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Research Article

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 ABRIG3, 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*

Kuzey İran Köy Tavuklarında *Salmonella* Enteritidis ve *Salmonella* Typhimurium'a Karşı Biyokontrol Stratejisi Olarak Lactobacilli İzolatlarının Taranması

Öz

Bu çalışmada, İran'daki köy tavuklarının barsaklarından Gram pozitif ve katalaz negatif çomak şekilli bakteriler izole edildi. Asit ve safra toleransı için bir ön taramanın ardından 14 *Lactobacillus reuteri izolatı* antibiyotik dirençli ile *Salmonella* Enteritidis ve *Salmonella* Typhimurium'a karşı antimikrobiyal aktivite için beta hemolizis şekillenmemesine göre seçildi. 14 izolatın tümü 3 saat süreyle pH 3'ü tolere edebildi ve %0.3 safra tuzlarına karşı direnci %50'den fazla idi. Bu 14 izolat içinde, *L. reuteri ABRIG22, L. reuteri ABRIG3, L. reuteri ABRIG18, L. reuteri ABRIG17, L. reuteri ABRIG8* ve *L. reuteri ABRIG9* 2 saat süreyle pH 2.3'e dayanabildi. *L. reuteri ABRIG17* 3 saat süreyle pH 2.3'te hayatta kalabilen tek izolattı. Beş izolat daha sert asidik ortama dayanabildi. Bunlar ayrıca daha az supernatant ile *Salmonella* Enteritidis ve *Salmonella* Typhimurium'u %90'dan daha fazla inhibe edebildi. Bu çalışmanın sonuçları, Kuzay İran'da yerel tavukların barsaklarından izole edilen *L. reuteri*'nin, *S.* Enteritidis ve *S.* Typhimurium enfeksiyonlarında potansiyel antimikrobiyal probiyotikler olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Probiyotik, Lactobacilli, Tarama, Yerel tavuk, 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

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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 antibiotic 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% CO_2 incubator; Binder). After the incubation time, the colonies were picked randomly from the surface of the agar plates and sub-cultured on fresh MRS agar plates for further purification. The isolates were preliminary identified by catalase (3% hydrogen peroxide), Gram stain and KOH (3%) tests. The broth media containing overnight cultures of the Gram positive and catalase negative isolates were stored with skim milk 10% and glycerol 60% (1/1) at -80°C.

Survival and Growth in the Presence of Bile Salts and Low pH: Rapid preliminary screening for the acid and bile tolerance of the lactic acid bacteria was performed on Gram positive and catalase negative isolated bacteria according to the method described by Ehrmann et al.[8] with minor modifications, and bile and acid tolerance assays were conducted according to the method described by Kumar and Kumar [9] and Yamazaki et al.[10], respectively, with some modifications.

Haemolysis: Haemolytic activity of the isolate was evaluated using blood agar (QUELAB), supplemented with 5% v/v of sheep blood, for 48 h incubation (37°C, 5% CO_2). 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 antibiotic 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

evaluated using the agar spot test as described by Toure et al.[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 *S.* Enteritidis and *S.* Typhimurium was assessed on 96-well microtitre plates. It was assessed according to a modified method described by Bian et al.[14] with the absorbance reader (BIO TECK ELx 808) at wavelength 630 nm as the minimum inhibitory concentration on (MIC90). The isolated *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.[15] and used immediately after preparation. Ten µL of the activated culture of *S.* Enteritidis and *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 OD_{630} change in the microtitre plate, in comparison with untreated controls.

