Characterization of Lactic Acid Bacteria Isolated from the Gastrointestinal Tract of Ethiopian Rhode Island Red Chicken and their Potential Use as Probiotics

Kibrnesh Tegenaw Tsega (kibiramare372@gmail.com)
University of Gondar https://orcid.org/0000-0003-1630-4212
Kagira Maina
Jomo Kenyatta University of Agriculture and Technology
Nega Berhane
University of Gondar

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Abstract

Background Chicken productivity and survival is affected by environmental stress and incidences of different diseases. After the ban of antibiotics growth promoters by different countries, the use of probiotics feed additives is mentioned as one alternative to antibiotics. The objective of this study was to isolate and identify potential probiotic lactic acid bacteria strains from the digestive tract content of Rhode Island Red chicken. Results The study was conducted in Gondar town of Amhara region, North West Ethiopia from November, 2018 to March, 2019. From the gastro intestinal content of 5 chicken 190 isolates were randomly selected. The isolates were identified and screened by their biochemical, morphological and 16S rRNA gene sequence. The in vitro probiotic potential of isolates was characterized by various tests. From 190 isolates 73 were found as gram positive, catalase negative and were able to grow at pH4. The bacteria were either rod (83.6%) or round (16.4%) shaped. Ten isolates were then randomly selected for further in vitro probiotic characterization due to higher cell surface hydrophobicity, good survival at pH2 and survival at 0.1% bile salt. From the 10 isolates 6 were able to survive at pH1 for 3hrs incubation. Five isolates (IS1, IS2, IS5, IS7 and IS8) were resistance to ampicillin, chloramphenicol, ciprofloxacin and erythromycin antibiotics. All the ten isolates showed antagonistic activity against Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Staphylococcus intermedius and Salmonella enteritidis. The optimum temperature for all ten isolates was 450C and all the isolates were able to grow at 0.69 mol/L of NaCl, and were able to ferment galactose, glucose, fructose, mannitol, sorbitol and sucrose. Using phylogenetic analysis of 16SrRNA gene sequence; IS3 was identified as Lactobacillus salivarius while IS4, IS6 and IS7 were identified as Lactobacillus reuteri. Conclusion The study concludes that the 10 selected isolates can be able to survive the stress conditions of gastrointestinal tract and can thus be considered as potential probiotics candidates for chickens.

Background

Globally, the poultry industry makes a substantial contribution to food security and nutrition. The preference of consumer for these high-quality products and their relatively low price stimulates the global production of poultry products [1]. In Africa, poultry are a major asset and key to poverty alleviation, for providing income and market participation particularly for small holders and the poor, both in rural and urban areas [2].

In Ethiopia chickens are the most widespread sources of meat, egg and income. In terms of breed, 97% are indigenouswhile the rest are exotic (2%) and hybrid (1%) [3]. In recent years exotic chickens particularly Rhode Island Red (RIR) have been distributed to farmers in order to improve the productivity of local birds through cross breeding. Unfortunately, the introduced breeds could not adapt well due to the hot climate, low quality feed incidence of disease and poor management [4]. Disease management for these exotic chicken have been mainly through use of antibiotics while other farmers have adopted use of antibiotics at sub-therapeutic levels in animals feed as Animal Growth Promoters (AGPs).

For many decades, sub-therapeutic level antibiotics improved growth and feed conversion rate in some animals such as poultry [5]. However, in recent years, the emergence of antibiotics resistant populations, the appearance of residual antibiotics in meats and an increasing public demand for organic production have increased interest in searching for alternatives to antibiotics [6].

The use of probiotics is described as one of the alternatives to antibiotics [7] and it have been advocated for prevention of disease, decrease of mortality, tolerate environmental stress and to enhance productivity of chicken [8]. Probiotics are “live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance”. These live microorganisms are nonpathogenic and nontoxic in nature, that are favorable to the host’s health when adequately administered through the digestive route [9].

Different microbial species have been used as probiotics including; species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species (mainly *Saccharomyces*), *Pediococcus* and undefined mixed cultures [10].

Lactobacillus species are commonly used to broiler chickens [11] in developed countries. Different strains belonging to similar species of *lactobacillus* have different properties and so effects or benefits can be different from one strain to another within the same species [12, 13]. As a result, potential probiotic bacteria can be isolated from the gastro intestinal tract (GIT) of well adapted chicken which can be used to formulate probiotics. The main objective of this study was to isolate and identify probiotic lactic acid bacteria (LAB) from the GIT of well adapted Rhode Island Red exotic chickens in Ethiopia.

Methods

Ethical Approval

Before the beginning of the study an ethical approval was obtained from Research Ethical Committee of University of Gondar. All animal work was conducted according to the institution guidelines for animal welfare.

Study Area

This study was conducted in Gondar Town of Amhara region, North West Ethiopia. Amhara Region is located between 8˚45'N and 13˚45'N latitude and 35˚46' E and 40˚25'E longitude in North West Ethiopia. The total area of the region is estimated at 156,960 km^2 and has a chicken population of 18,031,121 [39]. Despite the continuous distribution of exotic chickens (mainly RIR) to farmers by the regional state, the productivity of disseminated exotic chicken was far below the possible expectations to fulfill the regional as well as the national demand. In the region, the poultry farming mainly involves domestic chicken production due to less adaptability and high mortality rate of exotic chicken [40].

Isolation of Lactic Acid Bacteria from the Gastrointestinal Tract of Chicken

Five healthy and mature male RIR chickens were randomly selected and purchased from a local community in around Gondar zone, North West Ethiopia. The chickens were selected based on the information about their age and health status. A local agricultural extension expert confirmed the breed of chickens and a veterinarian confirmed their health status. After purchasing, the chickens were transported to Molecular Biology Laboratory (Department of Biotechnology), University of Gondar and killed humanely by cervical dislocation and slaughtered aseptically as described by [41] to isolate LAB. The gastrointestinal digestive tracts (crop, gizzard, small intestine and cecum) were used as LAB sources.

One gram of sample was collected from each sample site (crop, gizzard, small intestine and cecum) immediately after slaughter and put into sterile test tubes containing Phosphate-Buffered Saline (PBS) buffer. The sample buffer mixtures were vortexed for 5mins and serial dilutions were made. Afterward, samples were plated over sterilized deMan, Rogosa and Sharpe (MRS) agar medium (Himedia, India) and incubated in anaerobic condition at 37^0C for 48hrs. Anaerobic jar with anaerobic gas generating kit (Oxoid, UK) was used to maintain anaerobic condition. From each MRS agar plate, colonies were isolated based on their morphological differences (shape, size and color). Purification of colonies was made by sub-culturing each colony on MRS agar.

Initial Identification and Preliminary Screening of Isolates

Morphological examination of colonies, gram staining and catalase test were used for initial identification of the selected isolates. Overnight cultures of each isolate on MRS agar were used for these tests. Gram stained cells were examined under a light microscope for morphological characterization. For catalase test, 3% hydrogen peroxide was used to select catalase negative isolates. Based on the results, gram-positive and catalase-negative isolates were selected and stored at -20^0C in MRS broth provided with 20% glycerol for the remaining experiment.

