Heliyon 9 (2023) e17453

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Characterization of potential probiotics Lactobacillus species isolated from the gastrointestinal tract of Rhode Island Red (RIR) chicken in Ethiopia

Kibrnesh Tegenaw Tsega [a,c,*], Kagira J. Maina [b], Nega Birhan Tesema [c]

[a] Department of Molecular Biology and Biotechnology, Pan-African University of Institute of Basic Science, Technology and Innovation, P.O. Box 62000–00200, Nairobi, Kenya
[b] Department of Animal Sciences, Jomo Kenyatta University of Agriculture and Technology, P.O.Box 62000–00200, Nairobi, Kenya
[c] Institute of Biotechnology, University of Gondar, P.O. Box 196, Gondar, Ethiopia

ARTICLE INFO

Keywords:
Chicken
Lactobacillus
Probiotics
RIR

ABSTRACT

Background and aims: Nowadays, probiotic microorganisms are given high attention due to their potential for the improvement of animal production and productivity. The natural gut microflora of poultry can serve as an excellent source of optimum probiotic strains. Therefore, this study isolated, identified and characterized potential probiotic *Lactobacillus* strains from the digestive tract content of RIR chicken in Ethiopia.
Methods: A total of five RIR chickens were randomly taken and a sample was taken from each gastrointestinal content for further analysis. For further characterization, among 190 isolates only 10 representative isolates were randomly taken for further *in vitro* probiotic potential characterization. The selected isolates were screened and identified based on the biochemical, morphological, and 16S rRNA gene sequences.
Results: Survival isolates of IS3, IS4, IS6, and IS7 were significantly different ($P < 0.05$) at pH 2. IS3, IS4, IS6, and IS7 showed tolerance for 0.3% bile salt. Isolates of IS1, IS2, IS5, IS7, and IS8 were ampicillin-resistant, and chloramphenicol, ciprofloxacin, and erythromycin were used as antibiotics. All ten isolates showed antagonistic activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Staphylococcus intermedius,* and *Salmonella enteritidis*. The optimum temperature for all isolates was 45 °C, and all the isolates could grow at 0.69 mol/L of NaCl. Using phylogenetic analysis of the 16SrRNA gene sequence; IS3 was identified as *Lactobacillus salivarius,* while IS4, IS6, and IS7 were identified as *Lactobacillus reuteri.*
Conclusions: The results indicated that the selected *Lactobacillus* isolates can survive the stress conditions of the gastrointestinal tract and can thus be considered potential probiotic candidates for chickens.

1. Introduction

Probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance [1–3].

* Corresponding author. Department of Molecular Biology and Biotechnology, Pan-African University of Institute of Basic Science, Technology and Innovation, P.O. Box 62000–00200, Nairobi, Kenya.
E-mail address: kibiramare372@gmail.com (K.T. Tsega).

https://doi.org/10.1016/j.heliyon.2023.e17453
Received 17 October 2022; Received in revised form 12 June 2023; Accepted 18 June 2023
Available online 25 June 2023

These live microorganisms are nonpathogenic and nontoxic in nature, which is favourable to the host's health when adequately administered through the digestive route [1]. Probiotic microorganisms can be isolated from plants, food products, the environment, and human and animal sources [4]. Different studies have shown that the natural gut microflora of poultry serves as an excellent source of optimal probiotic strains [5].

In Ethiopia the main constraints for exotic chicken production includes feed shortage, hot climate and diseases [6]. In addition, the clean hatchery systems of exotic chick hatching process can lead to the acquisition of aberrant microbiota in the Gastrointestinal tract (GIT) of newly hatched chicks [7]. Due to this reason survival and adaptability of newly hatched chick after dissemination to local farmers and chicken production companies is low [8]. To fill these gaps and to improve the productive performance of exotic Rhode Island Red (RIR) chicken in Ethiopia, research and development of probiotics suitable for the local poultry industry may play a significant role. As alternative to antibiotics, supplementation of newly hatched chicken with microbial probiotic preparations is important to restore the protective gut microflora [9].

Different microbial species have been used as probiotics, including; species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, various yeast species (mainly *Saccharomyces*), and *Pediococcus* [10–13]. Different strains belonging to similar species have different properties and so effects or benefits can differ from one strain to another within the same species [14,15]. Depending on the host organism or animal species, ruminant, pig, or poultry, specific microorganisms are preferred as probiotics [16]. In developed countries, *Lactobacillus* species are commonly used in broiler chickens [17]. However, in developing countries like Ethiopia, isolation, and the use of *Lactobacillus* species have not yet been studied and are not to be given attention.

The use of probiotics is described as an alternative to antibiotics [18], and it has been advocated for use for chicken as it has roles in disease prevention, mortality reduction, environmental stress tolerance, and increased productivity [19,20]. For this purpose, potential probiotic bacteria can be isolated from the GIT of well-adapted (chickens grown in the study area from day old) chicken, which can be used to develop probiotics. Therefore, the main objective of this study was to isolate, identify and characterize probiotic *Lactobacillus* species from the GIT of Rhode Island Red (RIR) exotic chickens in Ethiopia.

2. Materials and methods

2.1. Ethical approval

All aspects of animal care and experimentation in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and followed the EEC directive of 1986 (86/609/EEC) and the ethical committee approved the study, Institute of Biotechnology, University of Gondar, Ethiopia.

2.2. Study area

This study was conducted in the University of Gondar animal production farm in Gondar town, Amhara Region, Ethiopia which is located between 8°45′N and 13°45′N latitudes and 35°46′ E and 40°25′E longitudes in northwest Ethiopia (Fig. 1) [21]. In this region, poultry farming mainly involves domestic chicken production due to less adaptability and the high mortality rate of exotic chickens [22].