The percentage of pathogen inhibition was calculated as:

% inhibition = OD untreated control - OD treatment/ OD untreated control ×100 [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 *Lactobacillus* genus *(Table 1)*. Twenty two isolated isolates from 26 LAB isolates were 99% similar to *L. reuteri* and 4 of them showed 99% similarity to *L. johnsonii*. The 16S rRNA gene sequences of the 26 *Lactobacillus* strains

Table 1. *Identification of the 26 lactic acid bacterial isolates from chicken intestine using 16S rRNA gene sequences*

Isolate	Source of Isolate	Accession Number	The Nearest Matched Species from GenBank	Similarity (%)
ABRIG1	Ileum	MF686461	*L. reuteri*	99
ABRIG2	Ileum	MF686462	*L. reuteri*	99
ABRIG3	Duodenum	MF686463	*L. reuteri*	99
ABRIG4	Duodenum	MF686464	*L. reuteri*	99
ABRIG5	Ileum	MF686465	*L. johnsonii*	99
ABRIG6	Duodenum	MF686466	*L. reuteri*	99
ABRIG7	Ileum	MF686467	*L. johnsonii*	99
ABRIG8	Cecum	MF686468	*L. reuteri*	99
ABRIG9	Ileum	MF686469	*L. reuteri*	99
ABRIG10	Duodenum	MF686470	*L. reuteri*	99
ABRIG11	Jejunum	MF686471	*L. reuteri*	99
ABRIG12	Duodenum	MF686472	*L. reuteri*	99
ABRIG13	Duodenum	MF686473	*L. reuteri*	99
ABRIG14	Ileum	MF686474	*L. johnsonii*	99
ABRIG15	Ileum	MF686475	*L. reuteri*	99
ABRIG16	Jejunum	MF686476	*L. reuteri*	99
ABRIG17	Jejunum	MF686477	*L. reuteri*	99
ABRIG18	Duodenum	MF686478	*L. reuteri*	99
ABRIG19	Ileum	MF686479	*L. reuteri*	99
ABRIG20	Jejunum	MF686480	*L. reuteri*	99
ABRIG21	Jejunum	MF686481	*L. reuteri*	99
ABRIG22	Duodenum	MF686482	*L. reuteri*	99
ABRIG23	Jejunum	MF686483	*L. reuteri*	99
ABRIG24	Ileum	MF686484	*L. johnsonii*	99
ABRIG25	Jejunum	MF686485	*L. reuteri*	99
ABRIG26	Jejunum	MF686486	*L. reuteri*	99