Probiotic Potential Characterization of Bacterial Isolates

Acid Tolerance: As pH tolerance is one of the important criteria for the selection of probiotic strain as it dictates the probability of survival of an exogenous culture in the GIT, isolates were subjected to acid tolerance test as described by [27]. MRS broth pH 6.5 was used as control. Each treatment was tested in triplicates.

Bile Salts Tolerance: The bile tolerance assay was done as described by [16] with modifications. One milliliter of overnight culture of each isolate at a final concentration of 7 to 8 log CFU/ml was inoculated into 9 ml of fresh MRS broth with 0.05%, 0.1% or 0.3% (w/v) of Difcooxgall (BD biosinces, USA) and incubated anaerobically at 37° C for 4hrs. MRS broth without bile salt (Oxgal) was used as control. After 4hrs incubation, 10-fold serial dilutions of up to 10^{-7} were prepared using PBS. Then 100µl of 10^{-5} to 10^{-7} dilutions from each sample was streaked on MRS agar plates and incubated anaerobically at 37° C for 24hrs, and colonies on the plates were counted and enumerated as CFU/ml. Bile tolerance was estimated by comparing viable cell counts in MRS with and without bile (Oxgall). The assay was performed in triplicates.

Antibiotic Sensitivity: Antibiotic susceptibility of selected LAB isolates was determined by using commercial antibiotic discs (Himedia, India) as described by [16]. One hundred microliter of lactic acid bacteria cell suspensions were spread over the entire surface of Muller Hinton agar (Himedia, India) containing plates. Then, paper discs containing antibiotics of; ampicillin (30µg), ciprofloxacin (30µg), chloramphenicol (30µg) and erythromycin (15µg) were placed on the plates. Subsequently, the plates were incubated anaerobically at 37°C for 24hrs. Antibiotic sensitivity of the isolates was determined by measuring the diameter of the clear zone around the antibiotics discs.

Temperature and Sodium Chloride Salt Tolerance: For the determination of growth at various temperatures, LAB isolates were inoculated into MRS broth and incubated at 25, 30, 35, 40, 45, and 50°C for 24hrs. The growth of the isolates on each specific temperature was evaluated by observing the turbidity of the culture medium. The test was performed in triplicates.

To determine sodium chloride (NaCl) tolerance of bacterial isolates, MRS broth adjusted with different concentration of NaCl (4%, 6% and 8%) was inoculated with fresh overnight culture of bacterial isolates. The test was done as described by [28].

Haemolytic Activity: Haemolytic activity of lactic acid bacteria isolates was determined by following the method described by [26]. *Streptococcus pyrogen* was used as positive control. The assay was performed in triplicate.

Antimicrobial Activity of the Selected Isolates: The antimicrobial activity of LAB isolates was determined by the well diffusion assay technique. For this test, the bacterial isolates were cultured in MRS broth and incubated overnight. On the other hand, the pathogens (*E. coli*, *S. aureus*, *S. typhimurium*, *S. intermedius* and*S.enteritidis*) obtained from Microbiology laboratory, College of Veterinary Medicine, University of Gonadr previously isolated from chicken were grown in Brain Heart Infusion (BHI) broth (Himedia, India). To determine the antimicrobial activity of isolates, 100µL of the test pathogens was spread onto the surface of Muller Hinton agar (Himedia, India) plates. McFarland (0.5%) was used to estimate the bacterial cell density. On these culture plates wells were punctured by using cork borer. To get the antimicrobial containing supernatant from the isolated bacterial culture, the cultures were centrifuged at 13,000rpm for 1min. Then 100µL of cell free supernatant (CFS) which pH was adjusted to neutral was added into each well [26, 33]. Thereafter, the plates were incubated at 37°C for 24hrs. The antimicrobial activity of the bacterial isolates was determined by the formation of inhibition zones around the wells. The diameter (in millimeter) of clear inhibition zone around the wells was measured and the size of the clear zone was directly proportionate to antagonistic activity of the isolate. Isolates that show wide spectrum of antimicrobial activity was selected for further analysis.

Cell Surface Hydrophobicity: Cell surface hydrophobicity was determined using the method of [42].

Carbohydrate Fermentation Profiles

The fermentation of different carbohydrates namely; starch, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, sucrose, sorbitol, trehalose and xylose was performed as described by [28].

Molecular Characterization of Selected Isolates (16S rRNA Gene Sequencing)

After *in vitro* characterization of lactic acid bacteria isolates, four isolates with best probiotic potential were randomly selected and subjected for 16S ribosomal Ribonucleic Acid (rRNA) gene sequencing in order to identify them at species level. For 16S rRNA gene sequencing, genomic DNA was extracted from overnight cultured LAB cells by using Bacterial Genomic Deoxy Ribonucleic Acid (DNA) isolation Kit

(Himedia) by following the manufacturer's instructions. After DNA isolation the 16S rRNA gene was amplified by using the universal primer, 27F [5'AGAGTTTGATCCTGGCTCAG 3'] and 1492R [5'TACGGCTACCTTGTTAGGACTT 3'] as described by [32]. The PCR amplification was performed as the following.A 40µl PCR mix was prepared by adding 8µl FIRE pol master mix, 2µl DNA (20 ng/µl), 0.4µl (100pm) of each forward and reverse primers and nuclease free water. The PCR amplification condition was initial denaturation at 95^0C for 3mins followed by 35 cycles consisting of denaturation at 95^0C for 30s, annealing at 60^0C for 45s and extension at 72 for 1min and final extension was at 72^0C for 7 mins. Confirmation of PCR products was done through agarose gel electrophoresis by using 1% agarose gel. The PCR product purification and sequencing were conducted by sending the PCR products to Macrogen (Europe).

Sequence Alignments and Phylogenetic Tree Construction: DNA sequence data of each isolates was obtained by sequencing the PCR products. The DNA sequences were compared with available sequences in the GenBank. Sequence similarity values were determined by using the Basic Local Alignment Search Tool (BLASTN) of the National Center for Biotechnology Information (NCBI). Greater than 96% sequences similarity value to the previously published sequences was used as a criterion to indicate species identity. A 16S rRNA gene multiple sequence alignment was done by using ClustalX2 and the aligned sequences were used to construct the phylogenetic tree by using Molecular Evolutionary Genetics Analysis (MEGA) version 6. The evolutionary history of sequences was inferred using the Neighbour-Joining method. Bootstrapping was performed for 1000 replicates. Computation of evolutionary distances was done using the Tamura 3-parameter method [43, 44].

Data Analysis

Data was entered into excel and then exported to SPSS for analysis. The quantitative data were analyzed by using One-way analysis of variance (ANOVA) with Duncan multiple range test ($P<0.05$) to distinguish the means between isolates using IBM SPSS statistics 21.0. The total bacteria cell colony forming unit (CFU/mL) counted was converted to the logarithmic value before statistical analysis.

