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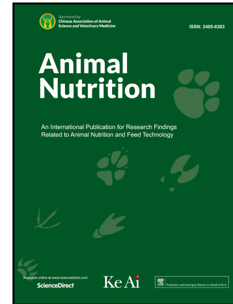

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Screening for Probiotic Characters in Lactobacilli Isolated from Chickens Revealed the Intra-Species Diversity of Lactobacillus brevis

Nasrin Noohi[1], Moslem Papizadeh[2], Mahdi Rohani[2], Malihe Talebi[3]*, Mohammad R. Pourshafie[2]*

1- Research Center for Conservation of Cultural Relics, Research Center of Iranian Cultural Heritage and Tourism Organization, Tehran, Iran

2- Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran

3- Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

Corresponding author's name:

Malihe Talebi: Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran. Phone number: 00982186703193, Email: talebi.m@iums.ac.ir

Mohammad R. Pourshafie: Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran. Phone number: 00982166405535, Email: pour@pasteur.ac.ir.

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2 **species diversity of *Lactobacillus brevis***
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4 Nasrin Noohi [a], Moslem Papizadeh[b], Mahdi Rohani [b], Malihe Talebi[c, *], Mohammad R.
5 Pourshafie[b, *]
6

7 [a] Research Center for Conservation of Cultural Relics, Research Center of Iranian Cultural
8 Heritage and Tourism Organization, Tehran, Iran

9 [b] Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran

10 [c] Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran,
11 Iran
12

13 *Corresponding author.

14 Email address: talebi.m@iums.ac.ir (M. Talebi); pour@pasteur.ac.ir (M. R. Pourshafie)
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16 **Abstract**

17 Considering the importance of the poultry industry and the increasing interest in alternative
18 growth promoters, probiotics are considered as a potential candidate for use in the poultry
19 industry. In this study, *Lactobacillus* species were isolated from 21 rectal swabs of 11 healthy 6-
20 day-old and 10 healthy 21-day-old chickens and their fecal and feed samples. The isolates were
21 characterized and their probiotic characteristics, including resistance to gastric acid and bile salts,
22 biofilm formation and adherence to epithelium or mucus, amylase and protease activity and
23 production of inhibitory compounds, were assessed. From 31 acid and bile resistant lactobacilli,
24 only 2 *Lactobacillus brevis* and 1 *Lactobacillus reuteri* strains showed significant probiotic
25 properties. These isolates indicated detectable attachment to Caco-2 cells and significant
26 antibacterial activities against Gram-positive and Gram-negative pathogens. Additionally,
27 phenotypic and genotypic diversity of lactobacilli isolates were studied by Phene Plate (PhP)
28 system (PhP-LB) and random amplified polymorphic DNA (RAPD)-PCR, respectively. PhP-LB
29 results of 24 *L. brevis* isolates showed a high phenotypic variation among the isolates. In
30 comparison, results of RAPD-PCR highlighted a low diversity. Therefore, it seems that
31 combination of the 2 techniques (PhP and RAPD-PCR) could result in a significant
32 discriminatory power than each of them used alone.

33

34 **Keywords**: Probiotic; Lactobacilli; Poultry; Feed; Fecal

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36 ## 1. Introduction

37 The Iranian poultry industry is the largest in the Middle East with nearly 1.2 million tons of
38 output (meat and eggs). Therefore, this industry has a special status in the Iranian industry
39 (Shariatmadari, 2000). The significant concerns related to this industry are health issues that
40 threaten not only animal production, but also the people using their products. (Griggs and Jacob,
41 2005; Nava et al., 2005; Zhang et al., 2018). Gastrointestinal diseases are one of the most
42 important threats, as they lead to lost productivity, increased mortality, and contamination of
43 poultry products for human consumption (Patterson and Burkholder, 2003).

44 The balance among the gastrointestinal microbiota plays a significant role in maintaining the
45 normal physiology of host animals. Gastrointestinal microbiota help direct the normal formation
46 or development of gut structure and morphology, support immune responses, offer protection
47 from intestinal pathogens, and play an active role in the digestion of nutrients (Slizewska, 2020;
48 Rodrigues, 2020). In the past, using antibiotics to promote the growth of animals and manage gut
49 microbiota was a norm. Feeding of antibiotics to food animals has been recognized as one
50 leading cause of the spread of antimicrobial resistance in human populations. The gradual
51 emergence of populations of antibiotic resistant bacteria has become a major public health
52 problem of global proportions. Due to this concern, since 2006 the European Union banned the
53 use of antibiotics as growth promoters in animal feed (de Souza, 2018). Therefore, several
54 alternative strategies have been proposed with some success that mimic the functions of
55 antibiotics. Probiotics have been widely studied because of their ability to modulate gut
56 microbiota and immunological systems in both humans and livestock. They have been used to
57 increase milk production and to reduce diarrhoea both in cattle and pigs, and to control the
58 colonisation of the intestinal tract by pathogenic bacteria (Alayande, 2020).