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

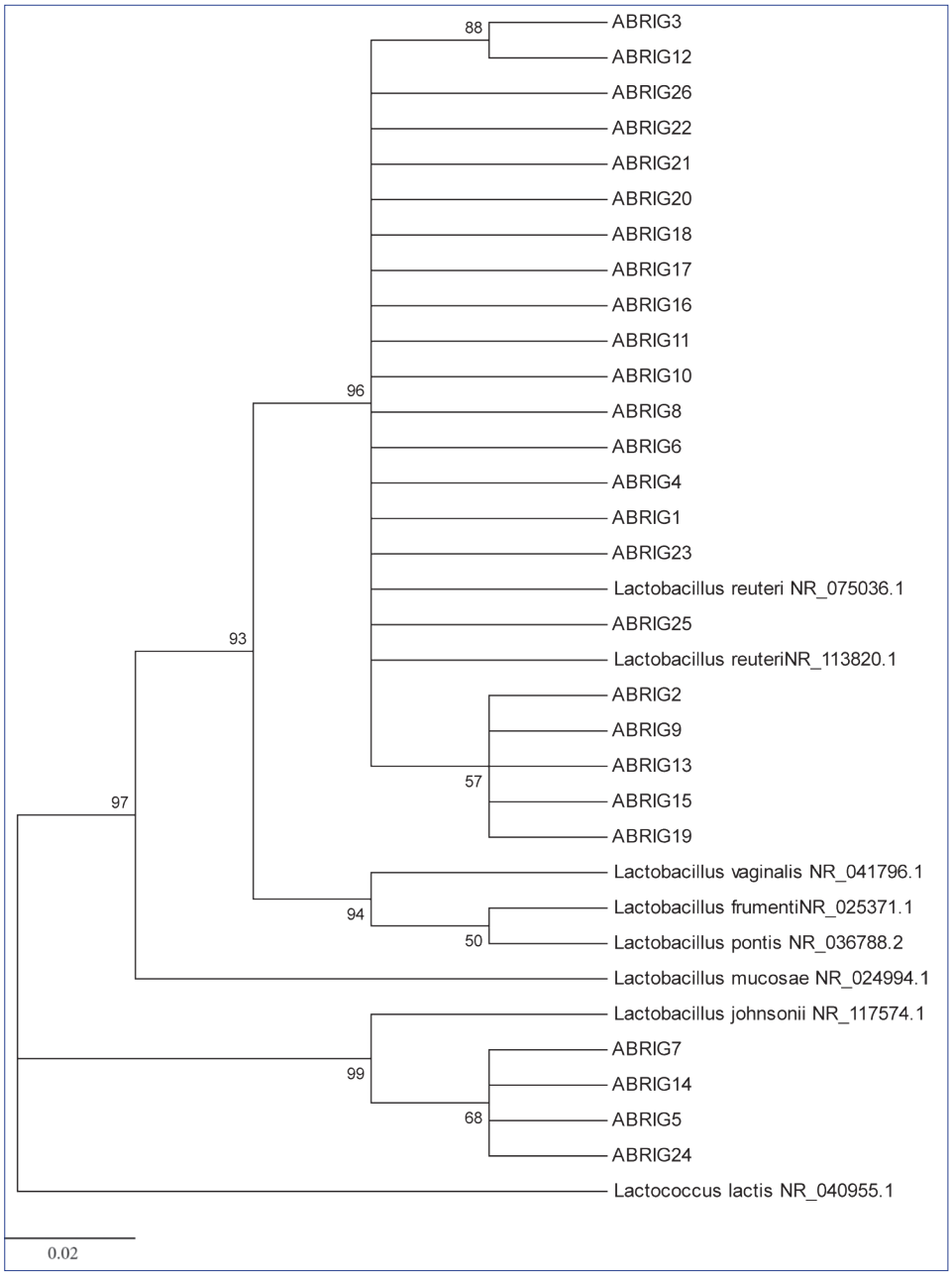

Fig 1. Phylogenetic tree based on the neighbor-joining method of 16S rRNA gene sequences. The analysis involved 26 sequences of *Lactobacillus* strains obtained in this study, 8 sequences of *Lactobacillus* species obtained from the GenBank, and the out group was *Lactococcus lactis* NR_040955.1. Bootstrap values above 50% are indicated at the nodes of the tree. The scale bar represents 0.02-nucleotide substitutes per position

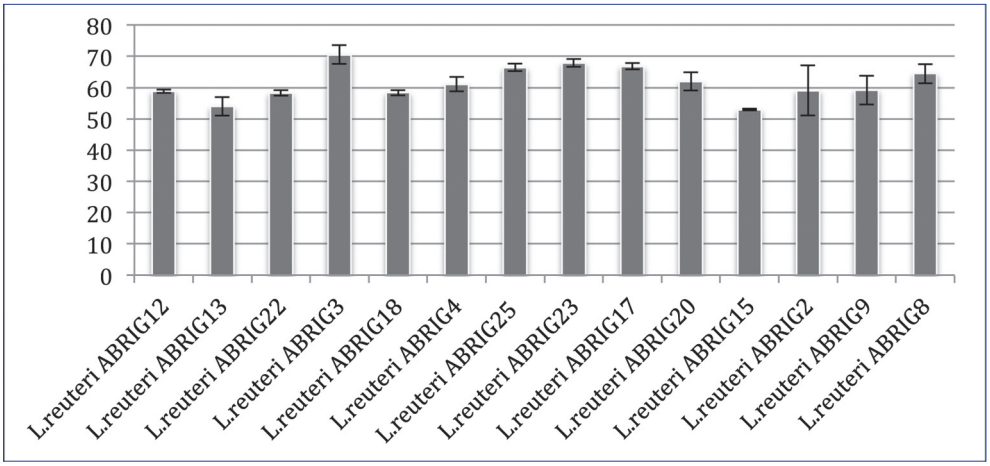

Fig 2. Growth of *Lactobacillus reuteri* isolates in MRS broth (control) and MRS broth containing 0.3% bile salt