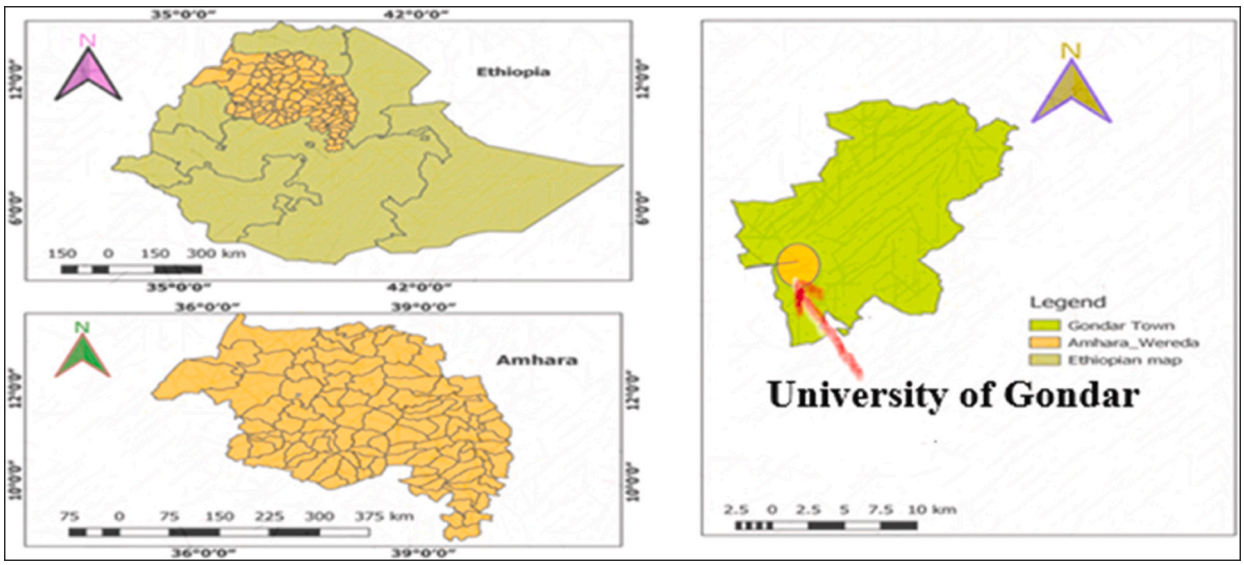

Fig. 1. Map of the study area.

2.3. Isolation of Lactobacillus species from the GIT tract of chickens

Five healthy and 10 to 12 months male RIR chickens were randomly selected and purchased from a local community living around the central Gondar zone. The chickens were killed manually by cervical dislocation and slaughtered aseptically as described by Ref. [23] to isolate *Lactobacillus*. The gastrointestinal digestive tracts (crop, gizzard, small intestine, and cecum) were used as *Lactobacillus* sources (Fig. 2).

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 (pH 6.8) for further processing. The sample buffer mixtures were vortexed for 5 min and serial dilutions were made. Afterwards, samples were plated over sterilized deMan, Rogosa, and Sharpe (MRS) agar medium (Himedia, India) and incubated under anaerobic conditions at 37 °C for 48 h. An anaerobic jar with an anaerobic gas generating kit (Oxoid Aneorgen Gas Kit, UK) was used to maintain the anaerobic condition. From each MRS agar plate, colonies were isolated based on their morphological differences (shape, size, and colour). Purification of colonies was made using sub-culturing selected colonies on MRS agar.

2.4. Initial identification and preliminary screening of isolates

Morphological examination of colonies, gram staining, and catalase test were used for the 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 the 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 °C in MRS broth provided with 20% glycerol for the remaining experiments [24].

2.5. Probiotic potential characterization of bacterial isolates

According to Refs. [25,26] some of the conventional criteria that can be used for the selection of microbial strains to be used as probiotics includes; biosafety, the choice of the origin of the strain, resistance to GIT *in vivo* conditions (lower pH, bile and pancreatic juice), adherence and colonization of intestinal epithelium/tissue, antimicrobial activity, stimulation of immune response and survival and resistance during processing and storage.

2.5.1. 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 an acid tolerance test as described by Ref. [27]. MRS broth pH 6.5 was used as a control. Each treatment was tested in triplicates.

2.5.2. Bile salt tolerance

The bile tolerance assay was performed as described according to Ref. [24] with modifications. One milliliter of an overnight culture of each isolate at a final concentration of 7–8 log CFU/ml was inoculated into 9 ml of fresh MRS broth with 0.05%, 0.1%, or 0.3% (w/v) of Difco oxgall (BD bioscience, USA) and incubated anaerobically at 37 °C for 4 h. MRS broth without bile salt (Oxgal) was used as a control. After 4hrs incubation, 10-fold serial dilutions of up to 10^{-7} were prepared using PBS. Then 100 μl of 10^{-5} to 10^{-7} dilutions from each sample was spread plated on MRS agar plates and incubated anaerobically at 37 °C for 24 h, 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

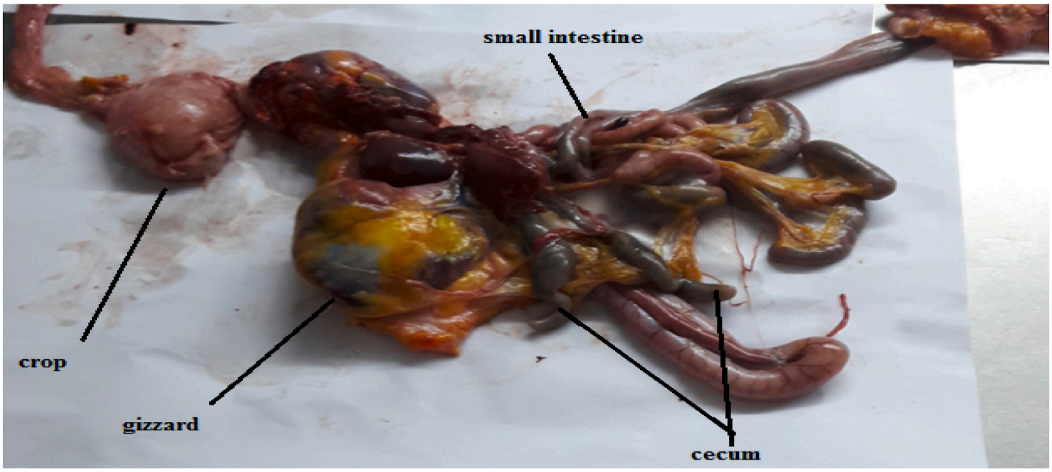

Fig. 2. Isolation of *Lactobacillus* from different parts of gastrointestinal digestive tracts (crop, gizzard, small intestine, and cecum).

without bile (Oxgall). The assay was performed in triplicates.

2.5.3. Antibiotic sensitivity

Antibiotic susceptibility of selected *Lactobacillus* isolates was determined according to Ref. [24] using commercial antibiotic discs (Himedia, India) 100 μl of cell suspensions were spread over the entire surface of Muller Hinton agar (Himedia, India) containing plates. Then, a paper disc containing antibiotics commonly used antibiotics for chicken of; ampicillin (30 μg), ciprofloxacin (30 μg), chloramphenicol (30 μg), and erythromycin (15 μg) were placed on the plates. Subsequently, the plates were incubated anaerobically at 37 °C for 24 h. The antibiotic sensitivity of the isolates was determined by measuring the diameter of the clear zone around the antibiotic discs.

2.5.4. Temperature and sodium chloride salt tolerance

For the determination of growth at various temperatures, freshly grown *Lactobacillus* isolates were inoculated into MRS broth and incubated at 25, 30, 35, 40, 45, and 50 °Cs for 24 h. The growth of the isolates at each temperature was evaluated by observing the turbidity of the culture medium. The test was performed in triplicates. To determine the sodium chloride (NaCl) tolerance of bacterial isolates, MRS Broth adjusted with different concentrations of NaCl (4%, 6%, and 8%) was inoculated with a fresh overnight culture of bacterial isolates. This test was performed as described by Ref. [28].

