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ORIGINAL ARTICLE

Isolation, functional evaluation of probiotic properties and molecular identification of strains isolated from Iranian poultry's gut

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ABSTRACT

Background and Objectives: The use of probiotics as an alternative to antibiotics in poultry diets has attracted a lot of interest recently. In this context, the determination of probiotic characteristics was evaluated on several isolates obtained from Iranian poultry's gut.
Materials and Methods: Probiotic characteristics such as hemolysis activity, acid, bile, gastric juice tolerate, *in vitro* adhesion assay, cell surface properties (hydrophobicity, auto-aggregation and co-aggregation) and antibiotic susceptibility test were evaluated. Finally, selected isolates identified molecularly after temperature-salt tolerance and extracellular enzyme activity (amylase, protease and cellulose).
Results: Out of 362 strains isolated from native poultry in three geographical regions of Iran, nine strains (belong to *Bacillus* sp., *Enterococcus* sp., *Pediococcus* sp., *Lactobacillus* sp., *Kluyveromyces* sp.) showed resistance against gastrointestinal physiological conditions, desirable surface properties, ability to adhere to epithelial intestine cell line and antibiotic susceptibility. Also, these strains were discovered to be temperature-salt tolerant but, only a small number of them were able to produce hydrolase enzymes.
Conclusion: According to the results, the selected strains can be introduced as native probiotic candidates for utilization in novel poultry feeds.

Keywords: Antibiotic; *Bacillus* sp; *In vitro* test; Poultry; Probiotic

INTRODUCTION

In many countries, the chicken industry is currently one of the most important food providers. Therefore, poultry disease prevention and control would be required to prevent significant financial losses. The use of subtherapeutic antibiotics as growth promoters for poultry and other livestock has been banned worldwide due to concerns about microbial antibiotic resistance (such as penicillin and tetracycline), and additional bans are anticipated in most nations. Probiotics have been studied as alternatives to antibiotic growth simulation as a means of addressing this issue in poultry production (1, 2). The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) define probiotics as live organisms that, when consumed in sufficient quantities, are known to have beneficial nutritional effects and growth-promoting effects. Potential probiotic strains can be isolated from humans, animals, plants, and

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the environment. Lactic Acid Bacteria (LAB) including *Enterococcus, Lactobacillus, Pediococcus, Streptococcus, Lactococcus* and *Leuconostoc*, several species of the *Bacillus* genus and yeasts are all probiotics that are isolated from traditional fermented products, dairy products (milk, yogurt, kefir), fruits, animal digestive tracts, feces and human breast milk (3-5). Beneficial bacteria that stop harmful bacteria from spreading through the gastrointestinal tract are known as probiotics. Coming up next is the overall system of activity of probiotics: Using competitive inhibition to prevent harmful germs from adhering to gut epithelial junctions, making organic acids and antimicrobial chemicals like hydrogen peroxide, bacteriocins, and defensins increases the secretion of digestive enzymes that help with normal digestion and affect T regulatory cells and antigen-containing cells to alter the host immune response (6-8). Concerns about the spread of antibiotic resistance genes to humans and the discovery of Iranian native probiotic strains suitable for poultry diets prompted this study. Even though there are a lot of commercially available probiotic strains, there is still a need for new strains that are better than the ones that are currently in use. In addition, because probiotic properties vary by strain, it is essential to identify and define each new probiotic strain in order to select the best ones. This research was carried out with the intention of isolating a number of different microbial groups from the gut sources of Iranian poultry, examining their probiotic characteristics, and selecting a few isolates for eventual use in poultry feed.

MATERIALS AND METHODS

Isolation and purification. Poultry collected from Mazandaran, Alborz and Lorestan provinces (chickens, roosters, ducks and geese) were sent to the slaughterhouse to use their meat, then their digestive system (small and large intestine). Poultry age of about one month was selected in this study. In order to obtain native probiotics, the food composition of the sampling area was desired and was not investigated in detail. a) For spore-forming bacteria isolation, 1 g of digestive system was transferred into 50 ml of Tryptic Soy Broth (TSB) and incubated for 3 days with shaking at 150 rpm at 39°C. Following incubation and before being used to inoculate new TSB at 1% (v v^{-1}), the enrichment medium was heated in a hot water bath (80°C) for 45 min to kill vegetable cells. The inoculated fresh broth medium was incubated at 39°C for three days (five times). Serial dilutions of each sample were made six times using the same media and grown on TSA, then incubated at 39°C (9, 10). b) To isolate LAB bacteria, 1 g of intestinal tissue and content was transferred into De Man, Rogosa and Sharpe Broth (MRS) broth and incubated for 48 h, followed by tenfold serial dilution and sub-culture on solid MRS and incubated at 39°C in 5% CO_2 conditions (2, 11, 12). c) Yeast was isolated and incubated at 25°C using Rose Bengal medium for screening and Yeast Extract–Peptone–Glycerol medium (YPG) medium for purification. Different colonies were purified after being isolated according to their morphology (color, form, and size). Gram staining, KOH, and catalase tests were carried out on the entire group of isolates. Following subcultures, purified isolates were stored in glycerol stock at -20°C for subsequent analysis (12).