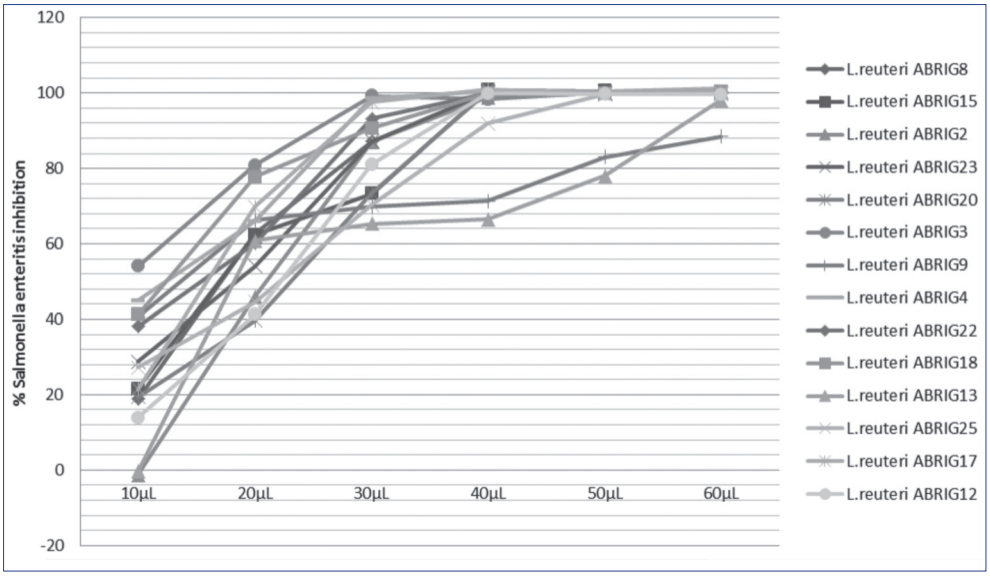

Fig 3. % *Salmonella* Enteritidis inhibition by different amounts of the *Lactobacillus reuterie* cell free supernatants after 24 h

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 survived 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 ABRIG20,* 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. reuterie, L. reuteri ABRIG4* and *L. reuteri ABRIG22* best inhibited this pathogenic bacteria. Thirty µL of their superntatant was able to inhibit *S.* Typhimurium more than 90%. However, the isolates *L. reuteri ABRIG9, L. reuteri ABRIG13*

Table 2. *Viability of Lactobacillus reuteri isolates (log CFU/ml) before and after 3 h exposure to pH 3, 2.5 , 2 and 2 h exposure to pH 2*