2.5.5. Haemolytic activity

The haemolytic activity of *Lactobacillus* isolates was determined by following the method described by Ref. [29]. *Streptococcus pyrogen* was used as a positive control. The assay was performed in triplicate.

2.5.6. Antimicrobial activity of the selected isolates

The antimicrobial activity of *Lactobacillus* isolates was determined using the good diffusion assay technique. For this test, the bacterial isolates were cultured in MRS broth and incubated overnight. Alternatively, the pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Staphylococcus intermedius,* and *Salmonella enteritidis*) obtained from the Microbiology laboratory, College of Veterinary Medicine, the University of Gondar previously isolated from chicken were grown in Brain Heart Infusion broth (Himedia, India). The selected pathogenic bacteria were common pathogenic bacteria for chicken. To determine the antimicrobial activity of isolates, 100 μL 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 using a cork borer. To get the antimicrobial-containing supernatant from the isolated bacterial culture, the cultures were centrifuged at 13,000 rpm for 1min. Then, 100 μL of cell-free supernatant (CFS) whose pH was adjusted to neutral, was added to each well [29,30]. After that, the plates were incubated at 37 °C for 24hrs. The antimicrobial activity of the bacterial isolates was determined based on the formation of inhibition zones around the wells. The diameter (in millimeters) of the clear inhibition zone around the wells was measured and the size of the clear zone was directly proportionate to the antagonistic activity of the isolate. Isolates that show a wide spectrum of antimicrobial activity were selected for further analysis [31].

2.5.7. Cell surface hydrophobicity

Cell surface hydrophobicity was determined using the method described by Ref. [32].

2.6. Molecular characterization of selected isolates (16S ribosomal Ribonucleic acid (rRNA) gene sequencing)

After *in vitro* characterization of *Lactobacillus* isolates, four isolates with the best probiotic potential were randomly selected and subjected to 16S rRNA gene sequencing to identify them at the species level. For 16S rRNA gene sequencing, genomic Deoxy Ribonucleic Acid was extracted from overnight cultured *Lactobacillus* cells using the Bacterial Genomic DNA Isolation Kit (Himedia, India) following the manufacturer's instructions. After DNA isolation, the 16S rRNA gene was amplified using the universal primer, 27F [5'AGAGTTTGATCCTGGCTCAG 3'] and 1492R [5'TACGGCTACCTTGTTAGGACTT 3'] as described by Ref. [31]. The PCR amplification was performed by optimizing the procedure described by Ref. [31]. That was; a 40 μl PCR mix was prepared by adding 8 μl FIRE pol master mix, 2 μl DNA (20 ng/μl), 0.4 μl (100pm) of each forward and reverse primer and nuclease-free water. The PCR amplification condition was initial denaturation at 95 °C for 3mins followed by 35 cycles consisting of denaturation at 95 °C for the 30s, annealing at 60 °C for 45s and extension at 72 for 1min, and final extension at 72 °C for 7 min. Confirmation of PCR products was done through agarose gel electrophoresis using 1% agarose gel. The PCR product purification and sequencing were conducted by sending the PCR products to Macrogen (Europe).

2.7. Sequence alignments and phylogenetic tree Construction

The DNA sequence data of each isolate were obtained by sequencing the PCR products. The DNA sequences were compared with available sequences in the GenBank. Sequence similarity values were determined using the Basic Local Alignment Search Tool (BLASTN) of the National Center for Biotechnology Information (NCBI). A 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 alignments were performed 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 performed using the Tamura 3-parameter method [33,34].

2.8. Data analysis

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

3. Results and discussion

3.1. Isolation and preliminary screening of Lactobacillus bacteria

A total of 190 bacterial colonies were randomly selected from the cultures of samples taken from the GIT of the five chickens. Initial screening was done based on different colony morphological characteristics such as shape, colour, appearance, and size. The initial Lactobacillus bacteria were identified based on Gram reaction, catalase tests, and acid tolerance. In the preliminary screening of the acid tolerance test, among the 190 isolates, 78 (41%) could grow at pH 4, of which 73 isolates were gram-positive. These gram-positive 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 tests revealed that 34 of the 73 isolates survived at pH 3. Of these 34 isolates, 17 (50%) isolates showed tolerance to 0.05% bile salt.

3.2. Probiotics potential characteristics tests

3.2.1. Acid and bile tolerance

Table 1 shows selected *Lactobacillus* isolates digestive tract parts, and pH and Bile salt tolerance. A survival assay under acidic conditions showed that 34 isolates survived at pH 3. However, viability was decreased at pH 3 and 2 compared with the control (pH 6.5). Fourteen isolates could survive at pH 2. *Lactobacillus* isolates survived at low pH (pH 2 and 3) and grew more slowly and the positive results were recorded after 48hrs incubation (Table 1).

The bile salt tolerance assay revealed that 10 isolates (isolated from cecum, small intestine and crop) survived at 0.1% bile salt concentration. However, the isolates taken from gizzard were not selected due to being unable to grow at pH2 and 0.1% bile salt concentration. After the acid and bile tolerance test, only 10 isolates that 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. Of these 10 isolates, only IS1, IS3, IS4, IS6, IS7, and IS9 showed resistance to 0.3% bile salt.

Probiotic microorganisms must survive under gastrointestinal stress factors to maintain their biological function within the host [35,36]. The ability of probiotic strains to act as probiotics is also determined by their ability to survive in the low pH of the stomach and the high concentration of bile salt in the gastrointestinal tract [37]. In this study, *Lactobacillus* isolates could grow up to pH 2. However, the bile salt tolerance assay showed that all isolates survived at 0.1% bile salt concentration and only 6 isolates were resistant to 0.3% bile salt. Similar results were reported in the study of [27] who found that the strains of *L. reuteri*, *L. salivarius,* and *L. animalis* that tolerate pH 2 for 4hr.

[24] also found different *L. salivarius* strains that survived at pH 2. (Jose, 2015 #13) found different *Lactobacillus* strains, including *L. reuteri* that exhibited 0.3% bile after 6hr incubation. Similarly [27], reported *L. reuteri* that resists 2% ox gall. Bile released in the small intestine damages bacteria by destroying the bacterial cell membrane. *Lactobacillus* has a bile salt hydrolase enzyme (BSH), which hydrolyzes bile salts and reduces their solubility [24].

Table 1
Selected *Lactobacillus* isolates digestive tract parts, and pH and Bile salt tolerance.

