Characterization of Probiotic Candidate Lactic Acid Bacteria to Overcoming Salmonellosis in Broilers

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ABSTRACT

Probiotic microorganisms are gaining popularity because they can improve feed nutrient absorption and feed conversion. The application of probiotics as an alternative to antibiotics can help improve chicken health naturally and safely. This can help reduce the risk of antimicrobial resistance and ensure safe consumption of chicken meat. The purpose of this research is to find, separate, and describe Lactobacillus probiotic strains believed to have their origins in the digestive systems of Indonesian broiler chickens. Five broiler chickens were randomly selected, and samples of their digestive contents were collected for additional investigation. To further characterize the in vitro probiotic potential, only ten isolates, randomly selected from 190 isolates, were used. Biochemical, morphological, and 16S rRNA gene sequences were used to test and identify specific isolates. All isolates (IS3, IS4, IS6, and IS7) showed tolerance to 0.3% bile salts at pH 2 with significant differences ($P < 0.05$). When ampicillin failed to cure IS1, IS2, IS5, IS7, and IS8 isolates, ciprofloxacin, chloramphenicol, and erythromycin were prescribed. These 10 isolates were shown to be in contact with Salmonella typhimurium, Salmonella enteritidis, Escherichia coli, Staphylococcus intermedius, and Staphylococcus aureus. The results showed that the chosen Lactobacillus liurarius can withstand the stress conditions of the gastrointestinal system, making them ideal probiotic options for broiler. Ten strains of probiotic Lactobacillus bacteria have been identified from the digestive tracts of broiler chickens in Indonesia, among others Lactobacillus reuteri NR_075036.1; Lactobacillus reuteri MF_686486.1; Lactobacillus reuteri NR_113820.1; Lactobacillus reuteri NR_1199069.1; Lactobacillus caviae NR_157747.1; Lactobacillus frumenti NR_025371.1; Lactobacillus aviarius NR_112691.1; Lactobacillus hayakitensis NR_041498.1; Lactobacillus salivarius NR_028725.2 and Lactobacillus salivarius NR_112759.1

Keywords: Putative Biological Agent, Lactobacilus issolate, gastrointestinal system.

INTRODUCTION

Probiotics are live microbial feed supplements that improve the host's gut microbial balance, resulting in beneficial benefits (Sanchez et al., 2017). When administered properly through the digestive tract, these live bacteria are nonpathogenic and nontoxic, which benefits the host's health (Szydlowska et al., 2022). Probiotic bacteria may be isolated from food, plants, the environment, as well as humans and animals (Shokryazdan et al., 2017). Several studies have found that the natural gut microbiota of chicken is a great source of appropriate probiotic strains (Jha et al., 2022).

In broiler farm, the primary restrictions for exotic chicken production include feed shortages, hot heat, and illness (Sebho et al., 2022). Furthermore, the pristine hatchery systems of the exotic chick hatching process might lead to the acquisition of abnormal microbiota in the gastrointestinal tract (GIT) of freshly hatched chicks (Pan et al., 2014). Because of this, the survival and adaptability of newly hatched chicks after

distribution to local farmers and poultry production firms is poor (Milkias et al., 2016). To close these gaps and increase the productive performance of exotic broiler chickens, research and development of probiotics tailored to the local poultry sector might be critical. As an alternative to antibiotics, supplementing freshly hatched chickens with microbial probiotic formulations is necessary to rebuild the protective gut microbiota (Donaldson et al., 2017).

In addition to environmental factors, commercial use of antibiotic growth promoters (AGPs) is limited due to drug resistance in enteric pathogens such as Escherichia coli. Antibiotic residues in feed and the environment endanger human and animal health (Carvalho and Santos., 2016. Gonzalez et al., 2017). One option to maximize the productivity and efficiency of raising broiler chickens is to add additional feed or probiotics as additional feed. This is consistent with the findings of Moniño et al., (2023) showed that the addition of probiotics impact on the performance of broiler chickens, including feed conversion, mortality, decreased feed consumption, increased body weight, and increased broiler carcass proportions.