Hemolysis activity. Isolates were cultured on blood agar (5% defibrinated sheep blood) and incubated at the right temperature as a result. The presence of a clear zone (hemolysis) was determined after 48 h. Hemolysis inactivity was shown by alpha-hemolysis (a greenish halo) and gamma-hemolysis (no halo) (2).

Acid tolerance. Suspension of isolates with 10^8 cells ml^{-1} were added to appropriate medium with pH 2.5 and 4 (1% v v^{-1}), as well as a control vial, incubated for 4 h. Following that, one ml of the incubated vial was put onto agar media and incubated. After counting, the survival rate of each isolate was calculated. The number of colonies formed should not be less than 10^6 when comparing inoculation concentrations (10).

Bile salt tolerance. From overnight cultured isolates was centrifuged at 3075×g for 15 min and reconstituted in appropriate medium with 0.3% and 0.5% (w v^{-1}) bile salt to achieve 10^8 cells ml^{-1}, and then incubated for 8 h with the negative control (broth medium with bile salt without microorganism). Bile salt tolerance was determined by measuring absorbance at OD_{620} and comparing it to the control using the equation below; T_8 control: OD_{620} after 8 h incubation without bile salt, T_0 control: OD_{620} at first without bile salt, T_8 treatment: OD_{620} after 8 h incubation with bile salt and T_0 treatment: OD_{620} at first with bile salt. $OD_{620} < 0.4$ shows bile salt resistance (13, 14).

$$((T8 - T0)\ \text{control} - (T8 - T0)\ \text{treatment}) / (T8 - T0)\ \text{control} \quad < 0.4$$

Gastric juice tolerance. The isolates overnight culture was centrifuged at 3075 × g for 15 min, washed, and a suspension of 10^8 cells ml^{-1} was prepared. The cell suspension was then inoculated with 2% of the medium pH 2 containing 2.5 mg/ml pepsin (250 unit mg^{-1}) and 2.5 mg ml^{-1} trypsin (1000-2000 unit mg^{-1}) and incubated for 4 h at the appropriate temperature. The strains' survival rates were measured following repeated dilution incubation. The number of colonies formed should not be less than 10^6 when comparing inoculation and control concentrations (15).

***In vitro* adhesion assay.** After culturing Caco-2 cells (IBRC C10094) in Dulbecco's Modified Eagle Medium (DMEM medium) containing 10% FBS and 1% L-glutamine 200 mM and so growing to 70% of the flask surface, the culture surface was removed, washed and added trypsin-EDTA, incubated at 37°C with 5% CO_2 for 3 min. To negate the effects of trypsin-EDTA was negated by centrifugation at 3075 × g for 15 min. Finally, 2 ml of cell suspension containing at least 5× 10^3 cells was placed into plate wells and for 24 h. After withdrawing culture medium, bacterial suspension with 0.5 McFarland concentration (control: IBRC-M 11018) was added and incubated for 3 h. So, the cells harboring bacteria adhering to the well surfaces were then removed from the well surfaces. First method: the suspension was diluted and the number of bacterial cells was counted and expressed as CFU ml^{-1} (10). Second method: after washing the unattached cells, 2 ml of methanol was applied to the cells and discarded after one min. Then cells were dyed by Giemsa and the number of adherent microbial cells to the epithelial cells were counted by phase contrast microscope (16).

Hydrophobicity. Isolates were grown overnight at 10^8 CFU ml^{-1} concentration, centrifuged at 3075 × g for 15 min, and resuspended in PBS buffer (pH 7.2) to achieve an OD_{600} of 1–1.2 for the cell surface hydrophobicity assay or Microbial Adhesion to Hydrocarbons assay (MATH assay). The cell suspension was mixed with an equal amount of each solvent (2 ml from suspension and 2 ml from each solvent) separately and vertexing for 2 min, before being stored at room temperature for 30 min. The absorbance was measured at 600 nm after the aqueous phase was removed (A1). The hydrophobicity of the isolates was determined by solvents such as n-hexane, ethyl acetate, xylene, and chloroform using this approach (17). The experiment was carried out three times. Hydrophobicity was calculated using the following equation, Where, $A_{30\ min}$ is the absorbance at t =30 min, and $A_{0\ min}$ is the absorbance at t = 0 min

$$(1 - A30 / A0\ min) \times 100$$

Auto-aggregation. As previously mentioned, isolates were pelleted in PBS (pH 7.2) and adjusted to yield 10^8 CFU ml^{-1} in the same medium. A precise volume of 5 ml of bacterial suspension was vortexed for 10 s, the absorbance at 600 nm (OD_i) was determined using a spectrophotometer, and then the sample was incubated for 2 h at 37°C. After 2 h of incubation, the supernatant's absorbance was measured (OD_{2h}). OD initial of the microbial suspension was compared with OD after 2 h incubation. For isolates with auto aggregation activity, OD after 2 h is decreased (15). The formula used to calculate the auto-aggregation coefficient (AC) is as follows. In this equation, OD_i: initial optical density of the microbial suspension at 600 nm and OD_{2h}: optical density of the microbial suspension at 600 nm after 2 h.