Isolates	Parts	Viable lactic acid bacteria isolates (Log_{10} CFU/mL)					
		pH Tolerance			Bile Salt Tolerance		
		pH 6.5	pH 3	pH 2	0.05%	0.1%	0.3%
IS1	Ce	8.01 ± 0.02*	7.24 ± 0.08	6.97 ± 0.21	7.96 ± 0.03	7.62 ± 0.11*	6.99 ± 0.11*
IS2	Ce	8.03 ± 0.02	7.36 ± 0.04*	6.93 ± 0.08	7.93 ± 0.04	7.09 ± 0.07	ND
IS3	Ce	8.02 ± 0.03	7.35 ± 0.09*	6.96 ± 0.12*	7.92 ± 0.04	7.59 ± 0.04*	7.02 ± 0.08*
IS4	Ce	8.02 ± 0.03	7.43 ± 0.08*	6.97 ± 0.03*	7.96 ± 0.04	7.58 ± 0.07*	7.22 ± 0.13*
IS5	Ce	8.01 ± 0.04	7.36 ± 0.07	6.92 ± 0.08	7.93 ± 0.03	7.19 ± 0.14*	ND
IS6	Ce	7.99 ± 0.06	7.39 ± 0.13	6.91 ± 0.12*	7.96 ± 0.01	7.65 ± 0.06*	7.03 ± 0.77*
IS7	SI	7.99 ± 0.05*	6.22 ± 1.94*	6.84 ± 0.32*	7.92 ± 0.02	7.69 ± 0.08	7.09 ± 0.69*
IS8	Ce	7.98 ± 0.02	7.39 ± 0.12	6.85 ± 0.22	7.91 ± 0.05*	7.10 ± 0.10*	ND
IS9	Cr	8.00 ± 0.04	7.36 ± 0.05	7.30 ± 0.47	7.92 ± 0.01	7.70 ± 0.06	6.51 ± 0.24*
IS10	SI	8.02 ± 0.02	7.35 ± 0.10	6.93 ± 0.92	7.97 ± 0.02	7.66 ± 0.06	ND

The values are expressed as mean ± SD. Means within the same column are significantly different ($P < 0.05$); ND=Not Detected; IS=Isolates; Ce=Cecum; SI=Small Intestine; Cr=Crop.

3.2.2. Antibiotic susceptibility

Antibiotic resistance assay of the *Lactobacillus* isolates was tested using ampicillin, chloramphenicol, ciprofloxacin, and erythromycin antibiotic discs (Fig. 3a and b). IS10 was found to be sensitive to all used antibiotics (diameter ≥21 mm) (Fig. 3a, b). Isolate IS1, IS2, IS5, IS7, and IS8 were resistant (diameter ≤15 mm) to all used antibiotics (Fig. 3b).

Isolates of IS4, IS6, and IS9 were shown to be sensitive to erythromycin (Table 2). The results of the antibiotics susceptibility test showed that isolates IS1, IS2, IS5, IS7, and IS8 were resistant to all used antibiotics and IS10 was found to be sensitive to all the tested antibiotics. Apart from IS10, all isolates were ampicillin-resistant. IS3 showed intermediate resistance to ciprofloxacin and chloramphenicol. Additionally, IS9 showed intermediate resistance to ciprofloxacin.

According to the reports of [29] the different *Lactobacillus* spp. patterns showed resistance and sensitivity to different antibiotics, including ciprofloxacin, erythromycin, ampicillin, and chloramphenicol [28]. reported that no influence of ampicillin on the growth of *Lactobacillus* population. Unlike the result of this study [38], reported different strains of *L. reuteri* were sensitive to erythromycin and chloramphenicol.

The resistance of the probiotics isolated to some antibiotics is considered an intrinsic property rather than transmissible, presenting no safety concerns in feed or food. Intrinsic resistance to some antibiotics is an advantage of probiotics [26]. Probiotic strains may be exposed to antibiotics in the animal gastrointestinal tract when antibiotics are used as animal health therapeutics. As a result, to be effective probiotics, the probiotic strains should possess non-transferable resistance, which aids them *in vivo* survival [29].

3.2.3. Antimicrobial activity of selected lactobacillus isolates

The antimicrobial activity of the isolates was tested against *E. coli*, *S. aureus*, *S. typhimurium*, *S. intermedius,* and *S. enteritidis*. All the isolates exhibited a variable range of inhibition against the growth of the selected pathogens (Fig. 4). The maximum size of the inhibition zone (17.83 mm) was shown to be against *S. typhimurium* by IS6. IS6 showed the maximum zone of inhibition against *E. coli* (17.66 mm), *S. aureus* (16.00 mm), *S. typhimurium* (17.83 mm), and *S. enteritidis* (14.50 mm). IS2 showed the minimum size of the zone of inhibition against all used pathogens except *E. coli.*

The antimicrobial activity of *Lactobacillus* isolates against selected pathogenic bacteria is shown in Fig. 5. The maximum size of the inhibition zone (17.83 mm) was shown to be against *S. typhimurium* by IS6. Antagonistic activity of probiotic microorganisms against pathogens is a characteristic of probiotics that maintain the gut microflora balanced and keep the gut rid of pathogens. Probiotics inhibit the growth of pathogenic bacteria through the production of nonspecific antimicrobial compounds such as short-chain fatty acid, hydrogen peroxide, and low-molecular-weight proteins [30]. The results of this study showed the antibacterial properties of isolates against *E. coli, S. aureus, S. typhimurium, S. intermedius,* and *S. enteritidis*. All the isolates exhibited a variable range of inhibition against the growth of the selected pathogens. The maximum size of the inhibition zone (17.83 mm) was shown against *S. typhimurium* by IS6. IS2 showed the minimum size of the zone of inhibition against all used pathogens except *E. coli*.

Different strains of *Lactobacillus* 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 [39]. [24] reported different *L. salivarius* strains that showed inhibition against *E. coli* and *S. enteritidis*. This antagonistic activity of probiotic microorganisms against pathogens is the role of probiotics to maintain the gut microflora balanced and to keep the gut rid of pathogens.

3.3. Temperature and sodium chloride tolerance

In this study, all isolates could survive from 25 to 50 °C. The optimum temperature for all isolates was 45 °C (Fig. 6). The isolated *Lactobacillus* species showed a variable capacity to survive at different concentrations of NaCl. All the isolates could grow at 4% (0.69

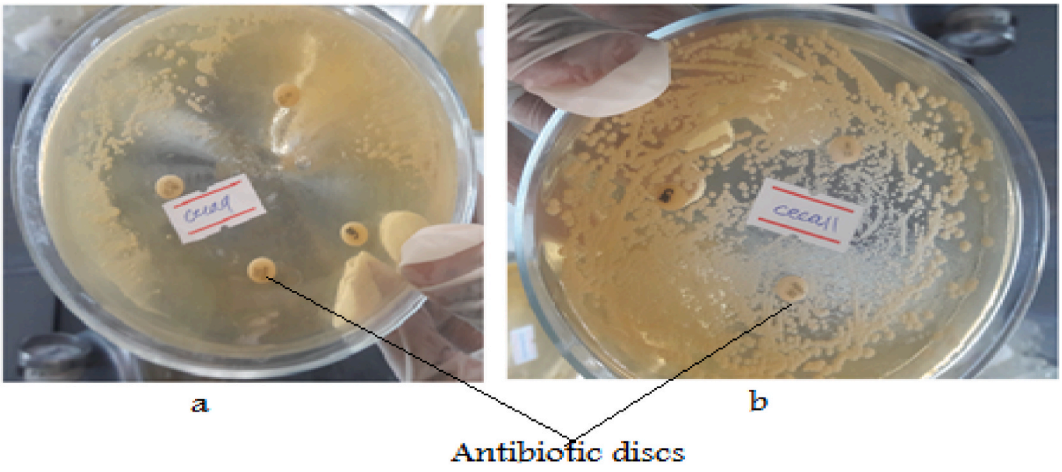

Fig. 3. Antibiotics susceptibility pattern of different Lactobacillus isolates to various antibiotics. (a) = antibiotic sensitive isolate and (b) = antibiotic resistance isolate.