Results

Isolation and Preliminary Screening of Lactic Acid Bacteria

A total of 190 bacterial colonies were randomly selected from the cultures of samples taken from the gastro intestinal tract of five chickens. Initial isolation was done based on different colony morphological

characteristics such as shape, color, appearance and size. The 190 isolates were from crop(40), gizzard (2), small intestine (71) and cecum (77). The highest number of lactic acid bacteria population was obtained in crop region and the smallest number was found in the gizzard. The total colony forming unites obtained from each sample site are presented in **Table 1.**

The initial identification of lactic acid bacteria was done based on gram reaction, catalase tests and acid tolerance. In the preliminary screening of acid tolerance test, among the 190 isolates, 78 (41%) were able to grow at pH 4, from which 73 isolates were gram positive. The bacterial cells were either rod (61 isolates, 83.6%) or round shape (12 isolates, 16.4%). All these gram positive isolates were catalase negative. Further pH tolerance test showed that 34 of the 73 isolates survived at pH3. Of these 34 isolates 17 (50%) isolates showed tolerance to 0.05% bile salt.

Probiotics Potential Characteristics Tests

Acid and Bile Tolerance: Survival assay under acid condition showed that 34 isolates survived at pH 3. However, viability was decreased at pH 2 and 1 comparing with the control (pH 6.5). Fourteen isolates were able to survive at pH2 and only six isolates survived at pH 1. Lactic acid bacteria isolates that survived at low pH (pH2 and 1) and grew more slowly and the positive results were recorded after 48hrs incubation.

Bile salt tolerance assay showed that 10 isolates survived at 0.1% bile salt concentration. After acid and bile tolerance test, only 10 isolates which tolerate 0.1% bile and pH2, were selected for detailed identification and these were the ones used in downstream analyses. These 10 isolates were designated as IS1, IS2, IS3, IS4, IS5, IS6, IS7, IS8, IS9 and IS10. From these 10 isolates, only IS1, IS3, IS4, IS6, IS7, and IS9 showed resistant to 0.3% bile salt (**Table 2**).

Antibiotic Susceptibility: Antibiotic resistance assay of the LAB isolates was tested by using ampicillin, chloramphenicol, ciprofloxacin and erythromycin antibiotic discs. Isolate IS1, IS2, IS5, IS7 and IS8 were resistant (diameter ≤15mm) to all used antibiotics. IS10 was found to be sensitive to all used antibiotics (diameter ≥21mm). Isolates of IS4, IS6 and IS9 were shown to be

Antimicrobial Activity of Selected LAB Isolates: Antimicrobial activity of isolates was tested against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Staphylococcus intermedius* and *Salmonella enteritidis*. All the isolates exhibited variable range of inhibition against the growth of the selected pathogens. Maximum size of inhibition zone (17.83mm) was shown to be against *S. typhimurium* by IS6. IS6 showed the maximum zone of inhibition against *E.coli* (17.66mm), *S.aureus* (16.00mm), *S. typhimurium* (17.83mm) and *S.enteritidis* (14.50mm). IS2 showed the minimum size of zone of inhibition against all used pathogens except *E. coli* (**Figure 1**).

Temperature and Sodium Chloride Tolerance: In this study all isolates were able to survive from 25-50^{0}c. The optimum temperature for all isolates was 45^{0}c (**Figure 2**). As shown in Table 5 the isolated lactic acid bacteria species showed a variable capacity to survive at different concentrations of NaCl. All the isolates were able to grow at 4% (0.69 mol/L) of NaCl, but none of the isolates could grow in 8% (1.36 mol/L) of NaCl (**Table 4**).

Molecular Identification of Selected Isolates

Genotypically, the four selected LAB isolates namely, IS3, IS4, IS6 and IS7 were identified based on sequence analysis of 16S rRNA gene. DNA fragments of PCR amplification products were about 1500bp (**Figure 4**). The 16S rRNA gene sequence results were successfully aligned and compared with known sequences obtained from GenBank. Based on the 16S rRNA sequence result, one isolate (IS3) was 98.4% similar to *Lactobacillus salivarius*NR_112759.1 and two isolates namely, IS4 and IS7 were 97 %, similar to *Lactobacillus reuteri* NR_075036.1. IS6 was 96.5% similar to *Lactobacillus reuteri* NR_113820.1. All accession numbers were obtained from the GenBank.

The 16S rRNA gene sequences of the four isolates (IS3, IS4, IS6 and IS7) were deposited in the GenBank database under the accession numbers **MK764683** to **MK764686** (**Table 6**).

Discussion

The poultry industry is an important economic activity across the world. Serious economic losses resulted when poultry are exposed to stressful environmental conditions and diseases. Emergences of a large variety of pathogens and antibiotics resistance bacteria are the main limiting factors for productivity of the poultry industry [14]. Inclusion of antibiotic growth promoters in chicken diets for disease management and growth promotion has been associated with the emergence of antibiotic resistance bacteria. As a result the global concern focuses on combating usage of antibiotics as growth promoter [15].

Chicken reared under tropical Africa conditions are considered to have a wide diversity of uncharacterized GIT microbiota which can be a good source of probiotics. Thus, isolation of endogenous probiotic microorganism is considered as a potential probiotic source to alleviate main problems related to chicken production [13].In the present study potential lactic acid bacteria strains were isolated from the GIT of RIR

chicken purchased from local farmers in Ethiopia. According to the farmers these RIR were pure breed and were doing healthy.

From the gastrointestinal tract content of the five chicken the lactic acid bacteria population was isolated from gizzard, cecum, small intestine and crop. The highest number of lactic acid bacteria population was found in the crop region (8.1 log CFU g^{-1}) and the smallest number was found in the gizzard (6.2 log CFU g^{-1}).

Similar to the present study, Janneh*et al*., (2014)[16] found from 7-9 log CFU g^{-1} lactic acid bacteria population in the crop, gizzard, small intestine and cecum regions of chicken. The variation in lactic acid bacteria population may caused by environment variation, producing facility, age of the chicken, feed and water [17]. The presence of gastric juices, pepsin and hydrochloric acid in the gizzard reduces the pH, resulting in lower bacterial population number [18].

In previous studies, various species of probiotic bacteria such as *L. reuteri*[19, 20], *L. salivarius*[19, 21, 16,22], *Enterococcus faecium* and *Enterococcus durans* [22]were isolated from chicken digestive tract. In the present study, four LAB isolates namely, IS3, IS4, IS6 and IS7 were identified based on sequence analysis of 16S rRNA gene. These isolates were identified as *L. salivarius*(IS3) and *L. reuteri*(IS4, IS6 and IS7). The sequence of these isolates is published in GenBank as *Lactobacillus salivarius* strain CEL1 (IS3), *Lactobacillus reuteri* strain CEC2 (IS4), *Lactobacillus reuteri* strain CEC3 (IS6) and *Lactobacillus reuteri* strain CEC4 (IS7).