$$AC_t\ (\%) = [1-(OD_{2h}/OD_i)] \times 100$$

Co-aggregation. Briefly, bacterial suspensions were made according to the instructions in the auto-aggregation section. Equal volumes of each isolate and pathogen (*E. coli* IBRC-M 10698, *Pseudomonas aeroginoa* IBRC-M 10828, *Staphylococcus aureus* IBRC-M 10917, *Salmonella* Typhimurium IBRC-M 10707) strain (1:1 v v^{-1}) were combined by vortexing for 15 s, and the mixture was then left to incubate at 37°C without agitation. At the same time, control tubes with 4ml of each bacterial solution were set up. After 5 h at room temperature of incubation, absorbance (A_{600}) was measured. The following equation was used to calculate the percentage of co-aggregation, Ax and Ay are the absorbance of the test strains and pathogen in the control tubes, respectively, and A (x + y) is that of the mixed bacterial suspensions (test strain and pathogen) for co-aggregation (18).

$$\text{Co-aggregation}\ (\%) = (Ax+Ay)A(x+y)/2\ Ax+Ay/2\times 100$$

Antibiotic susceptibility. Isolates (LAB and Bacillus) was tested against nine different antibiotics (vancomycin 30 µg, ceftazidime 30 µg, chloramphenicol 30 µg, erythromycin 15 µg, tetracycline

30 μg, ciprofloxacin 5 μg, penicillin 10 μg, imipenem 10 μg, gentamicin 10 μg) by using disk diffusion assay. 100 μl from each isolate (10^8 CFU ml^{-1}) was cultured on appropriate medium and one antibiotic disk was placed on it after drying. Then, plates in triplicate were incubated in appropriate temperature. The inhibit bacterial growth areas was evaluated by measuring the zone diameter around each disk that the strains were categorized as sensitive (≥20 mm), intermediate (15-19 mm), or resistant (≤14 mm) (19, 20).

Temperature-salt tolerance. Overnight isolates (1% v v^{-1}) were injected into the appropriate medium (MRS, YPG, or TSB) and incubated at 45, 50, 55, and 60°C (aerobic or anaerobic depending on strain) for the temperature tolerance test. After 24-72 h, their growth was measured with a spectrophotometer at 600 nm. Sub-culturing on-solid media without dilution will confirm their growth. Isolates were injected into their medium (1% v v^{-1}) overnight, comparable to the temperature tolerance test (MRS, YPG, or TSB containing a concentration of NaCl 0-10%). Before being sub cultured on solid medium, the isolates' growth was evaluated using a spectrophotometer on broth medium. The experiment was carried out three times (21).

Extracellular enzyme activity. The activity of extracellular enzyme was assessed based on the presence of a clear zone around the isolated colony after culture and incubation on enzyme production specialized media. The activity of protease, amylase, and cellulase was measured. Skim hydrolase as Protease activity (medium with 5% skim) was used as an indicator of protease activity in this approach. The appearance of a distinct halo after subculture on the medium and incubation suggests strain protease activity. Alpha amylase activity test was performed using the appropriate medium for isolates containing 2 g l^{-1} soluble starch. Plates were drowned in an iodine–potassium iodide solution for 5 min after incubation at room temperature. The solution was then withdrawn, and a clear halo was looked for. Cellulase activity was performed by a suitable medium containing 10 g l^{-1} carboxymethyl cellulose as an enzyme substrate was prepared for assaying cellulase activity. After culturing on the medium and incubation at the optimal temperature, the plate was floated by 0.2% Congo red and thus decolorized by NaCl 1 mol l^{-1} for 15 min. The existence of cellulase activity was indicated by a prominent halo surrounding the developing strain (22, 23).