Table 2
Antibiotics susceptibility test results of the selected *Lactobacillus* isolates for various antibiotics.

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

Were R = resistance; I = intermediate' S = sensitive.

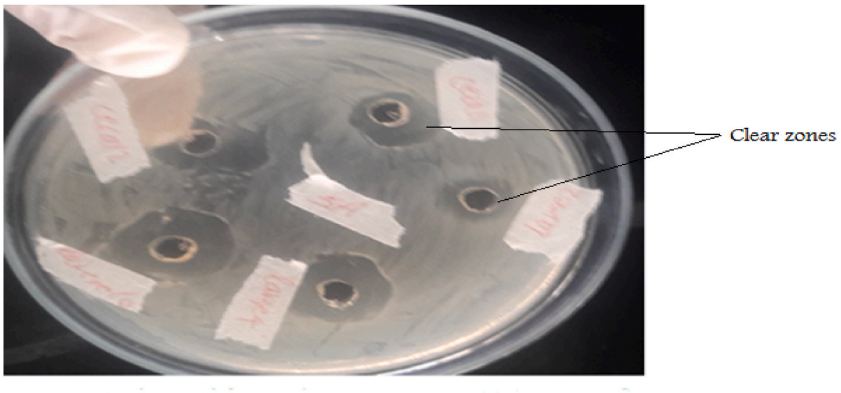

Fig. 4. Antagonistic activity of selected isolates against *S. aureus*.

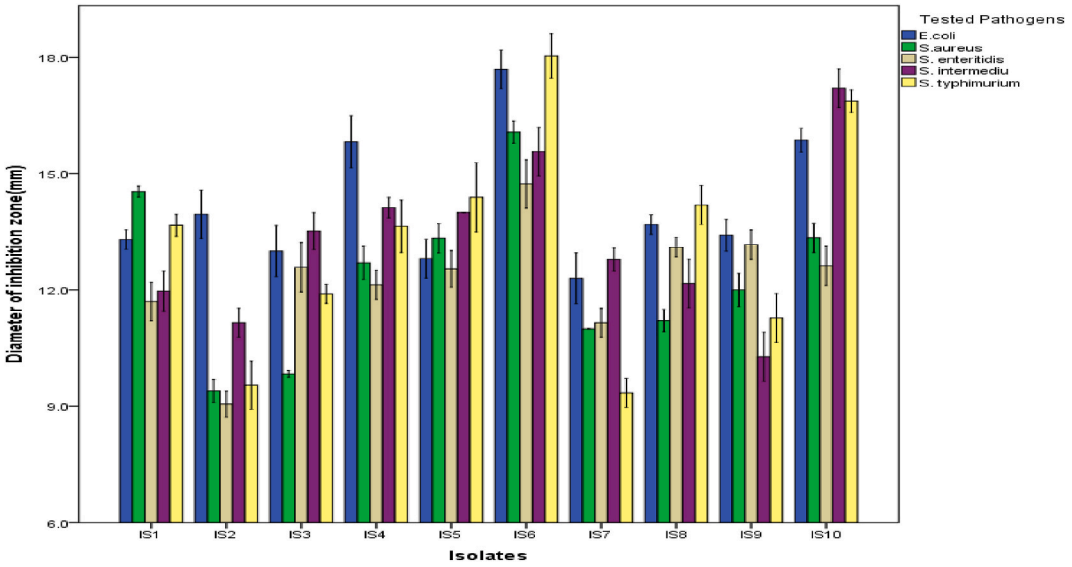

Fig. 5. Antimicrobial activity of *Lactobacillus* isolates against selected pathogenic bacteria.

mol/L) and 6% of NaCl, but none of the isolates could grow at 8% (1.36 mol/L) of NaCl.

In this study, all isolates could survive from 25 to 50 °C. The optimum temperature for all isolates was 45 °C. This ability of isolates will enable them to survive under various temperatures during processing, storage, and transport [36,40]. In this study, the isolated lactic acid bacteria spp. showed a variable capacity to survive at different concentrations of NaCl. All the isolates could grow at 4% (0.69 mol/L) of NaCl, but none of the isolates could grow at 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 to survive and grow in the gastrointestinal tract, which has an osmolarity equivalent to 0.3 mol/L [37].

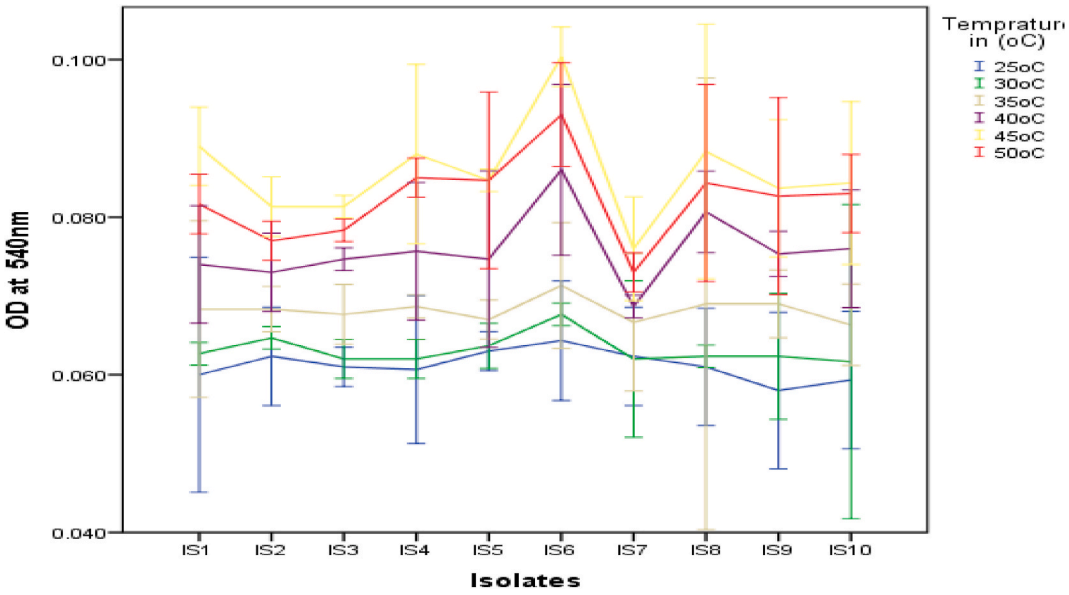

Fig. 6. Temperature tolerance of isolated *Lactobacillus* bacteria. The optimum temperature for all isolates was 45 °C.