Probiotics can lower mortality and disease rates in chicken by enhancing their health, which will lessen the financial losses brought on by unfavorable health circumstances. Probiotics supply the digestive system with a large number of lactic acid bacteria after consumption. It is well known that these microbes can change the environment in the stomach and introduce enzymes and other healthy materials (Anjana and Tiwari., 2022). adding considerably more L. acidophilus or mixed Lactobacillus cultures to chickens.

Numerous microbial species, including Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Streptococcus, numerous yeast species (mostly Saccharomyces), and Pediococcus (Silva et al., 2020). Diverse strains belonging to identical species have diverse features, and so impacts or advantages might vary from one strain to another within the same species (Bernardeau et al., 2013). Specific bacteria are selected as probiotics based on the host organism or animal species, whether ruminant, pig, or poultry (Masood et al., 2011). In developed countries, Lactobacillus species are commonly used in broiler chickens (Peng et al., 2016). However, in undeveloped nations, isolation and utilization of Lactobacillus species have not yet been explored and should not be given attention.

Probiotics are referred to as an alternative to antibiotics (Shin et al., 2008), it has been proposed for usage in chicken because of its involvement in illness prevention, mortality reduction, environmental stress tolerance, and higher production (Mahesh et al., 2021). Potential probiotic bacteria can be extracted from the GIT of well-adapted hens (chickens raised in the research location since day one) and utilized to manufacture probiotics. As a result, the primary goal of this work was to isolate, identify, and characterize probiotic Lactobacillus species from broiler chickens.

MATERIALS AND METHODS

Isolation Of Lactobacillus Species From Hens' Digestive Tract

Five strong male broiler chickens aged 10 to 12 months were randomly picked from a nearby hamlet in the Kediri District. The birds were euthanized aseptically by cervical dislocation according to the method described by Berg et al. (2015). Lactobacillus was found in the gastrointestinal tracts (Berg et al., 2015) crop, gizzard, small intestine, and cecum) (Fig. 2). One gram was obtained from each (crop, gizzard, small intestine, and cecum) immediately after slaughter and placed in sterile test tubes containing Phosphate-Buffered Saline (PBS) buffer (pH 6.8) for subsequent processing. The sample buffer mixtures were vortexed for 5 minutes before serial dilutions were performed. The samples were then plated on sterilized deMan, Rogosa, and Sharpe (MRS) agar medium and cultivated for 48 hours at 37°C in an anaerobic atmosphere. The anaerobic state was maintained using an anaerobic jar and an anaerobic gas generation kit (OXoid Aneorgen Gas Kit, UK). Each MRS agar plate had colonies that were classified morphological characteristics (shape, size, and color). To purify, selected colonies were subcultured on MRS agar

Initial Identification and Screening of Isolates

To identify the isolates, we performed the catalase test, morphological study of colonies, and Gram staining.

Each isolate was cultured overnight on MRS agar. Gram-stained cells were examined under a light microscope to determine morphology. Catalase-negative isolates were tested using 3% hydrogen peroxide. Based on these findings, gram-positive and catalase-negative isolates were selected, stored at -20°C in MRS broth with 20% glycerol (Jannah et al., 2014), and used for further tests.

The catalase test, morphological study of colonies, and gram staining were used to identify the selected isolates initially. For these experiments, each isolate was cultured overnight on MRS agar. A light microscope was used to examine Gram-stained cells and define their morphology. Catalase-negative isolates were chosen for the catalase test, which used 3% hydrogen peroxide. In following experiments, gram-positive and catalase-negative isolates were chosen based on findings and stored at 20 ◦C in MRS broth with 20% glycerol (Jannah et al., 2014).