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59 The microbes which are suitable for probiotic purposes in human and animals are mainly
60 members of a metabolically defined group of Gram-positive bacteria, known as lactic acid
61 bacteria (LAB) (Naidu et al., 1999). These microbes are widely distributed in the environment
62 and play a significant role in the gastrointestinal tract of a diverse array of animals (Bermudez-
63 Brito et al., 2012; Butel, 2014). A main part of the candidate strains, which have been introduced
64 for probiotic purposes, fall into the genus *Lactobacillus* which is a major genus of LAB and
65 harbor more than 200 species (Goktepe et al., 2005). *Lactobacillus* species, with a record of safe
66 use as probiotics in humans and animals, are among the common inhabitants of the broiler
67 gastrointestinal tract (Lu et al., 2003). In poultry, administration of the probiotic *Lactobacillus*
68 strains improves not only the feed digestion, but also the nutrient uptake. In addition, probiotics
69 increase the growth performance, neutralizing various enterotoxins and enhancing immune
70 responses (Ghadban, 2002; Al-Khalaifa, 2019). Additionally, probiotics reduce the risk of
71 gastrointestinal colonization by foodborne pathogens, such as *Campylobacter* (Ghareeb et al.,
72 2012; Khan et al., 2019; Neal-McKinney et al., 2012), *Clostridium* (Li, 2017) and *Salmonella*
73 (Kizerwetter-Swida and Binek, 2009; Tellez et al., 2012), and increase the safety of poultry-
74 based foods (Gaggìa et al., 2010). Such antagonistic activities against the pathogens is highly
75 linked to lactic acid produced by *Lactobacillus strains,* which can be toxic for many bacteria, can
76 compete for nutrients, and affect cell attachment capabilities of the beneficial microbes to the
77 intestinal epithelium (Patterson and Burkholder, 2003).

78 Despite the importance of the poultry industry in Iran, little attention has been given to the
79 isolation of probiotic bacteria and determination of their biological activities in this country. In
80 this study, we aimed to isolate and characterize *Lactobacillus* species from indigenous poultry
81 farms with a special focus on their probiotic properties.

82

83 ## 2. Materials and methods

84 ### 2.1 Isolation of lactic acid bacteria

85 A total of 21 rectal swabs of 11 healthy 6-day-old and 10 healthy 21-day-old chickens, their fecal
86 and their feed samples, were collected from 2 poultry farms near Tehran, Iran. The chicken breed
87 was Ross and diets were standard according to the breed requirements, containing maize and
88 soybean balanced with minerals, vitamins, and amino acids without growth promoting
89 antibiotics. To isolate LAB from fecal samples, 6 chicken feces from 2 poultry farms (each
90 sample was 1 g) were collected randomly in Man Rogosa and Sharpe (MRS) broth (Merck,
91 Germany). The samples were serially diluted in phosphate-buffered saline (PBS) pH 7.4 and
92 aliquots of them were plated on MRS agar medium in 8-cm plates. The plates were incubated for
93 48 h at 37 °C in microaerobic conditions. From each sample, different morpho-type colonies
94 were selected for further purification and then the LAB isolates were cryopreserved at -80 °C in
95 MRS broth containing 20% glycerol under defined designations.

96 ### 2.2 Resistance to low pH and bile salts

97 Tolerance of isolates to low acidity and bile salts were determined in triplicate experiments as
98 described by Cano Roca et al (2014). Briefly, exponentially growing cells in MRS broth were
99 washed by centrifugation (4,000 × *g* at 25 °C for 10 min) and re-suspended in PBS. After serial
100 dilutions, an initial dilution of the bacterial suspension was prepared for plating on MRS agar. To
101 investigate the reaction of the isolates to low pH values, 100 µL of the cell suspension (10^8
102 CFU/mL) was added to 900 µL of sterile PBS (pH = 3) in a 1.5-mL microtube. The endurable
103 cell counts were measured after 3 h of incubation at 37 °C. A similar procedure was performed
104 using PBS (pH = 7) as a control. To determine the tolerance of isolates to bile salts, 50 µL of

105 bacterial suspension was added into tubes containing 4,950 µL of MRS broth (Merck, Germany)
106 with 0.4% (wt/vol) of bile salts (Merck, Germany) and incubate at 37 °C for 6 h.
107 The harvested cells from the acid in both bile salt stress experiments were washed in PBS (pH =
108 7.4) and cultured on MRS agar and finally counting was performed. Based on 2, 2–4, 4–6 and >
109 6 log reduction in comparison to the initial suspension after 3 and 6 h of incubation in acid and
110 bile salts, isolates were grouped as strongly resistant, resistant, intermediate and susceptible,
111 respectively.

112 **2.3 Phenotypic classification**

113 All of the acid-bile resistant isolates were subjected to a biochemical fingerprinting with the
114 PhPlate system according to the manufacturer's instructions (PhPlate Micro-plate Techniques
115 AB, Stockholm, Sweden) which was modified for isolates typing (PhP-LB). The microplates
116 contained 4 sets of dehydrated reagents (23 different sugars including arabinose, xylose,
117 galactose, maltose, cellobiose, trehalose, palatinose, sucrose, lactose, melibiose, manose,
118 melezitose, inosin, mannitol, arbutin, sorbitool, gallac, sorbose, rhamnose, taghatose, amigdalin,
119 gluconate, salicin), which have been specifically selected for phenotypic typing of *Lactobacillus*
120 species. After the incubation of PhP-LB plates at 37 °C, the utilization of the substrates in each
121 well was measured by scanning the images after 24, 48, and 72 h. Scanned images were analyzed
122 by software package PhPWIN (PhPlate micro-plate techniques AB, Sweden). The mean
123 similarity between duplicate assays of all strains ± 2 SD was calculated as the identification level
124 (ID), which was 0.975 and strains with > 0.975 similarities were grouped into the same Phene
125 Plate (PhP) type.