Molecular identification. Isolates with favorable probiotic properties were selected for molecular identification (16S rRNA gene sequencing). Initially, DNA was extracted from freshly cultured cells using a kit, and the concentration and integrity of the collected DNA were determined using 0.8% agarose gel electrophoresis stained with ethidium bromide. 16S rRNA was amplified using universal primers (27f: 5′-AGAGTTTGATCCTGGCTCAG-3′, 1492r: 5′-TACGGYTACCTTGTTA CG ACTT-3′) to identify bacterial isolates using the PCR program outlined below: initial denaturation at 95°C for 0.30 min; 25 cycles of denaturation (95°C for 0.45 min), annealing (45-59°C for 0.45 min), and extension (72°C for 1.30 min); and a final extension step at 72°C for 10 min. In addition, yeast isolates were identified by sequencing of D1/D2 domain using NL1/NL4 primer (NL1:5′-GCATATCAATAAGCGGAGGAAAAG-3′, NL4: 5′-GGTCCGTGTTTCAAGACGG-3′) and by using the following PCR condition: initial denaturation at 95°C for 5 min; 35 cycles of denaturation (95°C for 0.30 min), anealing (52-62°C for 0.45 min), and extension (72°C for 1.20 min); and a final extension step at 72°C for 7 min. Gel electrophoresis was used to view the PCR result, which was subsequently sequenced using the Sanger method. BLAST (basic local alignment search tool) was used to compare the sequences to reference data from the GenBank databases EZbiocloud and NCBI (24). The sequences were considered to belong to an operational taxonomic unit (OTU) when sharing ≥97% sequence identity Neighbor-Joining (NJ) with 1000 bootstrap iterations. Genebank Accession nos: M PII (4): OM850326, LP7 (18): OM841513, MP2 (6): OM841511, AP12 (4): OM841506, 5ADL28: OM841496, 3H26: OM841495, Ch. q1 (7): OM841492, FM3 (26): OM841507, KF3 (4): ON140771

Statistical analysis. The independent experiment was determined in triplicate and the results are shown as means ±SD of at least two independent investigations. SPSS statistical studies were conducted to verify the significance of the findings. The p value (<0.05) was used to statistically validate the data. Differences were considered statistically significant when the p -value was <0.05.

RESULTS

Isolation and purification. We attempted to isolate various microbial groups and investigate their probiotic characteristics in this study. For isolation, MRS (Lactic Acid Bacteria), TSA (*Bacillus* strain) and YPG (yeast) media were utilized. Based on morphology, Gram, KOH and catalase, 362 isolates (10 yeast form, 99 spore-bacteria, 185 bacilli and 68 cocci form) were purified, preserved and utilized for probiotic characterization.

Hemolysis activity. A distinct halo around microbial growth on plate was seen in 46 of the 362 isolates, indicating hemolytic activity. For further probiotic testing, these strains were rejected.

Acid tolerance. For isolates with negative hemolysis, a low pH tolerance test was performed (316 strains). 84 isolates were able to develop at pH 4 and 2.5 (more than 10^6 CFU ml^{-1}) despite the acidic environment. *Bacillus* sp. accounted for 25 of the 84 isolates, yeast for two, and LAB for the rest.

Bile salt tolerance. According to the described procedure, the bile salt tolerance test was performed. After 8 h of exposure, 53 of the 84 tested isolates could survive bile salt concentrations of 0.3 and 0.5% (w v^{-1}) (according to the formula, results were >0.4).

Gastric juice tolerance. Of the 53 tested isolates, 13 isolates (24%) were able to tolerate pepsin and trypsin after 24 h. The results are plotted in Fig. 1 and are compared with the control sample. In our study, only 24% were able to tolerate pepsin and trypsin after 24 h.

***In vitro* adhesion assay.** Adhesion of isolates to epithelial cells was evaluated by using Caco-2 cells. *In vitro* adhesion assay is based on probiotic bacteria being close to and attaching to an epithelial cell line over time. The CFU ml^{-1} was determined after dilution and incubation, and all of the microbial cells adhering to the cell line were greater than 10^6. Also, attachment was confirmed by contrast phase microscopy.

Hydrophobicity. The hydrophobicity was conducted using the procedure described above, and the results were calculated using the formula. Surface hydrophobicity of isolates were compared in the (Fig. 2) Proteins, carbohydrates, and lipoteichoic acid are surface elements of a cell that interact during cell aggregation. The bacterial population in the gut is kept in check by the hydrophobicity and auto-aggregation. A number of greater than 50% implies that the cell surface is hydrophilic whereas a value of less than 50% suggests that the cell surface is hydrophilic.

Auto-aggregation. Results of auto-aggregation are shown in Table 1. This test was performed for nine isolates. According to the results, KF3 (4) and 5ADL28 showed excellent auto-aggregation ability 95.5 and 72.3, respectively. Adhesion ability of two isolates KF 3 (4) and 5ADL28 confirmed by auto-aggregation.

Co-aggregation. The results of co-aggregation of isolates with standard pathogens (*E. coli, P. aeruginosa, S. aureus* and *S. typhimurium*) were compared in Table 1. KF3 (4) and MP II (4) demonstrated the most and the least aggregation with the examined pathogens in this test, respectively. KF3 (4) had the greatest ability to attach to pathogens (almost 45%) while the 5ADL28 strain could adhere to pathogens after KF 3 (4). The remaining isolates had reduced ability to bind to pathogens. None of the pathogens could not adhere to MP II (4) at all.

Antibiotic susceptibility. For determination of safety of isolates, nine different antibiotics with various mechanisms were tested (Table 2). The diameter of lack of growth was measured that 5ADL28 was sensitive to total of tested antibiotic disk. Also, all nine tested isolates showed sensitivity to at least one of the antibiotic disks.