Probiotic Potential Characterization Of Bacterial Isolates

Traditional criteria for selecting which microbial strains to use as probiotics include biosafety, strain origin, resistance to GIT in vivo conditions (lower pH, bile, and pancreatic juice), adherence and colonization of intestinal epithelium/tissue, antimicrobial activity, immune response stimulation, survival and resistance during processing and storage (Khalil et al., 2018).

Acid tolerance

The pH tolerance of an external culture impacts its capacity to survive in the GI tract, which is a crucial factor to consider when picking probiotics. The isolates were evaluated for acid tolerance (Ehrmann et al., 2002). MRS broth served as a pH-6.5 control sample. Each treatment was tested three times.

Bile salt tolerance

With few modifications, the bile tolerance experiment was performed as described in Ref. (Jannah et al., 2014). Using 0.05%, 0.1%, or 0.3% (w/v) Difco oXgall (BD bioscience, USA), one milliliter of each isolate's overnight culture was introduced to nine milliliters of fresh MRS broth. The combination was then incubated under anaerobic conditions for four hours at 37 degrees Celsius. As a control, MRS broth without bile salt (OXgal) was used. Following a 4-hour incubation period, 10-fold serial dilutions up to 10-7 were created using PBS. The samples were split into 100 µl of 10-5 to 10-7 dilutions and spread-plated onto MRS agar plates. The plates were then incubated anaerobically at 37 °C for 24 hours before the colonies were enumerated and quantified as CFU/ml. Viable cell counts in MRS with and without bile (OXgall) were compared to determine bile tolerance. The assay run consisted of three copies.

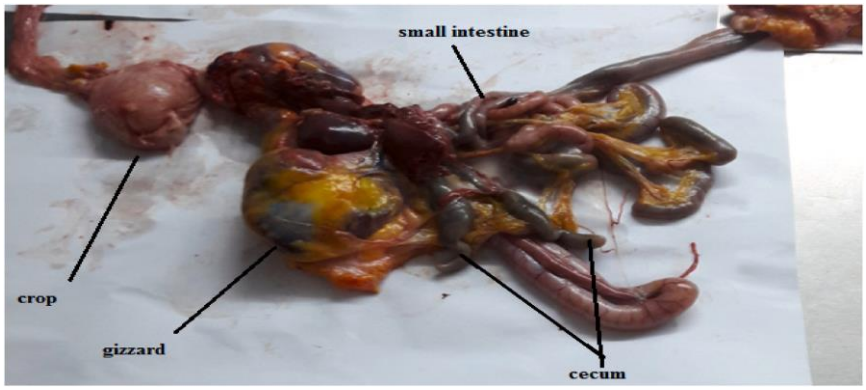

Fig. 2: Isolation of Lactobacillus from different parts of gastrointestinal digestive tracts (crop, gizzard, small intestine, and cecum).

Antibiotic sensitivity

Selected Lactobacillus isolates were tested for antibiotic susceptibility in accordance with Ref. (Jannah et al., 2014) using commercially available antibiotic CDs (Himedia, India). 100 microliters of cell suspension were

uniformly distributed throughout the Muller Hinton agar (Himedia, India)-containing plates. Next, a paper disc containing ampicillin (30 µg), ciprofloxacin (30 µg), chloramphenicol (30 µg), and erythromycin (15 µg), popular antibiotics for chicken, was placed on the plates. The plates were then anaerobically incubated for 24 hours at 37 degrees Celsius. The diameter of the clear zone surrounding the antibiotic discs was utilized to determine the isolates' antibiotic sensitivity.

Temperature and sodium chloride salt tolerance

Freshly generated Lactobacillus isolates were injected into MRS broth and incubated at various temperatures for 24 hours to assess growth. The turbidity of the culture medium was utilized to determine how well the isolates grew at different temperatures. Three copies of the test were distributed. A fresh overnight culture of bacterial isolates was injected into MRS Broth containing varied concentrations of NaCl (4%, 6%, and 8%) to determine the bacterial isolates' tolerance to sodium chloride (NaCl). This test was conducted in line with Ref's instructions (Ehrmann et al., 2017).