In general, microorganisms with potential probiotic advantage share common characteristics [23]. Lower pH tolerance, salt tolerance, bile acid resistance, utilization of different carbon sources (degradation of oligosaccharides), hemolytic properties, antibiotics sensitivity, antimicrobial activity and in vitro adherence properties are the major tests used to isolate probiotic microorganisms from different sources [24]. For this reason, different tests were performed to check whether the selected strains display the listed probiotic properties. Conventionally, probiotic microorganisms should be; safe to the host, resistance to GIT *in vivo* conditions, adhere and colonize to intestinal tissue, survive and resist during processing and storage and should have antimicrobial activity [25].

The probiotics strains should have a property to grow and survive in the host digestive system as they exposed to a range of stressful conditions in the GIT which includes lower pH and high bile salt [26].The ability of probiotic strains to act as probiotics is also determined by the ability to survive in the low pH of the stomach and in the high concentration of bile salt of the gastrointestinal tract [19]. In the present study, from 10 isolates 60% of the isolates were able to grow at pH1. On the other hand, bile salt tolerance

assay showed that all isolates survived at 0.1% bile salt concentration and only 6 isolates showed resistant to 0.3% bile salt.

Similar results were reported by Ehrmann *et al*., (2002) [27] who found the strains of *L. reuteri*, *L. salivarius* and *L. animalis* that tolerate pH 2 and 1 for 4h and 1h, respectively. Janneh *et al*., (2014) [16] also found different *L. salivarius* strains that survived at pH 2, and different *Lactobacillus* strains including *L. reuteri* that exhibited 0.3% bile after 6hrs incubation were found [26]. Similarly, Ehrmann *et al*., (2002) [27] reported *L. reuteri* that resist 2% ox gall. Bile released in the small intestine damages bacteria by destroying the bacterial cell membrane. Lactic acid bacteria had bile salt hydrolase enzyme (BSH), which hydrolyze bile salts and reduce their solubility [16].

The results of antibiotics susceptibility test showed that isolate IS1, IS2, IS5, IS7 and IS8 were resistance to all used antibiotics and IS10 was found to be sensitive to all the tested antibiotics. Apart from IS10, all isolates were resistance to ampicillin. IS3 showed intermediate resistance to ciprofloxacin and chloramphenicol. In addition IS9 showed intermediate resistance to ciprofloxacin. Jose *et al*., (2015) [26] also reported different *Lactobacillus* spp. pattern that showed resistance and sensitivity to different antibiotics including ciprofloxacin, erythromycin, ampicillin and chloramphenicol. Shakor *et al*., (2017) [28] reported that no influence of ampicillin on the growth of LAB population. Unlike the result of this study, Singh *et al*., (2014) [29] reported different strains of *L. reuteri* which were sensitive to erythromycin and chloramphenicol.

The resistance of the probiotics isolate to some antibiotics is considered as intrinsic property rather than transmissible, presenting no safety concerns in feed or food. Basically, intrinsic resistance to some antibiotics is regarded as an advantage of the probiotics [25]. Probiotics strains may be exposed to antibiotics in the animal gastrointestinal tract when antibiotics is used as animal health therapeutics. As a result to be an effective probiotics, the probiotics strains should possess non-transferable resistance which aids them *in vivo* survival [26].

The present study showed that all the isolates were able to ferment galactose, glucose, fructose, mannitol, sorbitol and sucrose. Except IS2, IS5 and IS10, all isolates utilize maltose as a carbon source. According to Shokryazdan *et al*., (2014) [21], *L.salivarius* species were able to ferment galactose, glucose, fructose, mannitol, sorbitol and sucrose. On the other hand, according to Jannah *et al*., (2014) [16], *L. salivarius* species were unable to metabolize starch. Carbohydrates especially, oligosaccharide metabolism is essential for ecological fitness of *lactobacilli*. *Lactobacillus* species have different intracellular enzymes to metabolize various carbon sources. However most of *lactobacilli* species are not

amylolytic [30]. Besides lack of appropriate enzyme, absence of intracellular transport system to take up the carbohydrate may prevent the bacteria to metabolize that carbohydrate [16]. Other studies also confirmed similar carbohydrate fermentation profiles of different *lactobacilli* species [21, 31]. Thus, it is difficult to accurately differentiate *lactobacilli* isolates through carbohydrate fermentation [32].

Antagonistic activity of probiotics microorganism against pathogens is regarded as a characteristic of probiotic to maintain the gut microflora balanced and to keep the gut rid of pathogens. Probiotics inhibits the growth of pathogenic bacteria through production of; nonspecific antimicrobial compounds such as short chain fatty acid, hydrogen peroxide and low-molecular-weight proteins [33].

The result of this study showed the antibacterial property of isolates against *E.coli*, *S. aureus*, *S. typhimurium*, *S. intermedius* and*S. enteritidis*. All the isolates exhibited variable range of inhibition against the growth of the selected pathogens. Maximum size of inhibition zone (17.83mm) was shown against *S. typhimurium* by IS6. IS2 showed the minimum size of zone of inhibition against all used pathogens except *E. coli.*

Different strains of lactic acid bacteria inhibit the growth of bacteria including *E. coli*, *S. typhimurium*, *S. aureus*, *C. perfringens*, *Klebsiella*spp. and *Proteus* spp. by bonding to the specific receptors and causing cell damage [34]. Janneh*et al*., (2014) [16] reported different *L. salivarius* strains that shown inhibition against *E.coli* and*S. enteritidis*. This antagonistic activity of probiotics microorganism against pathogens is a role of probiotic to maintain the gut microflora balanced and to keep the gut rid of pathogens.

As a safety requirement, probiotics should be harmless to the host[35] and one of the tests used to assess the safety of probiotics is haemolytic activity test [26]. In this study none of the isolates showed any sign of haemolysis when grown on blood agar. Similar results were found by [36]. This makes the isolates harmless to their host.

As shown from Figure 2, the isolates had different hydrophobicity result ranging from 26.4 to 79.3%. The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization [35]. Cellular hydrophobicity indicates the adhesion ability of LABs to enterocytic cellular lines. High adhesive ability of probiotic bacteria has the greatest beneficial effects to colonize the host gut [36].

In this study, all isolates were able to survive from 25-50^{0}c. The optimum temperature for all isolates was 45^{0}c. This ability of isolates will enable them to survive under a wide range of temperature during processing, storage and transport [37]. In the present study, the isolated lactic acid bacteria spp. showed a variable capacity to survive at different concentrations of NaCl. All the isolates were able to grow at 4% (0.69 mol/L) of NaCl, but none of the isolates could grow in 8% (1.36 mol/L) of NaCl. The most tolerant isolates to high NaCl concentrations could survive in the gastrointestinal tract of the animal. The ability to resist high salt concentrations is important for probiotic bacteria to maintain their osmotic balance in order to survive and grow in the gastrointestinal tract which has an osmolarity equivalent to 0.3mol/L [19].