Temperature-salt tolerance. Normally, all LAB and *Bacillus* sp. isolates can grow at 37°C, whereas yeast isolates grow at 25°C. After 24-48 h of incubation, only two *Bacillus* strains (5ADL28 and 3H26) could survive in environments above the temperature of 45°C (to 60°C). Also, while the remainder were able to withstand concentrations of 6-10% NaCl, MP II (4), LP 7 (18), and AP12 (4) could only tolerate 4% NaCl. Only the 3H26 strain was able to develop at a concentration of 10% salt whereas the majority of isolates were able to grow at a concentration of 6% salt.

Extracellular enzyme activity. Only 3H26 out of the examined isolates displayed Alpha-amylase activity, while both its protease and cellulase activities were inactive. The only isolate with cellulase

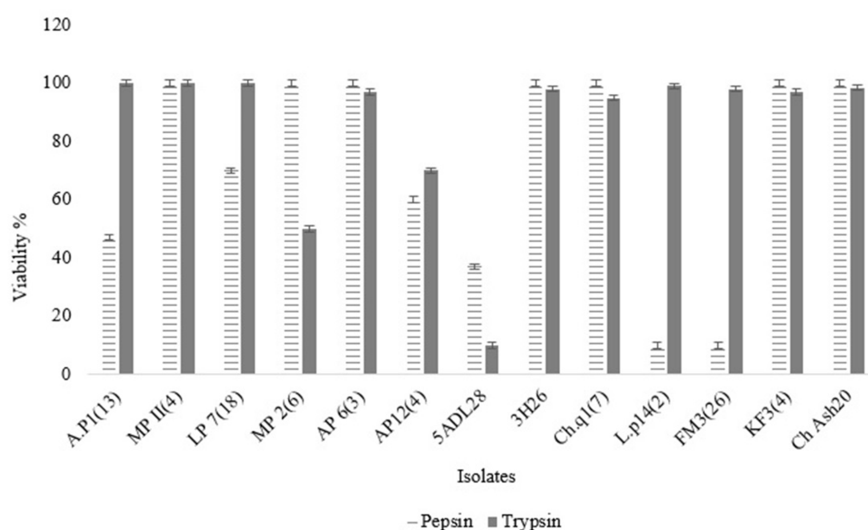

Fig. 1. Survival rate of isolates in pepsin and trypsin condition

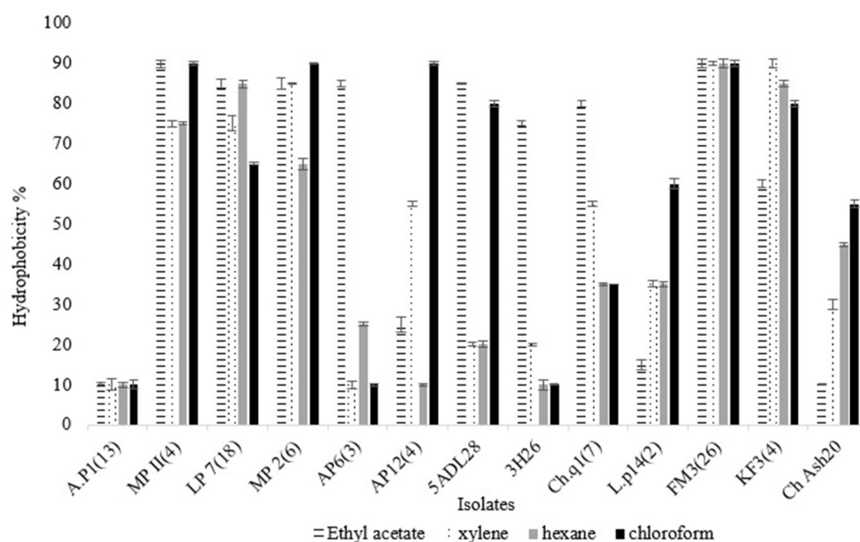

Fig. 2. Hydrophobicity of poultry strains to solvents

Table 1. Auto-aggregation and co-aggregation of isolates

Isolates	Auto-aggregation%	Co-aggregation%			
		E. coli	*P. aeroginoa*	*S. aureus*	*S. typhimurium*
3H26	30.5 ± 1	20.43 ± 0.49	15.86 ±1.15	17.05 ± 0.38	17.48 ± 0.6
5ADL28	72.5 ± 1	25.8 ± 0.92	31.98 ± 0.38	33.52 ± 59	30.6 ± 1.6
MP II (4)	23 ± 1	0	7 ± 0.24	0	0
LP7 (18)	28 ± 0.4	21.5 ± 1.22	14.86 ± 0.43	17.05 ± 0.54	11.47 ± 0.44
MP 2 (6)	18 ± 1.4	9.97 ± 0.45	8.07 ± 0.17	8.65 ± 0.64	5.26 ± 0.17
AP12 (4)	19 ± 0.3	12.33 ± 0.37	10.02 ± 0.1	6.08 ± 0.12	3.5 ± 0.77
Chq 1 (7)	23.5 ± 1	8.85 ± 0.45	7.16 ± 0.36	0	6.55 ± 0.46
FM3 (26)	45.5 ± 0.7	30.61 ± 0.45	29.01 ± 1.18	36.66 ± 0.55	13.18 ± 0.6
KF3 (4)	95.5 ± 1	47.78 ± 0.53	43.28 ± 0.95	45.5 ± 1	46.51 ± 0.97