126 **2.4 Molecular identification**

127 Total DNA of the acid-bile resistant isolates was extracted using a peqGOLD Bacterial DNA Kit
128 (peQlab, Germany) according to the manufacturer's instruction. Preliminary characterization of
129 lactobacilli was performed based on the phenotype. Then, molecular identification of the
130 *Lactobacillus* spp. was performed using primers which were specific for amplification of a 247
131 bp region of the 16S rRNA gene in the genus *Lactobacillus* (McOrist et al., 2002). The PCR
132 amplification program was as follows: a single initial denaturation cycle (5 min at 94 °C)
133 followed by 30 cycles (30 s at 94 °C [denaturation], 30 s at 57 °C [annealing], and 30 s at 72 °C
134 [elongation]), with a final extension of 7 min at 72 °C. As the next step, multiplex PCR
135 amplifications were used for *Lactobacillus* species identification. Hence, the previously designed
136 species specific primer pairs which were already confirmed for detection of *Lactobacillus*
137 *acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus*
138 *rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* were used to identify the
139 *Lactobacillus* isolates (Kwon et al., 2004). Multiplex PCR reactions for amplifications entailed a
140 cycle of 94 °C for 5 min, followed by 40 cycles (94 °C for 30 s, 51 °C for 40 s and 72 °C for 30
141 s), plus one additional cycle with a final 7 min chain elongation at 72 °C. The amplified genomic
142 regions of *Lactobacillus brevis* strains were sequenced. Additionally, species specific primers
143 were used for identification of *L. brevis* strains as described by Guarneri et al (2001).The
144 thermo-cycling conditions for the *L. brevis* specific PCR reaction was as follows: after one cycle
145 at 94 °C for 2 min, 25 cycles of 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1 min, followed
146 by an additional 10 min cycle of extension (Table 1).

147 **2.5 RAPD-PCR genotypic classification**

148 Acid-bile resistant isolates, which were identified as lactobacilli, were subjected to
149 Random amplified polymorphic DNA (RAPD)-PCR using a previously designed oligonucleotide

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150 by Tilsala et al (1998) with some modifications. The PCR amplification conditions were as
151 follows: 2 min at 94 °C for initial denaturation followed by 40 cycles of 30 s at 94 °C for
152 denaturation, 30 s at 37 °C for annealing, and 2 min at 72 °C for elongation. The final extension
153 at 72 °C was prolonged to 10 min. PCR reaction was performed for each primer in a separate
154 tube and run in the same well in 1.5% agarose gel to increase the discrimination. The UPGMA
155 method using the software Gel compare II version 4.0 was used to compare banding patterns
156 (Applied Maths, Sint-Martens-Latem, Belgium).

157 **2.6 Biofilm assay**

158 Biofilm formation of lactobacilli was studied as previously described by Lebeer et al (2007)
159 with minor modifications. For each strain, 200 µL aliquots of a modified tryptic soy broth (TSB)
160 medium (15 g/L TSB enriched with 20 g/L Bacto proteose peptone), which was already
161 inoculated by approximately 3×10^7 CFU of a *Lactobacillus* isolate, were added into 96-well
162 plates (8 wells for each strain) and incubated at 37 °C. After 72 h of incubation, the wells were
163 washed with PBS and stained for 30 min with 200 µL crystal violet (0.1%) in an isopropanol-
164 methanol-PBS solution (1:1:18). After washing with double distilled water, the wells were air-
165 dried for 30 min at room temperature. Extraction of the dye bound to the adherent cells was done
166 with 200 µL ethanol-acetone (80:20) solution. The optical density of 135 µL of each well was
167 measured at 570 nm. Data were normalized to the indicated positive control, which was taken as
168 100% to compare different experiments. The results are presented as means ± SD. Additionally,
169 the sterile medium and *Pseudomonas aeruginosa* were used as negative and positive controls,
170 respectively.

171 **2.7 Attachment to Caco-2 cells**

172 Detection of adhesion ability in lactobacilli isolates was performed according to Jacobsen et al
173 (1999). A monolayer of Caco-2 cells was cultured in Roswell Park Memorial Institute (RPMI)
174 medium (Gibco, Carlsbad, CA, USA), supplemented with 20% (vol/vol) fetal calf serum (Gibco,
175 Life Technology, USA), penicillin (100U/mL) and streptomycin (100 mg/mL), and incubated at
176 37 °C in 5% CO_2 atmosphere. At first, 3 mL of Caco-2 cells containing 1.5×10^5 cells/mL were
177 seeded on a 6-well cell culture plate and after confluency, the cells were washed twice with 3 mL
178 PBS. After adding 2 mL of RPMI (without antibiotics) to each well, the plates were incubated
179 for 3h at 37 °C. Overnight cultures of the isolates (cell concentration of approximately 10^9
180 CFU/mL) were suspended in 1 mL RPMI1640 medium (without antibiotics) and added to
181 different wells and incubated for 1 h at 37 °C. The wells were washed 4 times with PBS to
182 remove the unbound bacteria. Then, the cells were fixed with 3 mL of methanol and incubated
183 for 5 to 10 min at room temperature for the removal of methanol. Staining was made with 3 mL
184 of Giemsa stain solution (1:20) (Sigma-Aldrich Co., Mo, USA) and incubated for 30 min at room
185 temperature. After washing the plates with distilled water, the air-dried plates were examined
186 microscopically under oil immersion. Adherent isolates were counted in 20 random microscopic
187 fields. Cells showing < 40, between 40 and100, and > 100 attached bacteria were regarded as
188 non-adhesive, adhesive, and strongly adhesive, respectively.