According to Pundir*et al*., (2013) [38] LAB isolates were able to survive at temperature between 25 and 40^{0}C and 1.5-6% NaCl. According to Kobierecka *et al*., (2017) [19] different strains of *L. salivarius* showed variable resistance to 0.69 and 1mol/L NaCl and no *L. reuteri* strain showed survival on 0.69mol/L NaCl. To prevent excessive reduction of pH lactic acid bacteria pumps alkali outside and convert the free acid to its salt form. This causes elevation of the osmotic pressure on bacterial cells. For this reason, isolation of potential lactic acid bacteria strains especially for commercial production depends on high osmotolerance feature [19].

Conclusions

In the present study, four probiotic bacterial strains were isolated from the GIT content of Rhode Island Red chickens in Ethiopia and identified by 16sRNA gene sequence. One of the isolates (IS3) was identified as *L. salivarius* and IS4, IS6 and IS7 was identified as *L.reuteri*. All isolates demonstrated resistance to low pH and high bile salt, strong hydrophobicity to hydrocarbon and antagonistic activity against *E. coli*, *S. aureus*, *S. typhimurium*, *S. intermedius* and*S. enteritidis*.Considering the above potential probiotic characteristics, the isolates may be used as probiotics candidates in poultry farm. Further *in vivo* assays are recommended to evaluate the isolates as a probiotic supplement for chicken.

Abbreviations

AGP- Animal Growth Promoters

ANOVA- One-way Analysis of Variance

BLASTN- Basic Local Alignment Search Tool

CFU- Colony Forming Unit

CFS- Cell Free Supernatant

DNA-Deoxy Ribonucleic Acid

GIT- Gastro Intestinal Tract

LAB-Lactic Acid Bacteria

MEGA- Molecular Evolutionary Genetics Analysis

NCBI- National Center for Biotechnology Information

PCR- Polymerase Chain Reaction

RIR-Rhode Island Red

rRNA- ribosomal Ribonucleic Acid

Declarations

Ethics Approval and Consent to Participate

Before the beginning of the study an ethical approval letter was obtained from Research Ethical Committee of Institute of Biotechnology, University of Gondar. All animal work was conducted according to the institution guidelines for animal welfare. Consent to participate is not applicable for this study.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and the 16S rRNA gene sequences of isolates are deposited in the GenBank database under their accession numbers.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Kibrnesh T. organized the manuscript, performed all the laboratory works, analyzed and interpreted the data. Kagira J.M. and Nega B. were a major contributor in guiding the work, and revising and editing the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Total lactic acid bacteria population obtained from samples of gastrointestinal tract of Rhode Island Red Chicken incubated in MRS agar for 48hrs.

Sample site	Number of colony forming units (log CFU g^{-1}±SD (n=3))
Crop	8.15 ± 0.09
Gizzard	6.20 ± 0.35
Small intestine	7.64±0.10
Cecum	6.76± 0.32

Table 2: pH and Bile salt tolerance of selected LAB isolates.

Isolate	Viable lactic acid bacteria isolates (Log10CFU/mL)						
	pH Tolerance				Bile Salt Tolerance		
	pH6.5	pH3	pH2	pH1	0.05%	0.1%	0.3%
IS1	8.01±0.02*	7.24±0.08	6.97±0.21	ND	7.96±0.03	7.62±0.11*	6.99±0.11*
IS2	8.03±0.02	7.36±0.04*	6.93±0.08	ND	7.93±0.04	7.09±0.07	ND
IS3	8.02±0.03	7.35±0.09*	6.96±0.12	4.16±3.6*	7.92±0.04	7.59±0.04*	7.02±0.08*
IS4	8.02±0.03	7.43±0.08*	6.97±0.03	ND	7.96±0.04	7.58±0.07*	7.22±0.13*
IS5	8.01±0.04	7.36±0.07	6.92±0.08	4.26±3.7	7.93±0.03	7.19±0.14*	ND
IS6	7.99±0.06	7.39±0.13	6.91±0.12	6.46±0.24**	7.96±0.01	7.65±0.06*	7.03±0.77*
IS7	7.99±0.05*	6.22±1.94*	6.84±0.32	ND	7.92±0.02	7.69±0.08	7.09±0.69*
IS8	7.98±0.02	7.39±0.12	6.85±0.22	6.35±0.31**	7.91±0.05*	7.10±0.10*	ND
IS9	8.00±0.04	7.36±0.05	7.30±0.47	4.1±3.55*	7.92±0.01	7.70±0.06	6.51±0.24*
IS10	8.02±0.02	7.35±0.10	6.93±0.92	4.15±3.6*	7.97±0.02	7.66±0.06	ND

Note: Means in the same column with superscripts differ significantly(P<0.05); ND=not detected

Table 3: Antibiotics susceptibility test results of the selected LAB isolates to various antibiotics

Isolate	Antibiotic disc			
	Erythromycin	Chloramphenicol	Ampicillin	Ciprofloxacin
IS1	R	R	R	R
IS2	R	R	R	R
IS3	R	I	R	I
IS4	S	R	R	R
IS5	R	R	R	R
IS6	S	R	R	S
IS7	R	R	R	R
IS8	R	R	R	R
IS9	S	R	R	I
IS10	S	S	S	S

Note: R= resistance, I= intermediate, S= sensitive

Table 4: Tolerance of isolated *lactobacilli* to different concentrations of sodium chloride tolerance.

Isolate	Growth at different NaCl concentration		
	4%	6%	8%
IS1	+	+	-
IS2	+	+	-
IS3	+	+	-
IS4	+	+	-
IS5	+	+	-
IS6	+	+	-
IS7	+	+	-
IS8	+	+	-
IS9	+	+	-
IS10	+	+	-

Note: +: growth, -: absence of growth

Table 5: Carbohydrate fermentation pattern of selected lactic acid bacteria isolates

Carbon source (Substrate)	Carbohydrate Fermentation Pattern									
	IS1	IS2	IS3	IS4	IS5	IS6	IS7	IS8	IS9	IS10
Starch	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Maltose	+	-	+	+	-	+	+	+	+	-
Mannitol	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	+	+	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-

Note: +: positive reaction, -: negative reaction

Table 6: Identification of isolates using 16S rRNA gene sequencing

Isolate	Accession number	Nearest matched species from GenBank	Similarity (%)
IS3	MK764683	*Lactobacillus survilles*NR_112759.1	98.43
IS4	MK764684	*Lactobacillus reuteri* NR_075036.1	97.90
IS6	MK764685	*Lactobacillus reuteri* NR_113820.1	97.61
IS7	MK764686	*Lactobacillus reuteri* NR_075036.1	96.55

Figure 5 shows the phylogenetic tree based on the 16S rRNA gene sequence analysis, depicting the phylogenetic relationships among the four *Lactobacillus* strains and 10 *Lactobacillus* type strains obtained from the GenBank. *Clostridium perfringens* (M59103.1) was used as outgroup. Strains IS4 (**MK764684)**, IS6 (**MK764685)** and IS7 (**MK764686)** were closest to *Lactobacillus reuteri* NR_075036.1 with a bootstrap value of

96%. On the other hand, IS3 (**MK764683)** was clustered together with *Lactobacillus salivarius*NR_112759.1 with a bootstrap value of 72%.