According to Ref. [41] *Lactobacillus* isolates can survive at temperatures between 25 and 40 °C and 1.5%–6% NaCl. According to Ref. [37] different strains of *L. salivarius* showed variable resistance to 0.69 and 1 mol/L NaCl, and no *L. reuteri* strain showed survival on 0.69 mol/L NaCl. To prevent excessive reduction of pH lactic acid, bacteria pump alkali outside and convert the free acid to its salt form. This elevates the osmotic pressure on the bacterial cells. For this reason, the isolation of potential lactic acid bacteria strains especially for commercial production depends on the high osmotolerance feature [37].

3.3.1. Cell surface hydrophobicity and haemolytic activity

The isolates showed different hydrophobicity results ranging from 26.4 to 79.3% (Fig. 7). 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 hemolysis when grown on blood agar.

As a safety requirement, probiotics should be harmless to the host [42] and a test used to assess the safety of probiotics is the hemolytic activity test [29]. In this study, none of the isolates showed any sign of haemolysis when grown on blood agar. Similar results were recorded [43]. This makes the isolates harmless to their host.

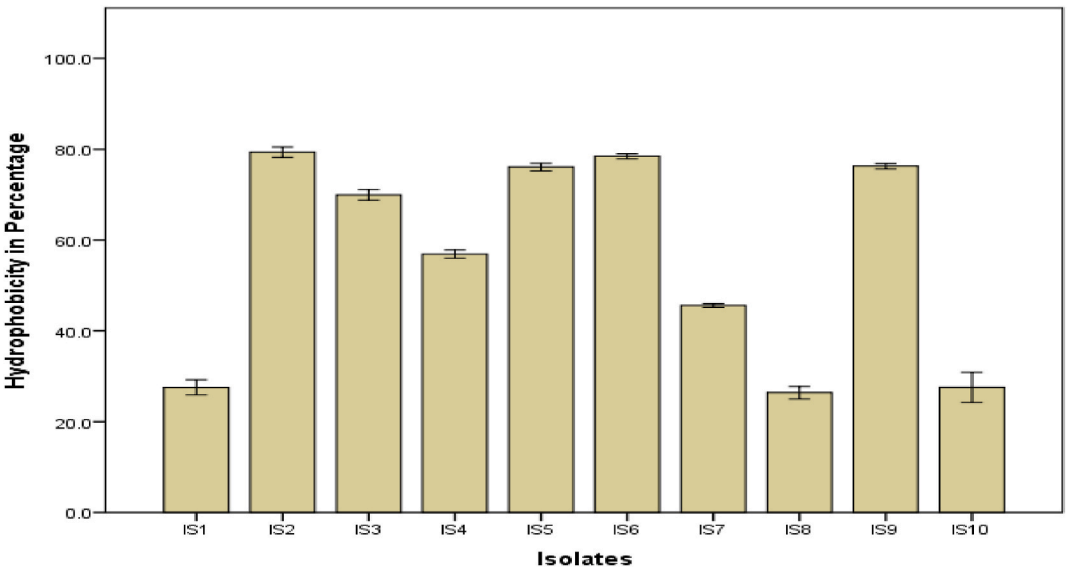

Fig. 7. Cell surface hydrophobicity assay of *Lactobacillus* isolates.

As shown in Fig. 2, the isolates showed different hydrophobicity results 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 a prerequisite for colonization [42,44]. Cellular hydrophobicity indicates the adhesion ability of *Lactobacillus* to enterocytic cellular lines. The high-adhesive ability of probiotic bacteria has the greatest beneficial effects on colonizing the host gut [43].

3.4. Molecular identification of selected isolates

The molecular identification of isolates using 16S rRNA gene sequencing is shown in Table 3. Based on the above results, IS3, IS4, IS6, and IS7 isolates were selected for molecular identification. These isolates showed the best probiotic properties based on their survival at pH 2 ($P < 0.05$), tolerance to 0.3% bile salt, cell surface hydrophobicity, and antimicrobial activity. Genotypically, the four selected *Lactobacillus* isolates, namely IS3, IS4, IS6, and IS7, were identified based on sequence analysis of the 16S rRNA gene.

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 GenBank. The 16S rRNA gene sequences of the four isolates (IS3, IS4, IS6, and IS7) are deposited in the GenBank database under the accession numbers MK764683 to MK764686 (Table 3). The sequences of these isolates are 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).

Fig. 8 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) were used as an outgroup. Strains IS4 (MK764684), IS6 (MK764685), and IS7 (MK764686) were closest to *Lactobacillus reuteri* NR_075036.1 with a bootstrap value of 96%. However, IS3 (MK764683) was clustered together with *Lactobacillus salivarius* NR_112759.1 with a bootstrap value of 72%.

Generally, microorganisms with potential probiotic advantages share common characteristics [46]. Lower pH tolerance, salt tolerance, bile acid resistance, use of different carbon sources (degradation of oligosaccharides), hemolytic properties, antibiotic sensitivity, antimicrobial activity, and *in vitro* adherence properties are the major tests used to isolate probiotic microorganisms from different sources [47].

This study shows, chickens reared under tropical African conditions are considered to have a wide diversity of uncharacterized GIT microbiota that can be a good source of probiotics. Thus, the isolation of endogenous probiotic microorganisms is considered a potential probiotic source to alleviate the main problems related to chicken production [15].

In previous studies, various species of probiotic bacteria such as *L. reuteri* [37,48], *L. salivarius* [4,24,37,49] *Enterococcus faecium* and *Enterococcus durans* [49] were isolated from the chicken digestive tract.

4. Conclusion

In this study, ten potential probiotic *Lactobacillus* bacterial strains were isolated from the GIT content of Rhode Island Red chickens in Ethiopia. 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*. Based on the above-indicated potential probiotic characteristics, the isolates may be used as probiotic candidates in poultry farms.

5. Limitations

The limitation of the present study was a small number of samples were taken to isolate bacteria from chickens for probiotics formulation. The other limitation was the lack of financial resources for further study on *in vivo* assays to evaluate the isolates as a probiotic supplement for chicken.

Declarations

Author contribution statement

Kibrnesh Tegenaw, Jone Kagira and Nega Birhan conceived and designed the experiments; Kibrnesh Tegenaw performed the experiments, analyzed and interpreted the data and contributed reagents; Kibrnesh Tegenaw, Jone Kagira and Nega Birhan wrote the paper.

Ethics approval

Ethical approval was obtained from the Research Ethical Committee of Institute of Biotechnology University of Gondar Ref. No: IOB/486/07/2019.