189 **2.8 Detection of amylase and protease activities**

190 Enzymatic activity of 31 acid-bile resistant lactobacilli was determined according to the method
191 described by Taheri et al (2009) with minor modifications. For assessment of the amylase
192 activity, the selected *Lactobacillus* strains were cultured on modified MRS broth described by
193 Taheri et al (2009) (0.25% starch instead of glucose), and inoculated on a medium containing
194 starch (2%), meat peptone (0.5%), yeast extract (0.7%), NaCl (0.2%), and agar (1.5%). After 48

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195 h of incubation at 37 °C, lugol's solution (5 g iodine [Merck, Germany] and 10 g potassium
196 iodide [KI] [Merck, Germany] in 100 mL distilled water) was poured over the agar for detection
197 of any clear zones as indicative of amylolytic activities.
198 For detection of proteolytic activity, *Lactobacillus* strains were inoculated into MRS broth and
199 were incubated at 37 °C for 24 h. Bacterial suspension (30 µL) was moved onto a disc placed
200 over a MRS agar containing 1% skim milk. Finally, the halo zone surrounding each disc was
201 measured.

202 **2.9 Antimicrobial activity**

203 The antimicrobial activity of the lactobacilli, which showed a detectable attachment to Caco-2
204 cells, was studied against *Shigella soneii* (ATCC 12022), *Pesudomonas aeruginosa* (ATCC
205 27853), *Salmonella typhi* (ATCC 19430), *Proteus mirabilis* (ATCC 25933), *Yersinia*
206 *enterocolitica* (ATCC 23715), *Streptococcus agalactiea* (ATCC 12386), *Listeria*
207 *monocytogenes* (ATCC 19113), wild types of *Escherichia coli* strains belonging to 3 pathotypes
208 i.e. enteropathogenic *E. coli* (EPEC) (ATCC 43887), enterotoxigenic *E. coli* (ETEC) and
209 enteroaggregative *E. coli* (EAEC). These strains were provided by the Microbial Collection of
210 Iran (Davoodabadi et al., 2015; Shahrokhi et al., 2011). The antimicrobial activity was observed
211 based on the well diffusion method as described by Fernandez et al (2003). Suspensions
212 containing approximately10^8 CFU/mL of the abovementioned pathogens were poured on Muller
213 Hinton agar medium in 8-cm plates, except for *L. monocytogenes* where brain heart infusion
214 (BHI) agar medium was used. Then, 100 µL of an overnight culture of the selected *Lactobacillus*
215 strains in MRS broth was poured into 6 mm agar wells created by punching in 8-cm plates. After
216 24 h of incubation at 37 °C, the antimicrobial activity was measured as the zone of growth
217 inhibition around the wells. The inhibition zones of 1, 2, 2 to 5 mm, and more than 5 mm were

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218 classified as strains of no (-), mild (+), strong (++), and very strong (+++) inhibition,
219 respectively.

220 **2.10 Antibiotic susceptibility testing**

221 The susceptibility of isolates to different antibiotics including penicillin G (10 µg), gentamicin
222 (120 µg), erythromycin (15 µg), tetracycline (30 µg), amoxicillin (25 µg), ciprofloxacin (5 µg),
223 chloramphenicol (30 µg), oxacillin (1 µg), and streptomycin (10 µg) (MAST Diagnostics, U.K.)
224 was determined by the agar disc diffusion method on MRS agar plates instead of the Muller
225 Hinton agar. Lactic acid bacteria require special growth conditions and conventional media, as
226 Mueller Hinton agar are not uniformly suitable for to susceptibility test them (Klare et al., 2005).
227 After incubation of plates at 37 °C for 48 h, the diameter of the inhibition zones was measured
228 and the results were expressed as sensitive or resistant according to CLSI standard (Institute,
229 2009).

230 **2.11 Plasmid profiles**

231 The isolation of plasmid DNA from the selected bacterial strains was performed by GF-1
232 plasmid DNA extraction kit (Vivantis, Malaysia). *E. coli* V517 was used as a positive control.
233 Electrophoresis of the extracted plasmids was performed in a 1% agarose gel and the plasmids
234 were visualized with UV trans-illumination in a Gel Doc apparatus.