Isolate	Cell Viability (log CFU/mL)				pH 3 (3 h)		pH 2.3 (2 h)		pH 2.3 (3 h)	
	Before Exposure to pH 3 and pH 2.3	After Exposure to pH 3 (3 h)	After Exposure to pH 2.3 (2 h)	After Exposure to pH 2.3 (3 h)	Reduction in Cell Viability (log units)	% Viability	Reduction in Cell Viability (log units)	% Viability	Reduction in Cell Viability (log units)	% Viability
L. reuteri ABRIG12	8.68±0.19	8.25±0.32	-	-	0.44	94.24	-	-		-
L. reuteri ABRIG13	8.89±0.05	8.85±0.005	-	-	0.04	99.53	-	-		-
L. reuteri ABRIG22	8.79±0.21	8.75±0.08	8.15±0.02	-	0.04	99.54	0.64	92.71		-
L. reuteri ABRIG3	8.95±0.12	8.90±0.10	7.43±0.09	-	0.06	99.37	1.52	83.01		-
L. reuteri ABRIG18	8.83±0.18	8.61±0.04	7.95±0.04	-	0.23	97.43	0.88	90.03		-
L. reuteri ABRIG4	8.89±0.00	8.51±0.02	-	-	0.38	95.78	-	-		-
L. reuteri ABRIG25	8.81±0.02	8.72±0.01	-	-	0.09	98.96	-	-		-
L. reuteri ABRIG23	8.73±0.120	8.66±0.09	-	-	0.07	99.20	-	-		-
L. reuteri ABRIG17	8.75±0.08	8.71±0.19	8.58±0.09	7.91±.01	0.04	99.58	0.17	98	0.84	90.4
L. reuteri ABRIG20	8.79±0.15	8.45±0.03	-	-	0.34	96.10	-	-	-	-
L. reuteri ABRIG15	8.80±0.152	8.71±0.05	-	-	0.09	98.94	-	-	-	-
L. reuteri ABRIG2	8.90±0.03	8.71±0.05	-	-	0.19	97.90	-	-	-	-
L. reuteri ABRIG9	8.78±0.16	8.73±0.00	7.22±0.22	-	0.05	99.43	1.56	82.23	-	-
L. reuteri ABRIG8	8.97±0.005	8.95±0.07	8.28±0.07	-	0.02	99.77	0.69	92.30	-	-

Values are means ± SD of two independent experiments, each with triplicate

Table 3. *Cell free supernatant pH and antagonistic activity of Lactobacillus reuteri isolates against S. Enteritidis and S. Typhimurium*

Isolate	Test Organism with IZD Mean(mm) ± SD		Cell Free Supernatant pH
	S. Enteritidis	***S.* Typhimurium**	
L. reuteri ABRIG12	12±0.58	12±0.58	4.06
L. reuteri ABRIG13	8.67±0.33	10.00±0.82	4.20
L. reuteri ABRIG22	3.66±0.33	10±0.58	4.06
L. reuteri ABRIG3	7±0.57	7.33±0.47	4.18
L. reuteri ABRIG18	8±1	6.67±0.47	4.10
L. reuteri ABRIG4	11.00±0.82	7.67±0.47	4.03
L. reuteri ABRIG25	9.66±0.34	10±0.58	4.22
L. reuteri ABRIG23	9.33±0.47	10.33±1.20	4.11
L. reuteri ABRIG17	8.66±0.66	9.00±0.58	4.17
L. reuteri ABRIG20	13.33±1.25	12.67±1.70	4.03
L. reuteri ABRIG15	9.00±0.82	8.33±0.94	4.04
L. reuteri ABRIG2	12.67±1.25	9.67±1.25	4.02
L. reuteri ABRIG9	4.33±0.33	4.7±0.34	4.15
L. reuteri ABRIG8	10.00±1.63	9.67±1.70	4.00