Figures

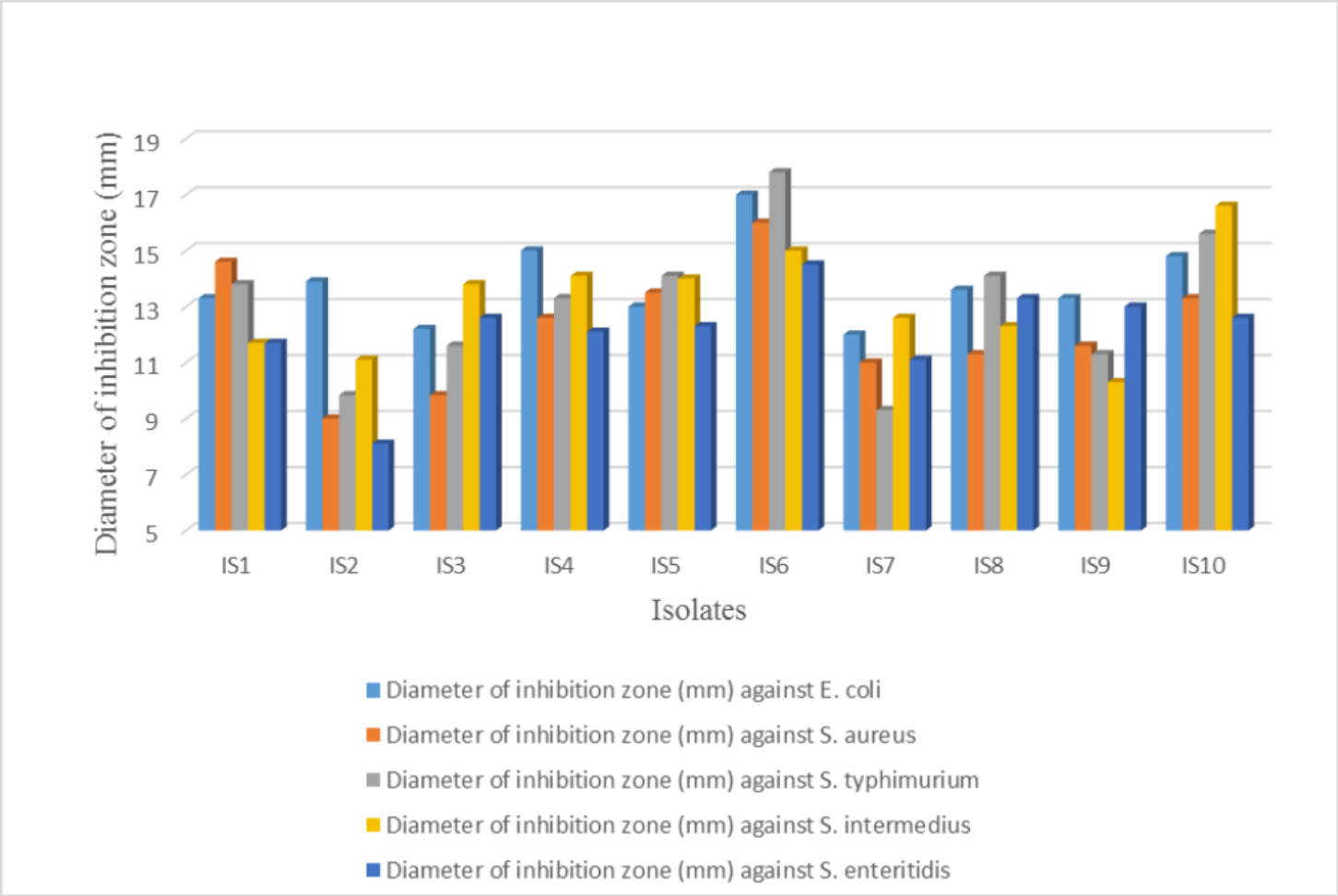

Figure 1

Antimicrobial activity of lactic acid bacteria isolates against selected pathogenic bacteria.