235 **3. Results**

236 **3.1 Isolation and identification**

237 A total of 168 LAB were isolated from rectal swabs, fecal and feed samples, among which 89
238 (53%) and 79 (47%) isolates were from 21 days and 6 days old chickens, respectively.
239 Furthermore, out of 168 LAB isolates, 51 (30.3%) isolates were resistant to low pH (pH 3.00)

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240 and bile salts (0.4%), among which 20 and 31 isolates were identified as members of the genera
241 *Pediococcus* and *Lactobacillus*, respectively.
242 The results of the molecular identification showed that 31 isolates belonged to the genus
243 *Lactobacillus*. Out of these, 24 (77.4%), 3 (9.6%), 2 (6.4%), and 2 (6.4%) were identified as *L.*
244 *brevis*, *L. plantarum, L. reuteri* and *L. vaginalis*, respectively (Table 2). Interestingly, a
245 considerable part of the isolates which originated from the rectal-swabs were identified as *L.*
246 *brevis*. The same results were observed in fecal samples.

247 **3.2 Phenotypic and genotypic classification**

248 Acid-bile resistant lactobacilli were typed using Phene Plate system (PhP-LB) followed by
249 genotype-based identification using RAPD-PCR. PhP-LB results of 24 *L. brevis* isolates showed
250 a high phenotypic diversity and most of the isolates gave unique phenotypes, denoted as single
251 types (3 common types with 10 isolates and 14 single types) (Fig. 1). On the other hand, the
252 results of RAPD-PCR showed a low genetic diversity in *L. brevis* species (3 common types with
253 20 isolates and 4 single types) (Fig. 2).

254 **3.3 Biofilm and Attachment to Caco-2 cells assay**

255 Out of the 31 acid-bile resistant lactobacilli strains examined in this study, *L. brevis* L2, L6, L31,
256 and *L. reuteri* L26 had the biofilm formation capability. Adhesion to Caco-2 cells showed that 22
257 (71%) isolates were non-adhesive (with less than 40 bacteria attached in 20 microscopic fields),
258 7 (22.5%) isolates (*L. brevis* L3, L29, L30, L31, *L. reuteri* L26, L27, and *L. vaginalis* L25) were
259 adhesive (with 41 to 100 bacteria) and 2 (6.4%) isolates (*L. brevis* L2 and L6) were strongly
260 adhesive (>101 bacteria) (Fig. 3).

261 **3.4 Antibacterial activity**

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262 The assay of antagonistic activity of lactobacilli with an acceptable ability of attachment to
263 Caco-2 cells showed that *L. brevis* strains L2, L6, L31 and *L. reuteri* strains L26 and L27 have a
264 strong inhibitory effect against different serotypes of *E. coli* (EAEC, EPEC, and ETEC) (Table
265 3). *L. brevis* strains L2 and L6 had antibacterial activities against all the tested pathogenic
266 bacteria with the exception of *L. monocytogenes* and *Staphylococcus aureus.*

267 **3.5 Detection of amylase and protease activities**

268 Measuring the size of the halo zones surrounding the colonies as an indicative of extracellular
269 enzyme level showed that all 31 lactobacilli isolates investigated here were protease positive but
270 extracellular-amylase negative.

271 **3.6 Plasmid profiles and antibiotic susceptibility testing**

272 No plasmid was found in *L. brevis* strain L6 but *L. brevis* L2 and *L. reuteri* L26 harbored a single
273 plasmid. Antibiotic susceptibility tests showed that the *Lactobacillus* isolates were sensitive to
274 augmentin, amoxicillin, erythromycin, penicillin G, chloramphenicol, and rifampin and were
275 resistant to ciprofloxacin, amikacin, tobramycin, oxacillin and streptomycin. *L. reuteri* L26 was
276 sensitive to tetracycline but *L. brevis* L2 and L6 were resistant to this antibiotic.

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277 **4. Discussion**

278 Probiotics have been emerging as a safe alternative to antibiotics for increasing performance in
279 livestock. Administration of probiotic strains may have a significant effect on absorption and
280 utilization of feed, and increase the body weight of various animals, including chicken, piglets,
281 sheep and cattle (Markowiak et al, 2018). Lactobacillus species with a record of safe use as a
282 probiotic in humans and animals is regarded as a significant part of chicken-GIT
283 (Gastrointestinal Tract) microbiota. (Wei et al., 2013; Yadav, 2019). Among such a diverse array
284 of *Lactobacillus* species, some defined species have been frequently reported as chicken-GIT