Haemolytic activity

The hemolytic activity of Lactobacillus isolates was tested using the technique described in Ref. (Jose et al., 2015). Streptococcus pyrogen was utilized as a positive control. The experiment was carried out in triplicate.

Antimicrobial activity of the selected isolates

Lactobacillus isolates' antibacterial activity was determined using an excellent diffusion assay method. The bacterial isolates were cultured in MRS broth and incubated during the test period. Alternatively, previously isolated pathogens (Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Staphylococcus intermedius, and Salmonella enteritidis) from chicken were grown in Diponegoro University Laboratory. The dangerous bacteria selected were often detected in hens. To examine the isolates' antimicrobial activity, 100 µL of each pathogen was coated on Muller Hinton agar plates. The bacterial cell density was calculated using McFarland (0.5%). These culture plates were perforated with a cork borer to create wells. The isolated bacterial culture was centrifuged at 13,000 rpm for one minute to collect the antimicrobial-rich supernatant. Then, 100 microliters of neutrally pH-adjusted cell-free supernatant (CFS) were added to each well (Karimi et al., 2008). The plates were then incubated for a full day at 37 degrees Celsius. Based on the development of inhibition, the antibacterial activity of the bacterial isolates was determined. Zones around the wells. The diameter (in millimeters) of the clear inhibitory zone surrounding the wells was measured, and it was shown to be directly proportional to the isolate's antagonistic activity. Isolates exhibiting a broad range of antibiotic activity were chosen for further investigation (Dowarah et al., 2018).

Cell surface hydrophobicity

The hydrophobicity of the cell surface was evaluated using the method described in reference (Lee et al., 2015).

Molecular analysis of selected isolates (16S ribosomal Ribonucleic acid (rRNA) gene sequencing)

Following in vitro characterization, four Lactobacillus isolates with the highest probiotic potential were randomly selected and sequenced using the 16S rRNA gene to determine species. The Bacterial Genomic DNA Isolation Kit (Himedia, India) and manufacturer's instructions were used to extract genomic DeoXy Ribo- nucleic acid from overnight-cultured Lactobacillus cells for 16S rRNA gene sequencing. Following DNA isolation, the universal primers 27F [5'AGAGTTTGATCCTGGCTCAG 3'] and 1492R [5'TACGGCTACCTTGTTAGGACTT 3'] were used to amplify the 16S rRNA gene the methodology indicated in Reference. The PCR amplification was carried out by improving the process provided in Reference (Dowarah et al., 2018). To make a 40 µl PCR miX, add 8 µl FIRE pol master miX, 2 µl DNA (20 ng/µl), 0.4 µl (100pm) of forward and reverse primers, and nuclease-free water. The PCR amplifi- cation condition was initial denaturation at 95 ◦C for 3 minutes, followed by 35 cycles of denaturation at 95 ◦C for 30 seconds, annealing at 60 ◦C for 45 seconds, extension at 72 ◦C for 1 minute, and final extension at 72 ◦C for 7

minutes. The PCR results were confirmed using 1% agarose gel electrophoresis. The PCR products were purified and sequenced by submitting them to Macrogen (Europe).

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 pre- viously 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-param- eter method (Irawan et al., 2015).

Data analysis

After entering the data into Excel, it was exported to SPSS for analysis. The quantitative data was analyzed using IBM SPSS statistics 21.0, using One-way ANOVA and Duncan multiple range tests ($P < 0.05$) to estimate means among isolates. Before statistical analysis, the total number of bacteria (measured in CFU/mL) was converted to a logarithmic value.

RESULTS AND DISCUSSION

Lactobacillus Bacterial Isolation and First Screening.