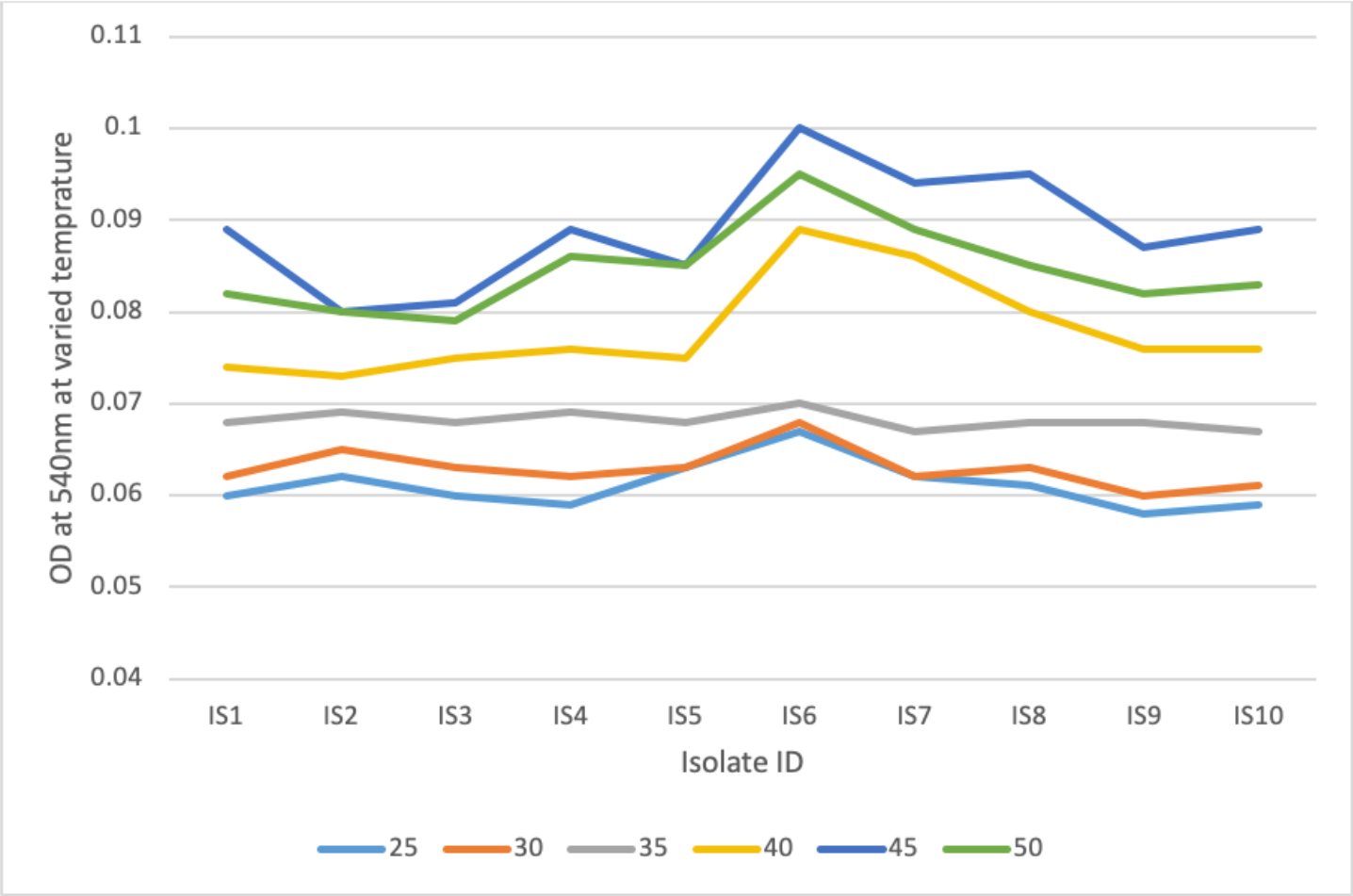

Figure 2

Temperature tolerance of isolated lactobacilli Cell Surface Hydrophobicity and Haemolytic Activity: The isolates showed a different hydrophobicity result ranging from 26.4 to 79.3% (Figure 3). The IS2 isolate showed the highest ($P<0.05$) hydrophobic activity against toluene. IS1, IS8 and IS10 exhibited showed less than 30% hydrophobicity. In this study none of the isolates showed any sign of haemolysis when grown on blood agar.

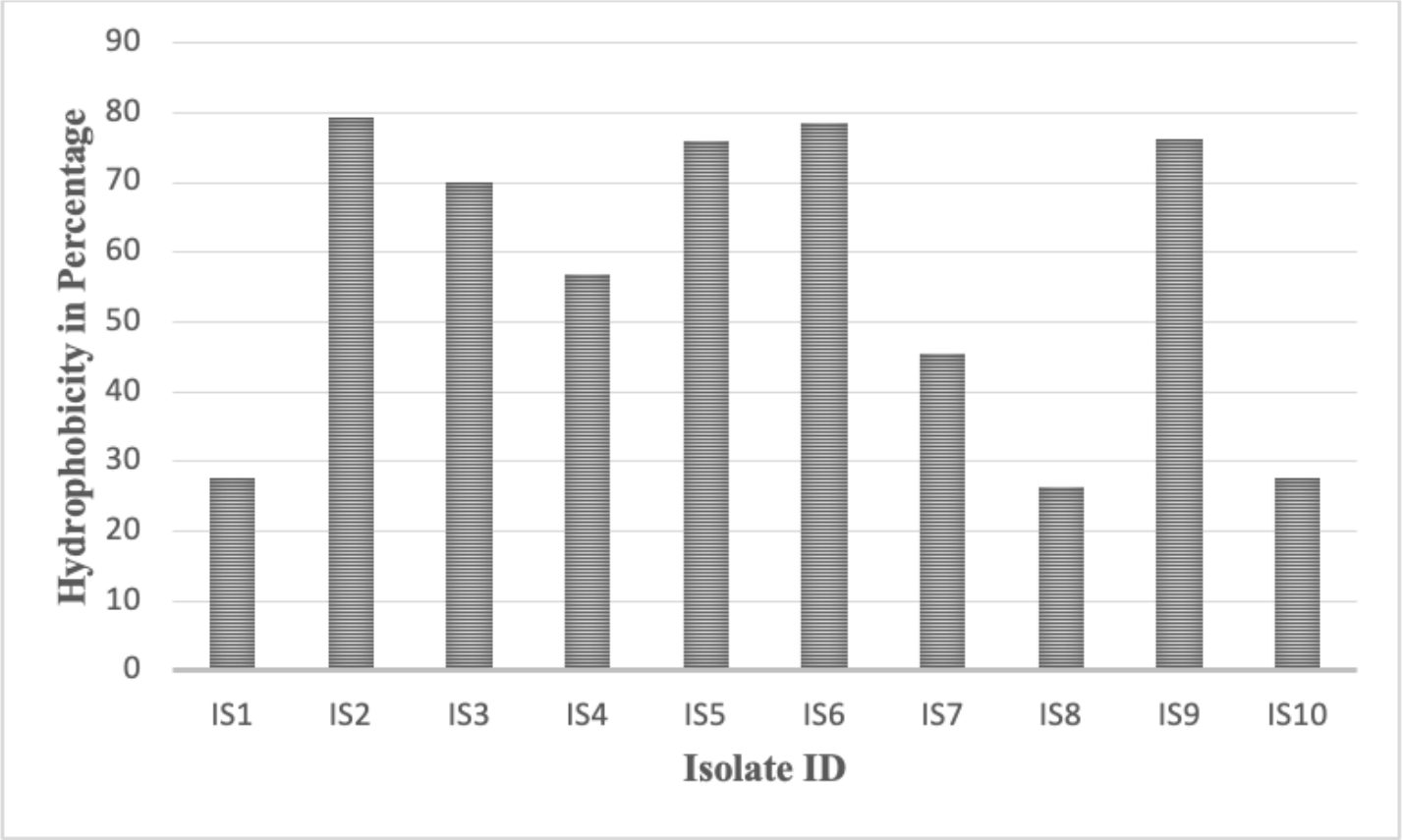

Figure 3

Cell surface hydrophobicity assay of lactic acid bacteria isolates Carbohydrate Fermentation: The result of carbohydrate fermentation profiles of the isolates is presented in Table 5. All the isolates were able to ferment galactose, glucose, fructose, mannitol, sorbitol and sucrose. Apart from IS2, IS5 and IS10, all the isolates were able to ferment maltose and only IS4 and IS5 wereable to ferment inositol. On the other hand, no isolate were able to ferment starch, arabinose, ribose cellobiose, glycerol and rhamnose.