285 inhabitants. *L. reuteri*, *L. salivarius* and *L. johnsonii* are among the most detected lactobacilli in
286 chicken-GIT samples (Adhikari and Kwon, 2017; Dec et al., 2016; Pan and Yu, 2014).
287 Interestingly, *L. reuteri*, *L. salivarius* and *L. johnsonii* have been repeatedly isolated from the
288 GIT samples of a wide range of hosts and this harsh environment is among their preferred
289 ecological niches (Lebeer et al., 2008; Walter, 2008; Pokorná, 2019). However, *L. brevis* have
290 also been found in GIT samples in a diverse range of warm-blooded animals (Feyereisen, 2019,
291 Fraunhofer, 2018). Considering the results of this study, 3 lactobacilli strains were isolated,
292 which showed promising probiotic characteristics. Those strains fall into *L. brevis* (L2 and L6)
293 and *L. reuteri* (L26) species. *L. brevis* shows a significant prevalence in the GIT of chickens
294 although the GIT is not the preferred ecological niche for this species. The species *L. brevis* falls
295 into a *Lactobacillus* phylogenetic group which harbors mostly foodborne species (Papizadeh et
296 al., 2017). Strains of this species have been isolated from a diverse array of samples, including
297 water, feces of various animals, and various food-associated samples (Feyereisen, 2019). Hence,
298 the findings of this study shed more light on the ecological distribution of *L. brevis*.
299 Considering the results, *L. brevis* species were isolated from the rectal swabs of both 21- and 6-
300 day-old chickens and also from their feed and fecal samples. Hence, it can be inferred that this
301 species has the ability to survive on a wide range of substrates (Ramos et al., 2013).
302 Phenotypic characterization of the isolates indicated a high intra-species diversity among *L.*
303 *brevis* isolates. In comparison, results of RAPD-PCR highlighted a low diversity. Therefore, it
304 seems that combination of 2 techniques (PhPlate and RAPD-PCR) could result in a more
305 significant discriminatory power than each of them used alone. In this study, biochemical
306 fingerprinting of lactobacilli was used primarily for the screening of biodiversity in lactobacilli
307 strains to reduce their number for the next tests; a significant number of single types indicated

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308 that PhP system alone cannot serve as a method for determining relationships between
309 *Lactobacillus* strains (Skelin et al., 2012).
310 Adhesion to mucosal surfaces has been used as a criterion for the selection of probiotic bacteria
311 because this character has a major role in the colonization of the gastrointestinal tract (GIT) by
312 these bacteria (Broderick and Duong, 2016; Kosin and Rakshit, 2006). Additionally, mucosal
313 adhesion is important for pathogenic antagonism, modulation of the immune system and healing
314 of damaged gastric mucosa (Oelschlaeger, 2010; Ohland and MacNaughton, 2010; Monteagudo-
315 Mera, 2019). In this study, 2 strongly adhesive strains (*L. brevis* strains L2 and L6) showed the
316 highest biofilm formation capacity. Furthermore, we observed specific correlation between
317 adhesion to Caco-2 cells and biofilm formation by lactobacilli isolates.
318 Antimicrobial resistance poses a serious global threat of growing concern to human, animal and
319 environmental health. This is due to the consumption of antibiotics in animals (raised for food or
320 kept as pets) and humans (Aslam et al., 2018). Therefore, probiotics with antibacterial activity
321 against pathogens are a promising alternative to antibiotics (Baldwin et al., 2018). The strong
322 antibacterial activity of *L. brevis* strains L2, L6, L31 and *L. reuteri* strains L26 and L27 against
323 various serotypes of *E. coli* (EAEC, EPEC and ETEC), which is shown in Table 3, have
324 highlighted the probiotic capabilities of these strains. Interestingly, such a capability among the
325 isolates of this study was highly detectable in cases of *L. brevis* strains L2 and L6, which showed
326 significant antibacterial activities against all the tested pathogenic bacteria (except for *L.*
327 *monocytogenes* and *S. aureus*). Such an antibacterial activity of *Lactobacillus* isolates is
328 essentially associated with the production of bacteriocins, H_2O_2, lactic acid and other metabolites
329 which inhibit the growth of pathogens (Vieco Saiz, 2019). Considering the fact that various
330 serotypes of *E. coli* (EAEC, EPEC, and ETEC) are considered as the most important cause of

331 enteric bacterial infections in poultry, the use of such isolates with functional probiotic
332 competence can significantly reduce the infection rate. Another finding was that all 31
333 lactobacilli isolates investigated in this study, were protease positive with no extracellular
334 amylase activity and this has shed light on the importance of these isolates since amylase, lipase,
335 and protease enzymes play very important roles in the digestion of nutrient materials.
336 The probable existence of transferable resistant genes in the 3 probiotic *Lactobacillus* strains was
337 observed by the determination of antibiotic resistance patterns and plasmid profiling, but no
338 plasmid was detected in *L. brevis* strains L2 and L6 and only a single plasmid was detected in *L.*
339 *reuteri* strain L26. Furthermore, *L. reuteri* L26 was sensitive to tetracycline, but *L. brevis* L2 and
340 L6 were resistant to this antibiotic. The properties of antibiotic resistance in various
341 Lactobacillus species seem to be associated with drug resistant genes which are mainly located
342 on the chromosome.
343 According to the criteria, the potential probiotic strains, which are assumed for animal or human
344 applications, have to be non-pathogenic and from the same origin (host). Additionally, such
345 strains should resist intestinal tract, gastric and bile acids, adhere to the epithelium or mucus, and
346 produce inhibitory compounds. Among the lactobacilli isolated in this study, we found 3
347 lactobacilli strains with probiotic characteristics, L. brevis (L2 and L6) and L. reuteri (L26),
348 which could be considered probiotic strains for use in the poultry industry.

349 **5. Conclusion**

350 In this study, the most common acid and bile resistant lactobacilli strains isolated from chickens
351 belonged to the *L. brevis* species, with a high intra-species phenotypic diversity. In vitro
352 evaluation in this study showed that 4 Lactobacillus strains (3 *L. brevis* strains and 1 *L. reuteri*
353 strain) could be considered as probiotic. Further in vivo evaluation for determination of the

354 beneficial effects of our isolates in natural conditions could be highly advantageous to the Iranian
355 poultry industry.