As many 190 bacterial colonies in all were chosen at random from the cultures of samples extracted from the five hens' gastrointestinal tracts. The first screening was conducted using many colony morphological traits, including size, shape, color, and appearance. Based on the Gram reaction, catalase tests, and acid tolerance, the original Lactobacillus bacteria were identified. Of the 190 isolates tested in the acid tolerance test preliminary screening, 78 (41%) could grow at pH 4, with 73 of those isolates being gram-positive. There were two shapes of these gram-positive bacterial cells: round (12 isolates, 16.4%) or rod (61 isolates, 83.6%). These isolates, all gram-positive, tested negative for catalase. 34 of the 73 isolates were found to survive at pH 3 in further testing for pH tolerance. Seventeen (or fifty percent) of these 34 isolates demonstrated tolerance to 0.05% bile salt.

Probiotics Potential Characteristics Tests

Acid Tolerance

The acid tolerance of Lactobacillus isolates was tested at various pH levels. Table 1 presents the results of the acid tolerance experiment and bile salt tolerance. Among the 34 isolates tested, only a few survived at pH 2. Overall, all isolates showed decreased viability at pH 2 and 3 compared to the control pH of 6.5. At pH 2, fourteen isolates were able to persist. After 48 hours of incubation, Lactobacillus isolates showed favorable results, although their growth slowed at low pH levels (pH 2 and 3) (Table 1). These results indicate that lactic acid bacteria can endure low pH conditions, making them potential probiotics for the digestive system (Manshur and Hidayat, 2019).

Bile Salt Tolerance

The bile salt tolerance assay evaluated the ability of isolates to survive at different bile salt concentrations. Ten isolates (from the cecum, small intestine, and crop) were able to survive at a bile salt concentration of 0.1%. However, isolates from the gizzard could not grow at pH 2 and 0.1% bile salt concentration, and thus were not selected. Only the ten isolates that passed both the acid and bile tolerance tests and could tolerate

0.1% bile salt and pH 2 were chosen for further identification and subsequent analysis. These isolates were labeled IS1, IS2, IS3, IS4, IS5, IS6, IS7, IS8, IS9, and IS10. Among these ten isolates, only IS1, IS3, IS4, IS6, IS7, and IS9 exhibited resistance to 0.3% bile salt.

Discussion and Comparison

To function physiologically within the host, probiotic bacteria must be able to withstand gastrointestinal stresses (Kosin et al., 2006). The ability of probiotic strains to survive low pH in the stomach and high bile salt concentrations in the gastrointestinal tract determines their probiotic efficacy (Kobierecka et al., 2017). The Lactobacillus isolates from this study were able to grow at pH 2. In the bile salt tolerance assay, only six isolates were resistant to 0.3% bile salt, whereas all isolates survived at 0.1% bile salt. Similar results were observed in the study by Ehrmann et al. (2002), which found that strains of L. reuteri, L. salivarius, and L. animalis can endure pH 2 for 4 hours. Jannah et al. (2014) also discovered some L. salivarius strains that survived at pH 2. Jose (2015) reported several Lactobacillus strains, including L. reuteri, that survived 0.3% bile salt after 6 hours of incubation. Similarly, Ehrmann et al. (2002) demonstrated that L. reuteri is resistant to 2% oXgall. Bile secreted in the small intestine harms bacteria by disrupting their cell membranes. Lactobacillus contains bile salt hydrolase (BSH), an enzyme that hydrolyzes bile salts and reduces their solubility (Jannah et al., 2014).

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

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.

Antibiotic susceptibility

IS10 was found to be responsive to all tested medicines, however isolates IS1, IS2, IS5, IS7, and IS8 were found to be resistant to all used antibiotics, the results of antibiotic susceptibility tests. Save for IS10, every isolate was ampicillin-resistant. Intermediate resistance was shown by IS3 to ciprofloxacin and chloramphenicol. Additionally, IS9's ciprofloxacin resistance was modest.

The various Lactobacillus spp. patterns shown sensitivity and resistance to various antibiotics, such as ampicillin, erythromycin, ciprofloxacin, and chloramphenicol (Shakoor et al., 2014) (Jose et al., 2015). shown that ampicillin has no effect on the population expansion of Lactobacillus. In contrast to the findings of this investigation (Singh et al., 2014), it was reported that distinct strains of L. reuteri exhibited erythromycin and chloramphenicol sensitivity.