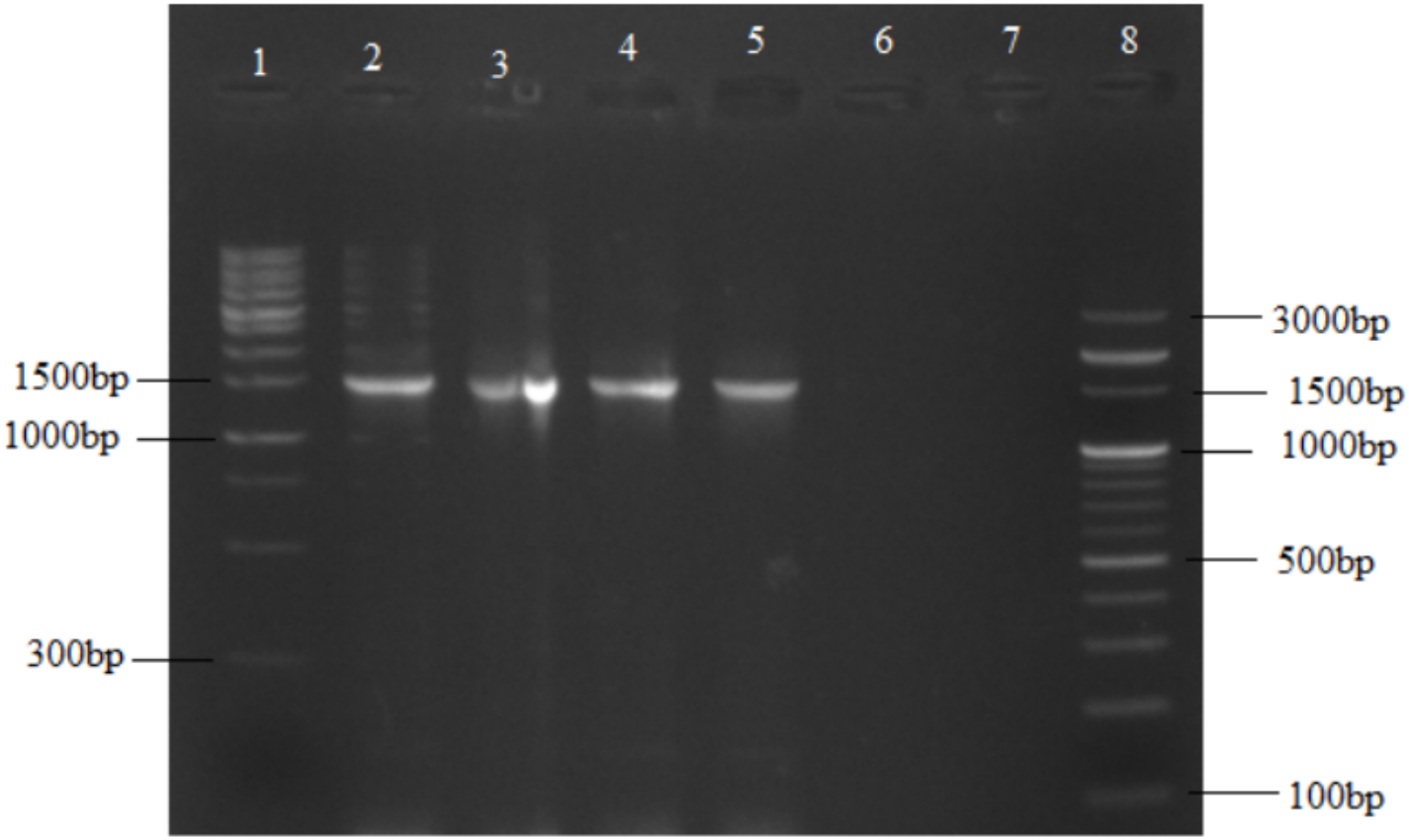

Figure 4

1% agarose gel analysis of PCR amplification products of 16S rRNA gene of lactic acid bacteria isolates. Lane 1: 1kb DNA ladder, lane 2, 3, 4 and 5: PCR amplification products of 16S rRNA gene of isolates (which are around 1500bp), lane 6 and 7: PCR negative controls and lane 8: 100bp DNA ladder

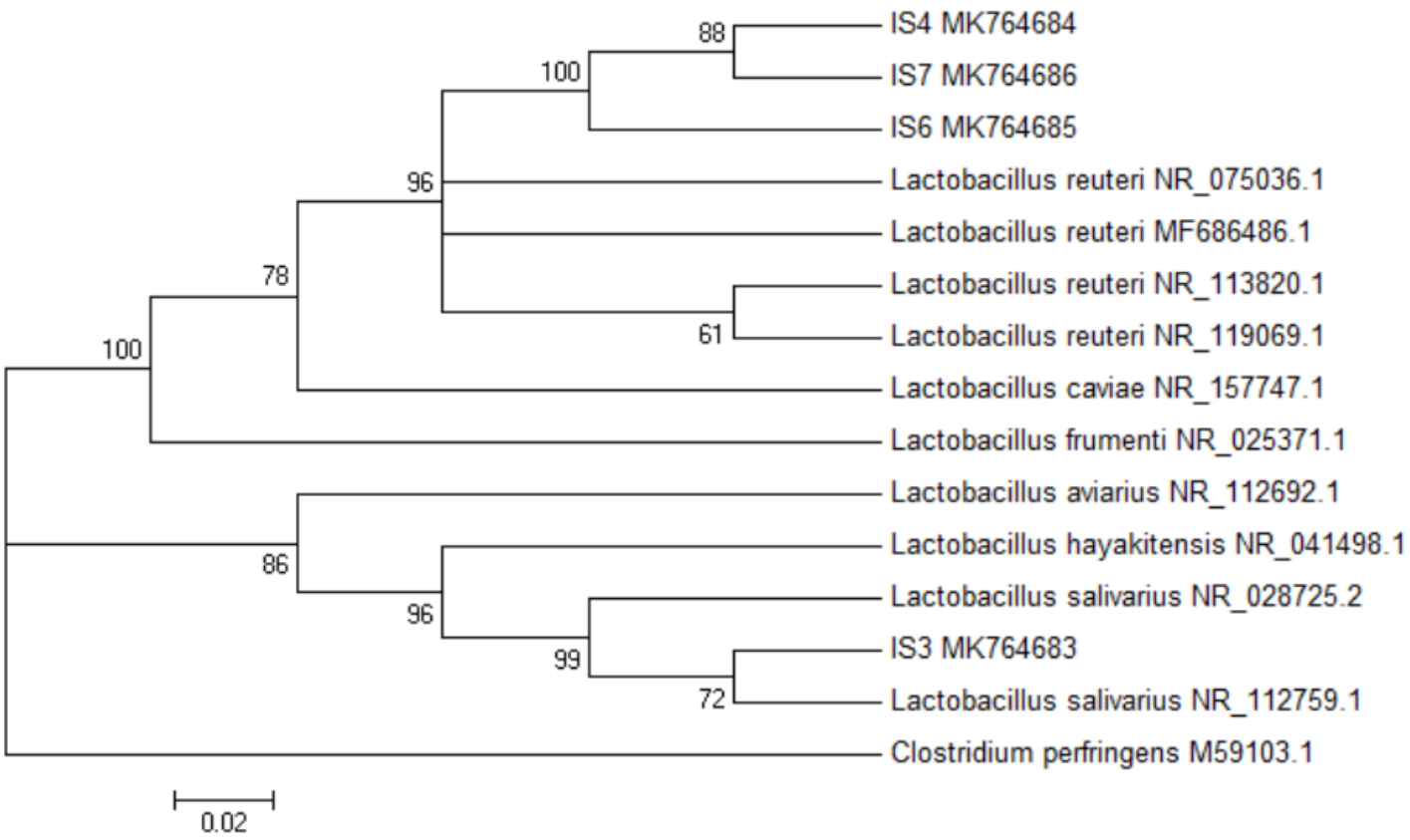

Figure 5

Phylogenetic tree based on 16S rDNA gene sequences analysis of LAB isolates. The position of IS3 (MK764683), IS4 (MK764684), IS6 (MK764685) and IS7 (MK764686) among selected lactobacilli is presented in the figure. Neighbor-joining method was used to generate the tree. The out group was C. perfringens. Bootstrap values based on 1000 replications are indicated at the nodes of the tree. The scale bar indicates 0.02 substitutions per nucleotide position. Accession numbers for sequences obtained from NCBI database.

Supplementary Files

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