, respectively. Garriga et al.\textsuperscript{[22]} reported that pH 3 did not exert any negative effect on the survival of isolated bacteria from the chicken gastrointestinal tract. Also Jin et al.\textsuperscript{[23]} reported that chicken intestinal isolated lactobacilli exhibited moderate to good resistance at pH 3 and reduction in survivability in pH lower than 2. Taheri et al.\textsuperscript{[24]} reported that viability of isolated Lactobacillus strains from chicken intestines reduced at pH 2 relative to pH 3. Musikasang et al.\textsuperscript{[25]} reported a decrease of approximately 1-2 log cfu/mL in the lactic acid bacteria viability in pH 2.5.

The acidity of the L. reuteri ABRIG2 (pH=4.02) and L. reuteri ABRIG8 (pH=4.22) was higher than the other isolated L. reuteri ABRIG8 (pH=4) was lower than other isolated L. reuteri. In this study, the pH of the L. reuteri ABRIG13 (pH=4.20), and L. reuteri ABRIG25 (pH=4.22) was higher than the other isolated L. reuteri isolates. Additionally, these two isolates were able to inhibit S. Typhimurium growth more than 90% with higher amounts of supernatant 60 and 50 μL, respectively. But inhibition of S. Enteritidis by cell-free supernatants of these isolates revealed that isolates with a higher pH compared to the other isolates were able to inhibit this pathogen more than 90% with lower amounts of their supernatant, 30 μL.

The results of this in vitro study indicated that the 14 antibiotic susceptible L. reuteri isolates have different abilities to survive in the gastrointestinal tract’s harsh condition. All 14 antibiotic susceptible L. reuteri isolates
showed antagonistic activities against two food borne pathogens. The results of the in vitro co-incubation assay that tested the 14 isolated L. reuteri isolates indicated that the 5 isolates among 6 isolates that were able to withstand stronger acidic conditions were able to inhibit S. Enteritidis and S. Typhimurium more than 90% with less of their supernatants. The isolates L. reuteri ABRIG22, L. reuteri ABRIG3, L. reuteri ABRIG18, L. reuteri ABRIG17, and L. reuteri ABRIG8 showed stronger antagonistic activities against two food borne pathogens and could be considered as good potential probiotic candidates for in vivo studies. These isolates should be further studied for their ability to produce other active and bactericidal compounds.

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