There are no safety issues with feed or food due to the probiotics' innate resistance to some antibiotics, which is thought to be a characteristic rather than a transmissible one. One benefit of probiotics is their inherent resistance to some antibiotics (Khalil et al., 2018). Antibiotics used as therapies for animal health may reveal probiotic microorganisms in the gastrointestinal system of the animal. Because non-transferable resistance helps probiotic strains survive in vivo, it is necessary for them to be successful (Jose et al., 2015).

Antimicrobial activity of selected lactobacillus isolates

Tests were conducted on the isolates' ability to inhibit the growth of E. coli, S. aureus, S. typhimurium, S. intermedius, and S. enteritidis. Figure 4 shows that the isolates all shown varying degrees of inhibition against the development of the chosen pathogens. IS6 demonstrated that the highest size of the inhibition zone (17.83 mm) was against S. typhimurium. The maximal zone of inhibition against S. aureus (16.00 mm), S. typhimurium (17.83 mm), S. enteritidis (14.50 mm), and E. coli (17.66 mm) was demonstrated by IS6. With the exception of E. coli, IS2 displayed the least size of the zone of inhibition against all employed pathogens.

Figure 5 illustrates the antibacterial efficacy of isolated Lactobacillus against certain pathogenic microorganisms. IS6 demonstrated that the inhibition zone's maximal size (17.83 mm) was against S. typhimurium. One feature of probiotics that helps to keep the gut microbiota balanced and free of infections is their antagonistic action against pathogens. By producing non-specific antimicrobial substances such short-chain fatty acids, hydrogen peroxide, and low-molecular-weight proteins, probiotics prevent the development of harmful bacteria (Karimi et al., 2008).

The antibacterial characteristics of the isolates against E. Coli, S. aureus, S. typhimurium, S. intermedius, and S. enteritidis were demonstrated by the study's results. The spectrum of inhibition that each isolate showed against the development of the chosen pathogens varied. In the case of S. typhimurium, IS6 demonstrated the largest size of the inhibition zone (17.83 mm). With the exception of E. coli, all used pathogens demonstrated a minimal zone of inhibition size in IS2.

Lactobacillus bacteria come in various strains that bind to certain receptors and harm cells to prevent the growth of bacteria, such as E. Coli, S. typhimurium, S. aureus, C. perfringens, Klebsiella spp., and Proteus spp. (Cisek et al., 2022). Different strains of L. salivarius were reported by (Jannah et al., 2014) to exhibit inhibition against S. enteritidis and E. coli. The function of probiotics is to keep the gut microbiota balanced and free of infections by means of the antagonistic activity of probiotic bacteria against viruses.

Antibiotic Resistance

The antibiotic sensitivity testing revealed that among the ten selected isolates, only IS1, IS3, IS4, IS6, IS7, and IS9 demonstrated resistance to 0.3% bile salt. This subset of isolates was also tested for antibiotic sensitivity to commonly used antibiotics, including ampicillin, ciprofloxacin, chloramphenicol, and erythromycin. The presence of resistance or susceptibility to these antibiotics can have significant implications for the practical use of these probiotics.

Antibiotic resistance in probiotics is a critical factor to consider. Probiotics with resistance to certain antibiotics may pose a risk of transferring resistance genes to pathogenic bacteria in the gastrointestinal tract, which could complicate treatment protocols and contribute to the spread of antibiotic resistance (O'Connor et al., 2006; Cummings et al., 2018). Conversely, the susceptibility of these probiotics to antibiotics can be advantageous in clinical settings, where probiotics are used alongside antibiotic therapy. Susceptibility ensures that probiotics do not contribute to the development of resistance and are compatible with ongoing treatments (Ganzle, 2015).