Table 3
Molecular identification of isolates using 16S rRNA gene sequencing.

Isolate	Accession number	Nearest matched species from GenBank	Similarity [45]
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

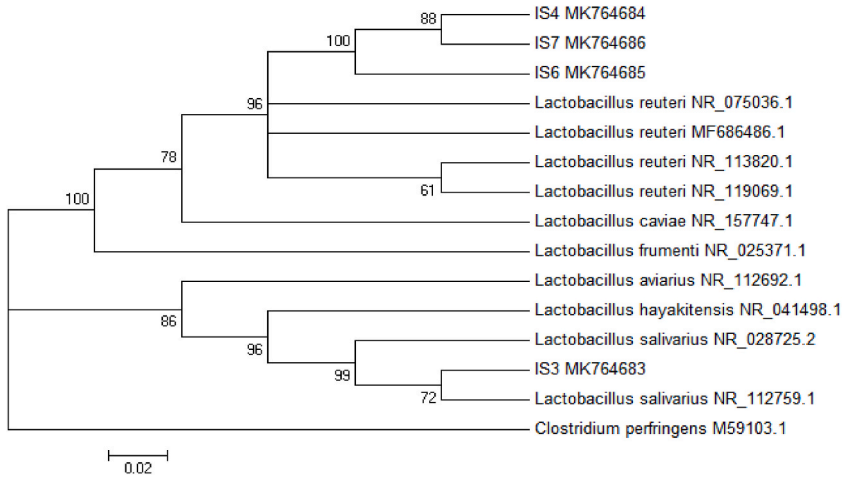

Fig. 8. Phylogenetic tree based on 16S rDNA gene sequence analysis of *Lactobacillus* isolates. The neighbour-joining method was used to generate the tree. The out group was *C. perfringens*. Bootstrap values based on 1000 replications are indicated at the nodes of the tree. The scale bar indicated 0.02 substitutions per nucleotide position. Accession numbers for sequences obtained from the NCBI database.

Data availability statement

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 (MK764684 to MK764686).

Funding statement

This work was funded by the Pan African University Institute for Basic Sciences, Technology and Innovation (PAUSTI) with reference number PAU/ADM/PAUSTI/004/17.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to give special thanks to Pan African University for the sponsorship of.
this research work was funded. We are also grateful to the University of Gondar, Ethiopia, for this research laboratory work.

References

[1] A. Szydłowska, B. Sionek, Probiotics and postbiotics as the functional food components affecting the immune response, Microorganisms 11 (1) (2022) 104, https://doi.org/10.3390/microorganisms11010104.
[2] B. Sánchez, et al., Probiotics, gut microbiota, and their influence on host health and disease, Mol. Nutr. Food Res. 61 (1) (2017), 1600240, https://doi.org/10.1002/mnfr.201600240.
[3] R. Nourizadeh, et al., Impact of Probiotics in Modulation of Gut Microbiome. Microbiome-Gut-Brain Axis: Implications on Health, Springer Nature Singapore, 2022, pp. 401–409, https://doi.org/10.1007/978-981-16-1626-6_20.
[4] P. Shokryazdan, et al., Probiotics: from isolation to application, J. Am. Coll. Nutr. 36 (8) (2017) 666–676, https://doi.org/10.1080/07315724.2017.1337529.
[5] R. Jha, et al., Probiotics (direct-fed microbials) in poultry nutrition and their effects on nutrient utilization, growth and laying performance, and gut health: a systematic review, Animals 10 (10) (2020) 1863, https://doi.org/10.3390/ani10101863.