356 **Author contributions**

357 Nasrin Noohi: writing - original draft, visualization, investigation, formal analysis; Moslem
358 Papizadeh: writing - review & editing; Mahdi Rohani: investigation; Malihe Talebi: project
359 administration, methodology; Mohammad R. Pourshafie: conceptualiza, supervision.

360 **Conflict of interest**

361 The authors declare that there is no conflict of interest.

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524 **Figures and tables**

525

526 Table 1. Sequence of primers used to identify different species of *Lactobacillus* bacteria.

527

Target bacteria	Sequence (5' to 3')	References
All *Lactobacillus*	5´ TGGAAACAGGTGCTAATACCG 3´ 5´ CCATTGTGGAAGATTCCC 3´	McOrist et al., 2002
L. casei-group *L. acidophilus* *L. delbrueckii* *L. gasseri* *L. reuteri* *L. plantarum* *L. rhamnosus*	5' CCACCTTCCTCCGGTTTGTCA 3' 5' AGGGTGAAGTCGTAACAAGTAGCC 3' 5' TGGTCGGCAGAGTAACTGTTGTCG 3' 5' AACTATCGCTTACGCTACCACTTTGC 3' 5' CTGTGCTACACCTAGAGATAGGTGG 3' 5' ATTTCAAGTTGAGTCTCTCTCTC 3' 5' ACCTGATTGACGATGGATCACCAGT 3' 5' CTAGTGGTAACAGTTGATTAAAACTGC 3' 5' GCCAACAAGCTATGTGTTCGCTTGC 3'	Kwon et al., 2004
L. brevis	5' CTTGCACTGATTTTAACA 3' 5' GGGCGGTGTGTACAAGGC 3'	Guarneri et al., 2001

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530 Table 2. Determination of acid and bile resistant *Lactobacillus* species isolated from different
531 samples.
532
533

Item	*L. brevis*		*L. plantarum*		*L. reuteri*		*L. vaginalis*	
	21-day-old	6-day-old	21-day-old	6-day old	21-day-old	6-day-old	21-day-old	6-day-old
Feed sample	0	1	3	0	0	0	0	0
Rectal swab	14	6	0	0	1	1	0	2
Fecal sample	3	0	0	0	0	0	0	0

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534
535 Table 3. Spectrum of antimicrobial activity exhibited by Lactobacillus strains L2, L3, L6, L25, L26, L 27, L 29, L 30 and L 31.

Item	L2 (*L. brevis*)	L3 (*L. brevis*)	L6 (*L. brevis*)	L25 (*L. vaginalis*)	L26 (*L. reuteri*)	L27 (*L. reuteri*)	L29 (*L. brevis*)	L30 (*L. brevis*)	L31 (*L. brevis*)
ETEC	+	-	+	+	+	+	-	-	+
EPEC	++	-	++	-	++	++	-	-	++
EAEC	++	-	++	-	+	+	-	-	++
Salmonella entritidis	++	-	++	+	+	+	-	-	+
Salmonella typhi	+	-	+	-	-	-	-	-	-
Pseudomonas aeruginosa	+	-	+	-	-	-	-	+	-
Shigella flexneri	+	-	+	-	+	+	-	-	-
Klebsiella pneumonia	+	-	+	-	-	-	-	-	-
Citrobacter freundii	+	-	+	-	-	-	+	+	-
Proteus mirabilis	+	-	+	-	-	-	+	+	-
Yersinia. enterocolitica	+	-	+	-	-	-	-	-	-
Listeria monocytogenes	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-
Staphylococcus saprophyticus	+	-	+	-	-	-	-	-	-
Streptococcus group A	+	-	+	-	-	-	-	-	-

536 ETEC = enterotoxigenic E. coli; EPEC = enteropathogenic E. coli; EAEC = enteroaggregative E. coli.
537 Note: no (-), mild (+), and strong (++) inhibition.

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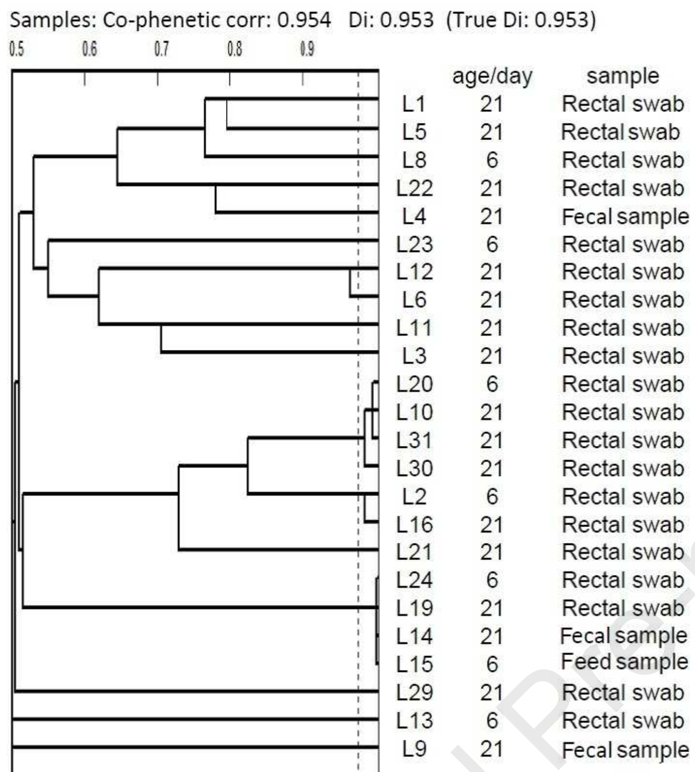

539
540 Fig. 1. Cluster analysis of Phene plate (PhP) assay of 24 bile and acid resistant *Lactobacillus*
541 *brevis*.