The effectiveness of probiotics in preventing or treating infections partly depends on their ability to outcompete pathogenic bacteria. If probiotics are resistant to antibiotics, they may persist in the gut longer, potentially interfering with the effectiveness of antibiotic treatments (Vinderola et al., 2019). On the other hand, probiotics that are susceptible to antibiotics may help in maintaining a balance in gut microbiota without promoting antibiotic resistance (Hammer et al., 2019).

In summary, while the acid and bile tolerance of our Lactobacillus isolates suggest they could be effective probiotics, their antibiotic resistance profiles require careful consideration. Future research should focus on the clinical implications of these findings, particularly concerning the risks associated with using these probiotics in conjunction with antibiotic therapies.

Temperature and sodium chloride tolerance

All of the isolates in this investigation were able to endure temperatures between 25 and 50 ◦C. Figure 6 shows that 45 ◦C was the ideal temperature for every isolate. Different amounts of NaCl were shown to have varying effects on the survival capacity of the isolated Lactobacillus species. The maximum growth rate for each isolate was 4% (0.69 mol/L) and 6% of NaCl, but none of the isolates could grow at 8% (1.36 mol/L) of NaCl..

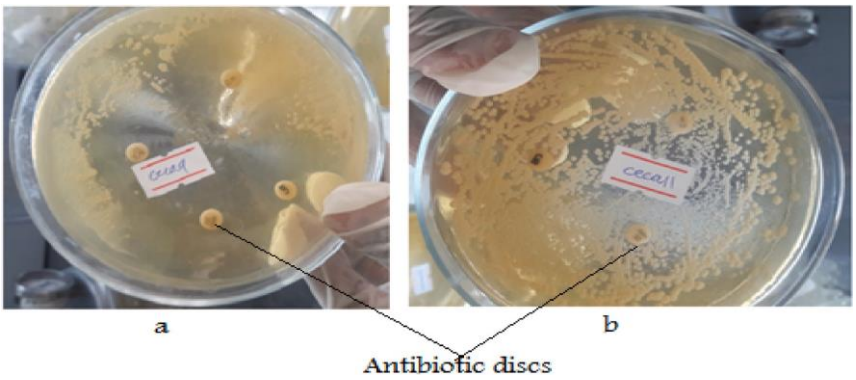

Fig. 3: Antibiotics susceptibility pattern of different Lactobacillus isolates to various antibiotics. (a) = antibiotic sensitive isolate and (b) = anti-biotic resistance isolate.

Table 2: Results of the chosen Lactobacillus isolates' antibiotic susceptibility tests for different 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.