[6] H.K. Sebho, Exotic chicken status, production performance and constraints in Ethiopia: a review, Asian J. Poultry Sci. 10 (1) (2016) 30–39, https://doi.org/10.3923/ajpsaj.2016.30.39.
[7] D. Pan, Z. Yu, Intestinal microbiome of poultry and its interaction with host and diet, Gut Microb. 5 (1) (2014) 108–119, https://doi.org/10.4161/gmic.26945.
[8] M. Milkias, Review on exotic chicken status, production performance and constraints in Ethiopia, J. Biol. Agr. Heal. 6 (15) (2016).
[9] E.E. Donaldson, et al., The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs, PeerJ 5 (2017) e3587, https://doi.org/10.7717/peerj.3587.
[10] S. Doron, D.R. Snydman, Risk and safety of probiotics, Clin. Infect. Dis. 60 (suppl_2) (2015) S129–S134, https://doi.org/10.1093/cid/civ085.
[11] D.R. Silva, et al., Probiotics as an alternative antimicrobial therapy: current reality and future directions, J. Funct.Foods 73 (2020), 104080, https://doi.org/10.1016/j.jff.2020.104080.
[12] S. Fijan, Microorganisms with claimed probiotic properties: an overview of recent literature, Int. J. Environ. Res. Publ. Health 11 (5) (2014) 4745–4767, https://doi.org/10.3390/ijerph110504745.
[13] G.E.S. Negm, Classification, antimicrobial potential, industrial applications and probiotic capability of lactic acid bacteria: a review article, Res. J. Appl. Sci. 13 (12) (2018) 742–757. https://www.researchgate.net/publication/330006181.
[14] M. Bernardeau, J.-P. Vernoux, Overview of differences between microbial feed additives and probiotics for food regarding regulation, growth promotion effects and health properties and consequences for extrapolation of farm animal results to humans, Clin. Microbiol. Infection 19 (4) (2013) 321–330, https://doi.org/10.1111/1469-0691.12130.
[15] S. Lahtinen, et al., Lactic Acid Bacteria: Microbiological and Functional Aspects, Crc Press, 2011.
[16] M.I. Masood, et al., Beneficial effects of lactic acid bacteria on human beings, Crit. Rev. Microbiol. 37 (1) (2011) 91–98, https://doi.org/10.3109/1040841X.2010.536522.
[17] Q. Peng, et al., Effects of dietary Lactobacillus plantarum B1 on growth performance, intestinal microbiota, and short chain fatty acid profiles in broiler chickens, Poultry Sci. 95 (4) (2016) 893–900, https://doi.org/10.3382/ps/pev435.
[18] M. Shin, et al., Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use, J. Appl. Microbiol. 105 (6) (2008) 2203–2212, https://doi.org/10.1111/j.1365-2672.2008.03935.x.
[19] S. Yadav, R. Jha, Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry, J. Anim. Sci. Biotechnol. 10 (1) (2019) 1–11, https://doi.org/10.1186/s40104-018-0310-9.
[20] M. Mahesh, R.K. Mohanta, A.K. Patra, Probiotics in livestock and poultry nutrition and health, Adv. Prob. Sustain. Food Med. (2021) 149–179, https://doi.org/10.1007/978-981-15-6795-7_7.
[21] S.A. Mekuria, et al., Small ruminant fattening practices in Amhara region, Ethiopia, Agric. Food Secur. 7 (1) (2018) 1–9, https://doi.org/10.1186/s40066-018-0218-9.
[22] T. Sisay, K. Alemayehu, G. Zeleke, Trends of exotic chicken dissemination in north western Amhara, Ethiopia: challenges and opportunities, J. Biol. Agr. Heal. 7 (24) (2017) 41–51.
[23] C. Berg, M. Raj, A review of different stunning methods for poultry—animal welfare aspects (stunning methods for poultry), Animals 5 (4) (2015) 1207–1219, https://doi.org/10.3390/ani5040407.
[24] S.N. Jannah, et al., Characteristics of lactic acid bacteria isolated from gastrointestinal tract of Cemani chicken and their potential use as probiotics, Media Peternakan 37 (3) (2014) 182–189, https://doi.org/10.5398/medpet.2014.37.3.182.
[25] R. Fuller, Probiotics in man and animals, J. Appl. Bacteriol. 66 (5) (1989) 365–378.
[26] E.S. Khalil, et al., Probiotic characteristics of exopolysaccharides-producing Lactobacillus isolated from some traditional Malaysian fermented foods, CyTA - J. Food 16 (1) (2018) 287–298, https://doi.org/10.1080/19476337.2017.1401007.
[27] M.A. Ehrmann, et al., Characterization of lactobacilli towards their use as probiotic adjuncts in poultry, J. Appl. Microbiol. 92 (5) (2002) 966–975, https://doi.org/10.1046/j.1365-2672.2002.01608.x.
[28] G. Shakoor, et al., Isolation of lactic acid bacteria from chicken gut and its probiotic potential characterization, Int. J. Biosci. 11 (3) (2017) 1–9, https://doi.org/10.12692/ijb/11.3.1-9.
[29] N.M. Jose, et al., Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and, Microorganisms 3 (2076–2607) (2015) 198–212, https://doi.org/10.3390/microorganisms3020198.
[30] M.A. Karimi Torshizi, et al., Screening of indigenous strains of lactic acid bacteria for development of a probiotic for poultry, Asian-Australas. J. Anim. Sci. 21 (2008), https://doi.org/10.5713/ajas.2008.80081.
[31] R. Dowarah, et al., Selection and characterization of probiotic lactic acid bacteria and its impact on growth, nutrient digestibility, health and antioxidant status in weaned piglets, PLoS One 13 (3) (2018), e0192978, https://doi.org/10.1371/journal.pone.0192978.
[32] H.K. Lee, et al., Screening and characterization of lactic acid bacteria strains with anti-inflammatory activities through in vitro and Caenorhabditis elegans model testing, Kor. J. Food Sci. Anim. Resour. 35 (1) (2015) 91–100, https://doi.org/10.5851/kosfa.2015.35.1.91.
[33] B.G. Hall, Building phylogenetic trees from molecular data with MEGA, Mol. Biol. Evol. 30 (5) (2013) 1229–1235, https://doi.org/10.1093/molbev/mst012.
[34] M.I. Irawan, S. Amiroch, Construction of phylogenetic tree using neighbor joining algorithms to identify the host and the spreading of SARS epidemic, J. Theor. Appl. Inf. Technol. 71 (3) (2015) 424–429.
[35] M. Mbye, et al., Updates on understanding of probiotic lactic acid bacteria responses to environmental stresses and highlights on proteomic analyses, Compr. Rev. Food Sci. Food Saf. 19 (3) (2020) 1110–1124, https://doi.org/10.1111/1541-4337.12554.
[36] B. Kosin, S.K. Rakshit, Microbial and processing criteria for production of probiotics, Rev. Food Tech. Biotech. 44 (2006) 371–379.
[37] P.A. Kobierecka, et al., In vitro characteristics of Lactobacillus spp. strains isolated from the chicken digestive tract and their role in the inhibition of Campylobacter colonization, MicrobiologyOpen 6 (5) (2017), e00512, https://doi.org/10.1002/mbo3.512.
[38] T.P. Singh, R.K. Malik, G. Kaur, Safety assessment and evaluation of probiotic potential of Lactobacillus reuteri strains under in vitro conditions, Int. J. Curr. Microb. Appl. Sci. 3 (2) (2014) 335–348.
[39] A.A. Cisek, et al., Selection and optimization of high-yielding DNA isolation protocol for quantitative analyses of methanogenic archaea, Microorganisms 10 (3) (2022) 523, https://doi.org/10.3390/microorganisms10030523.
[40] M. Cabello-Olmo, et al., Influence of storage temperature and packaging on bacteria and yeast viability in a plant-based fermented food, Foods 9 (3) (2020), https://doi.org/10.3390/foods9030302.
[41] R.K. Pundir, S.R.N. Kashyap, A. Kaur, Probiotic potential of lactic acid bacteria isolated from food samples: an in vitro study, J. Appl. Pharmaceut. Sci. 3 (3) (2013) 85–93, https://doi.org/10.7324/JAPS.2013.30317.
[42] E. Demir, G.B. Kılıç, D. Özbalcı, Biosafety assessment of probiotics “Probiotics”, Turk. J. Agr. Food Sci. Tech. 7 (4) (2019) 639–645, https://doi.org/10.24925/turjaf.v7i4.639-645.2327.
[43] R. Balamurugan, et al., Probiotic potential of lactic acid bacteria present in home made curd in southern India, Indian J. Med. Res. 140 (3) (2014) 345–355.
[44] R. Gupta, K. Jeevaratnam, A. Fatima, Lactic acid bacteria: probiotic characteristic, selection criteria, and its role in human health (A review), J. Emerg Tech. Innov. Res.(JETIR) 5 (10) (2018) 411–424. https://ssrn.com/abstract=3462244.
[45] A. Adámková, et al., Nutritional Potential Of Selected Insect Species Reared On The Island Of Sumatra, 14(5), 2017, p. 521, https://doi.org/10.3390/ijerph14050521.
[46] M.E. Koenen, et al., Development and validation of a new in vitro assay for selection of probiotic bacteria that express immune-stimulating properties in chickens in vivo, FEMS Immunol. Med. Microbiol. 40 (2) (2004) 119–127, https://doi.org/10.1016/s0928-8244(03)00306-7.

[47] A. Sanni, et al., Characterization and technological properties of lactic acid bacteria in the production of "Sorghurt," a cereal-based product, Food Biotechnol. 27 (2) (2013) 178–198, https://doi.org/10.1080/08905436.2013.781949.
[48] M. Nakphaichit, et al., The effect of including Lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens, Poultry Sci. 90 (12) (2011) 2753–2765, https://doi.org/10.3382/ps.2011-01637.
[49] J. Buahom, S. Siripornadulsil, W. Siripornadulsil, Feeding with single strains versus mixed cultures of lactic acid bacteria and Bacillus subtilis KKU213 affects the bacterial community and growth performance of broiler chickens, Arabian J. Sci. Eng. 43 (2018) 3417–3427, https://doi.org/10.1007/s13369-017-3045-6.