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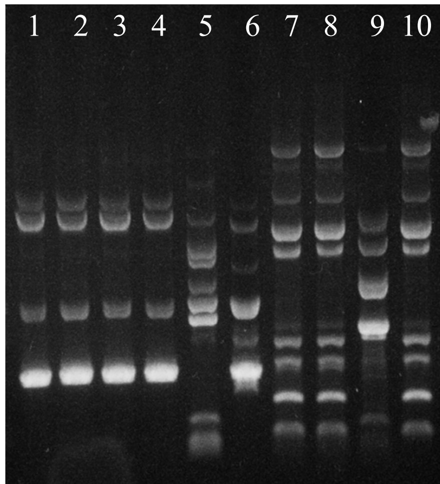
542
543



544
545
546 Fig. 2. Agarose gel electrophoreses of RAPD-PCR products of *Lactobacillus brevis* strains.
547 RAPD = random amplified polymorphic DNA.
548
549

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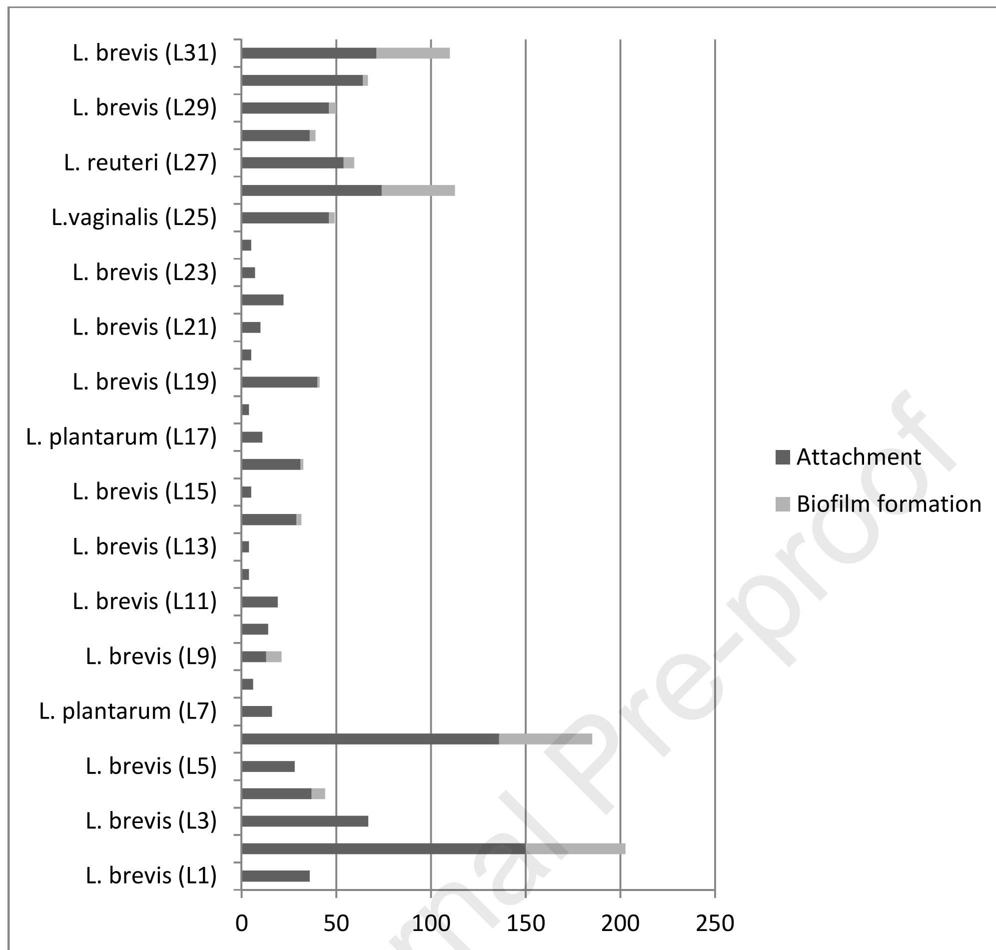

550
551
552 Fig. 3. Biofilm formation and attachment in 31 bile and acid resistant lactobacilli. Dark gray bars
553 show the number of attached lactobacilli in 20 microscopic fields to Caco-2 cell line and light
554 gray bars show the percentage of biofilm formation.
555
556

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Nasrin Noohi: Writing - Original Draft, Visualization, Investigation, Formal analysis **Moslem Papizadeh**: Writing - Review & Editing, **Mahdi Rohani**: Investigation, **Malihe Talebi**: Project administration, Methodology, **Mohammad R. Pourshafie**: Conceptualiza, Supervision.

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