Table 2. Antibiotic susceptibility of isolates

Antibiotic designation	V	CAZ	C	AN	TOB	E	TE	CP	P
3H26	S	R	S	I	I	S	S	S	S
5ADL28	S	S	S	S	S	S	S	S	S
MPII (4)	R	R	S	R	R	S	R	R	S
MPII (6)	R	R	S	R	R	S	R	R	S
Chq 1 (7)	S	R	S	R	R	R	S	R	S
AP12 (4)	R	R	S	0	0	S	R	R	S
FM 3 (26)	R	R	26	16	15	S	R	R	S
LP7 (18)	I	R	22	R	R	S	S	R	S

activity was MP II (4). Apart from 3H26 and KF3 (4), all isolates had indicated protease activity. Also, 5ADL28 displayed only protease activity. A distinct halo was found around six of the nine strains (67%) studied, showing that they have the potential to hydrolyze casein (milk protein). Only 12% of *Bacillus* strains (abundance of amino acids and proteins) and 53% of strains (deficiency of proteins and amino acids) demonstrated protease activity in a similar study. Among the strains, only MP II (4) was able to show poor ability to hydrolyze cellulose (11%).

Molecular identification. Nine isolates with probiotic features, were chosen for molecular identification. By blasting the sequence results in GenBank databases NCBI and EZ taxon, it was discovered that two strains belong to the genus *Bacillus*, one to the genus *Kluyveromyces*, three to the genus *Lactobacillus*, two to the genus *Pediococcus* and one to the genus *Enterococcus*. M PII (4), LP7 (18), MP2 (6), 3H26 and FM3 (26) from Mazandaran, AP12 (4) from Alborz and 5ADL28, Ch. q1 (7) and KF3 (4) from Lorestan were isolated. Also, the blast results are listed. BioEdit and MEGA version 11 were used to create phylogenetic tree based on ITS and 16S rRNA is shown in (Fig. 3) and (Fig. 4).

DISCUSSION

In the current investigation, the spore-forming bacteria were shown to have stronger hemolytic activity than *Lactobacillus* and *Lactococcus.* The most of LAB strains are non-hemolytic, as reported earlier. Hemolytic activity is considered a virulence factor by FAO recommendations. On the other hand, hemolytic activity may be less significant because blood cells do not interact as much with the digestive system (15, 25). In tolerance to low pH test, According to the results reported by Rajoka et al. (26), the survival ratio of isolates at pH 4 was higher than pH 2.5. In our study, only about 15% of the isolates were able to survive for 4 h. Only 15% of the bacteria that could tolerate pH 4 continued to exist at pH 2.5. The findings in a study (2) showed that different LAB probiotic strains have different chances of surviving in simulated gastric juice (pH 2.0). Additionally, strains that have been successful to live within this setting may get through the tough conditions of the chicken gut and connect to intestinal cells, causing positive effects. The capacity of strains to survive in the acidic environment of the gastrointestinal tract is a critical element of employing them as poultry probiotics. Bile salts are amphipathic chemicals that emulsify fat. Bile salt tends to disrupt cell membrane structure and have a direct impact on the survival of bacteria in the digestive tract, particularly in the small intestine, which is why bile salt tolerance is a critical trait of any strain that can survive in the intestine. Although the concentration of bile salt in the GIT is between 0.175 and 0.008%, 3% to 5% of bile salt is examined in several articles. 63% of the isolates assessed in this study were able to withstand this quantity of bile salt (2). However, all isolated strains were able to withstand bile salts in two studies (2, 9). The results reported in study (2) showed that 46% of the isolates were able to tolerate gastric juice with pH 2. Moreover, in a study (27) evaluating pepsin at pH 2 for 45 min of incubation, the strains were reported to have a 98-100% survival rate. In another investigation (28), all of the strains (isolation from dairy products) were found to be very viable in both acidic and alkaline gastric juice. In a study (10) the capacity of different isolates to adhere to Caco-2 cells ranged from 70% to 90%, which was significantly better than the previously discovered isolates (29). The capacity of probiotic bacteria to attach to Caco-2 cells is also a significant parameter for their selection. This property has positive consequences such as immune system regulation and harmful microorganism exclusion. The hydrophobicity of a probiotic isolate's cell surface is an important characteristic. This demonstrates the isolate's capacity to adhere to an enterocyte cell line which is a highly desirable trait in probiotics. FM 3 (26), M P II (4), MP 2 (6), and KF 3 (4) had the highest adhesion capacity among the isolates evaluated in this investi-