Values are means ± SD of two independent experiments, each with triplicate

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

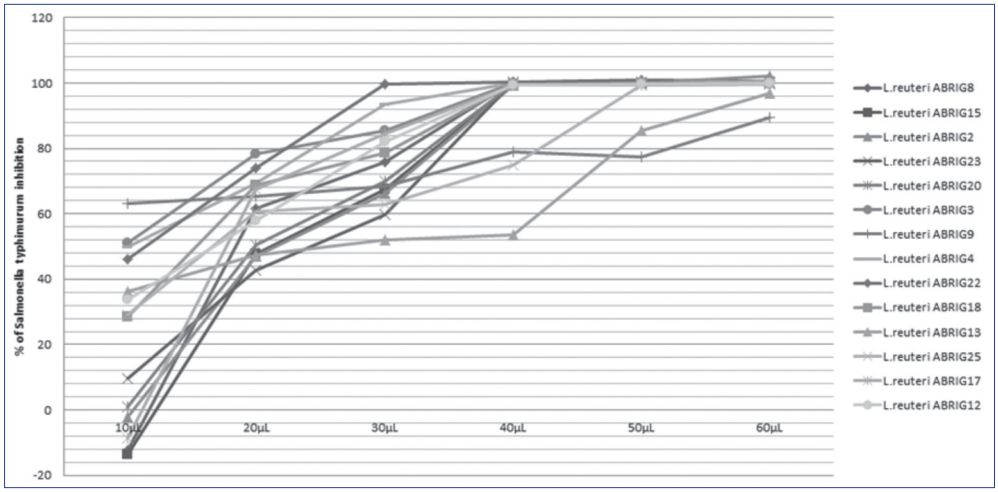

Fig 4. % *Salmonella* Typhimurium inhibition by different amounts of the *Lactobacillus reuterie* cell free supernatants after 24 h

gut [16]. In the present study, as in many previous studies, the ability to tolerate isolated isolates relative to the 0.3% bile salt were measured [17-19]. In our screening study, all of the 14 antibiotic susceptible *L. reuteri* isolates exhibited a tolerance to 0.3% w/v bile salt (oxgall) of more than 50% after 8 h incubation in MRS broth containing oxgall. Gilliland *et al*.[18] found that 0.3% oxgall exerted an inhibitory effect on the chicken intestinal *Lactobacillus*. Taheri *et al*.[20] also found that isolated lactobacilli could withstand bile that had concentrations lower than 0.3% bile salts (0.15 and 0.075%). They concluded bile salts at concentrations 0.3 and 1% (w/v) lead to inhibition of *Lactobacillus* growth. Shokryazdan *et al*.[17] found that among 23 isolated *Lactobacillus* from mulberry silage, only 10 isolates could grow more than 50% in 0.3% bile salts compared to the control. Also Shokryazdan *et al*.[21] reported a reduction in the viability by *L. salivarius* strains isolated from chicken intestines after 3 h exposure to 0.3% bile salts.

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*.[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*.[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*.[20] reported that viability of isolated *Lactobacillus* strains from chicken intestines reduced at pH 2 relative to pH 3. Musikasang *et al*.[24] reported a decrease of approximately 1-2 log cfu/mL in the lactic acid bacteria viability in pH 2.5. Yamazaki *et al*.[10] reported that all *L. salivarius,* and *L. kitasatonis* strains and each of the *L. ingluviei* strains cannot survive in a low pH environment (pH 2) after 1 h of incubation time.

In the present study, all the 14 antibiotic susceptible *L. reuteri* isolates inhibited *S.* Enteritidis, and *S.* Typhimurium growth to different degrees. The result of our study is in agreement with the results of previous studies that have reported that isolated lactobacilli from chicken intestines are capable of inhibiting intestinal pathogenic bacteria growth [10,25]. Yamazaki *et al*.[10] reported isolated *Lactobacillus* strains from layer feces can inhibit *S.* Enteritidis and *S.* Typhimurium in different diameter lengths. In their study, a larger inhibition zone was observed in several *L. salivarius* strains. However, other *Lactobacillus* strains such as *L. reuteri, L. delbruechii,* and *L. intestinalis* produced smaller inhibition zones. Van Coillie *et al*.[26] reported pH reduction resulted in the production of organic acids from lactic acid bacteria. In our study, 14 antibiotic-susceptible *L. reuteri* isolates reduced pH levels of their medium to 4.00-4.22 during the 24 h incubation period, which indicates the production of organic acids from these strains in accordance with previous studies.

The results of our study suggest parts of the antibacterial activity isolated *L. reuteri* had against *S.* Enteritidis and *S.* Typhimurium in the co-incubation assay was due to the production of organic acids, produced by these bacteria. This is indicated by a decrease in the pH of cell-free supernatant from 4.00 *(L. reuteri* ABRIG8) to 4.22 *(L. reuteri* ABRIG25) relative to normal pH of the MRS broth. The acidity of the *L. reuteri* ABRIG2 (pH=4.02) and *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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