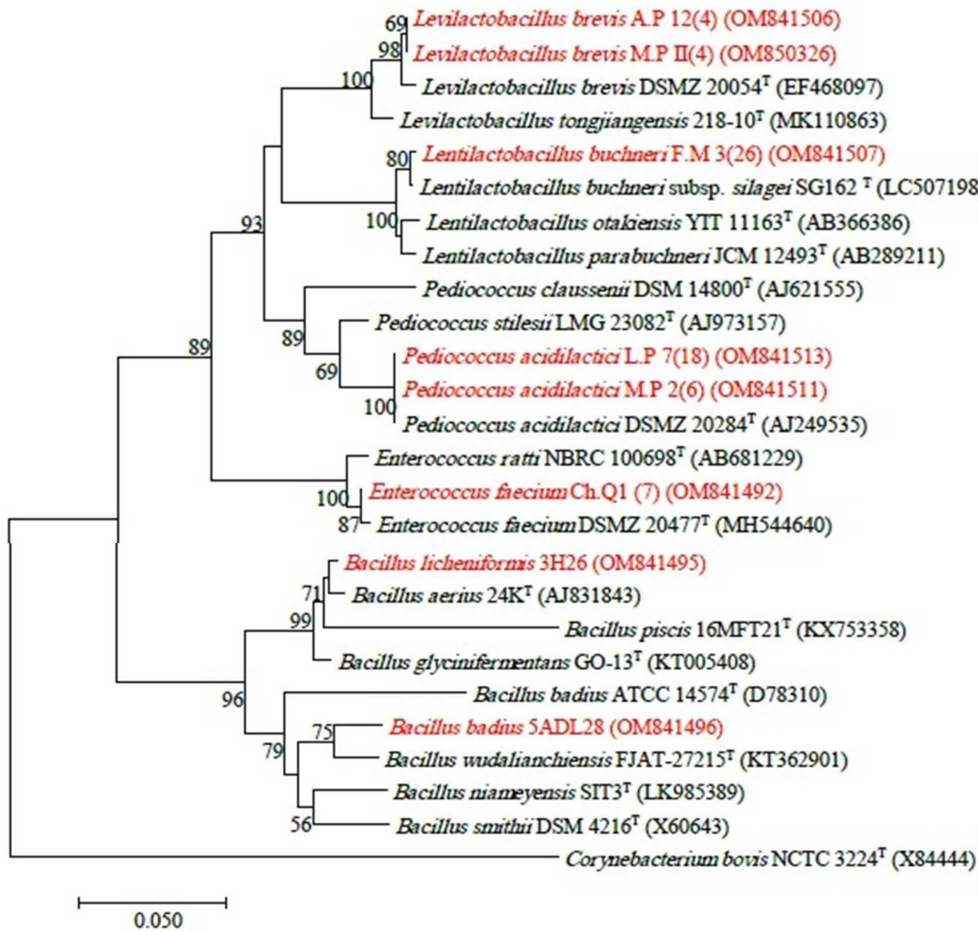

Fig. 3. Phylogenetic tree of *Bacillus, Lactobacillus, Enterococcs* and *pediococcus* strains. Numbers above the nodes are the bootstrap values above 50%.

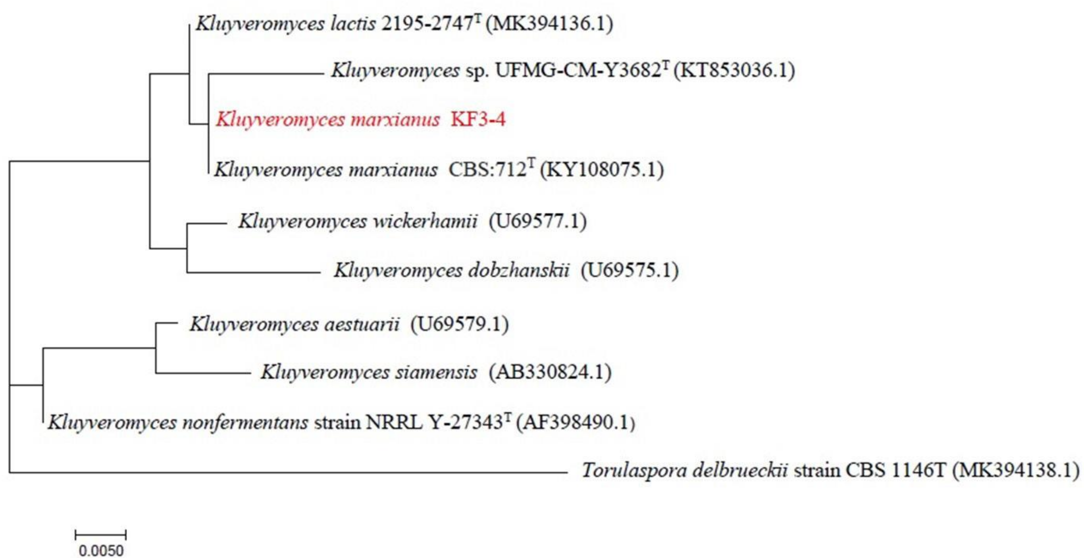

Fig. 4. Phylogenetic tree of *Kluyveromyces* strain. Numbers above the nodes are the bootstrap values above 50%.

gation. Compared to the previous report, *Bacillus* spp. had a lower adhesion capacity (17). 50% of the tested LAB exhibit high hydrophobicity with values ranging from 50.0-90.0% while at two different study and, recorded hydrophobicity of between 3.6-93.53 and 30-71.1%, from LAB taken from poultry (30). According to our findings, the adhesion capacity of 5ADL28 was 80% in chloroform while it was lower in xylene and hexane. 3H26 strain didn't show satisfactory results for hydrophobicity. Solvents such as hexane, xylene and chloroform are non-polar and are usually used to assay the cell surface hydrophobicity. The use of n-hexane has been mentioned in various articles. Ethyl acetate is polar solvent compared to the others and it is expected that cell hydrophobicity in this solvent will show different results compared to non-polar solvents. But in our study, the expected difference in the use of polar and non-polar solvents were not reported in some of the tested strains. Aggregation ability is usually related to cell adherence properties and confirms the hydrophobicity result. Cells aggregation between the microorganisms of the same strain (auto aggregation) is of considerable importance in several ecological niches. Only one of the two yeast isolates were able to adhere. In a study, LAB isolated from poultry showed almost 50% auto-aggregation (15). Cell-to-cell adhesion across genetically distinct strains is known as co-aggregation, and it has also been linked to the capacity to engage with pathogens. As a result, co-aggregation gauges the test strain's adherence to the enteric fever pathogen. Probiotic strains' capacity for co-aggregation may make it possible to create a barrier that hinders the colonization of harmful bacteria. However, probiotic strains with a track record of success in the digestive system may still exist. *Lactobacillus plantarum* MBTU- HK1 possessed higher level (62.6%) of co-aggregation with *S.* Typhi (18). In another investigation, enteric pathogens *S.* Typhi, *Salmonella* Paratyphi A, and *Vibrio cholera* could co-aggregate with the probiotic *Bacillus* subtilis isolated from cow's milk (31). Despite the fact that the proportions of co-aggregation varied based on specific strain combinations. When choosing new probiotic strains, it's also important to look at how susceptible they are to the antibiotics used in antimicrobial therapy. Antibiotic resistance in bacteria can be inherent or acquired through chromosomal mutation or horizontal gene transfer. Transferring the isolates to pathogenic strains will be possible because they contain antibiotic resistance genes.

The temperature range was used to see if isolated cultures were able to grow within the normal body temperature range. *Bacillus* strains and LAB were able to tolerate 45°C in our study; however, in agreement with other findings, one of the yeasts was unable to grow at this temperature (13). In addition, a study found that LAB isolated from food samples could withstand temperatures of 40°C (21).

NaCl concentrations of 1.0-9.0% can be tolerated by LAB from various sources, including meat, chicken feces, milk, and products derived from milk (32). Probiotics' extraordinary capacity to encourage the growth and synthesis of metabolites is demonstrated by these findings. The ability to grow at high salt concentrations is important for industry. Additionally, probiotic bacteria must be able to withstand high salt concentrations in order to thrive in the gastrointestinal system, where the osmolarity of the environment is 2% NaCl (33).

Our finding about extracellular enzyme is in line with previous findings that bacillus species (Their amylase activity (50 percent in our study) plays a significant role in starch fermentation. The ability of probiotic strains to hydrolyze starch is an important characteristic because polysaccharides like starch make up a significant portion of chicken diets. Hydrolyzing protein is advantageous in chicken diets devoid of amino acids and containing complex protein. This suggests that bacteria's inability to produce an extracellular protease enzyme is a constraint that can be used to reduce protein abundance (9). Using the method, the cellulase activity of the strains was evaluated. The percentage of cellulose in poultry feed varies. Probiotics' ability to digest and break down cellulose is desirable for lowering costs and increasing cellulose uptake in poultry because of the gastrointestinal tract's deficiencies in cellulose digestion (23).

CONCLUSION

Due to the growing need for meals that are nourishing and beneficial, or foods that do more than just offer basic nutrition, probiotics have attracted a lot of interest in the food and animal feed sectors in recent decades. In this study, various microbial species were examined, and nine strains belonging to *Bacillus* sp., *Enterococcus* sp., *Pediococcus* sp., *Lactoba-*

cillus sp., *Kluyveromyces* sp. with probiotic characteristics were found. Isolation of *B. badius* 5ADL28 with favorable probiotic properties without antibiotic resistance is considered as the strength of this study. According to the results, the selected strains can be introduced as native probiotic candidates for utilization in novel poultry feeds. Of course, whole genome analysis and in vivo tests are suggested to confirm and complete the laboratory results of this study.

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