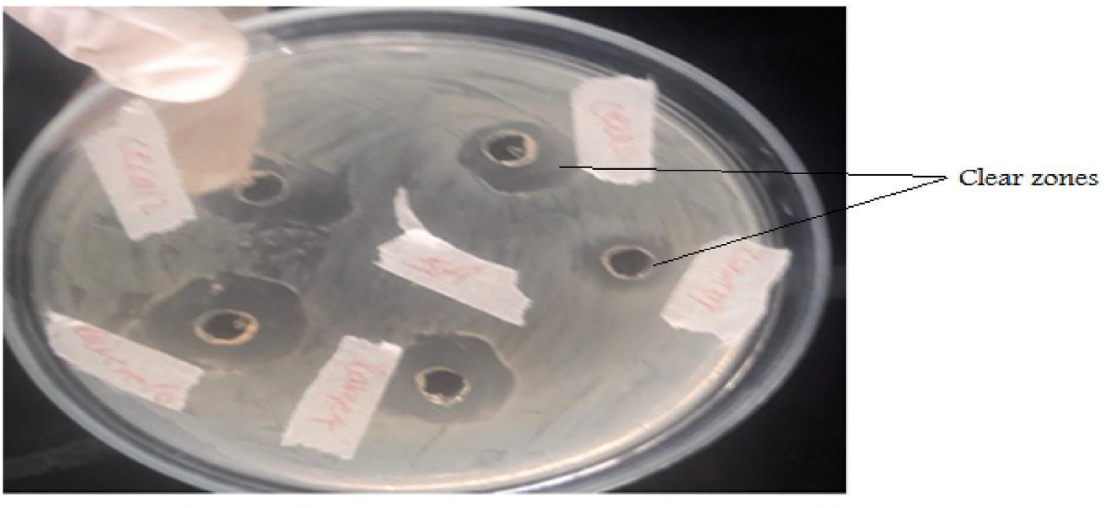

Fig. 4: Some isolates have antagonistic action against S. Aureus

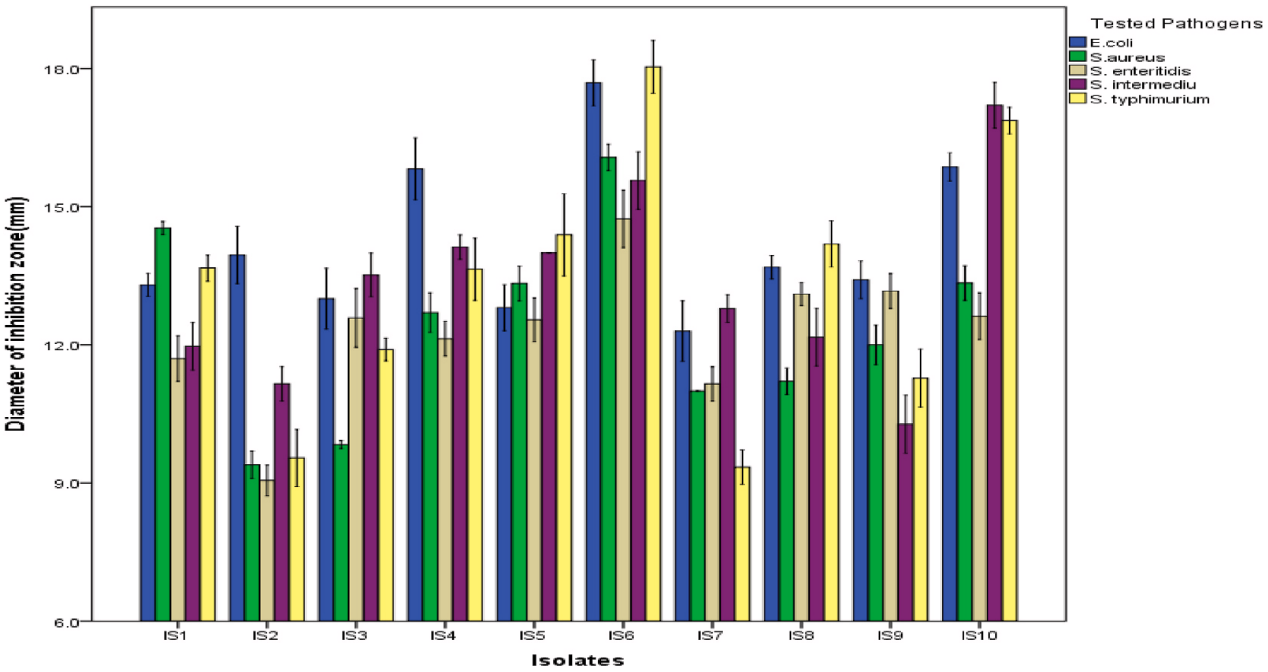

Fig. 5: The ability of isolated Lactobacillus to fight certain harmful microorganisms.

All of the isolates in this investigation were able to endure temperatures between 25 and 50 ◦C. For every isolate, a temperature of 45 °C was ideal. Because of their capacity to withstand different temperatures, isolates will be able to withstand processing, storage, and transportation (Cabello et al., 2020). Different amounts of NaCl were found to have varying effects on the survival capacity of the lactic acid bacteria species isolated in this investigation. At 4% (0.69 mol/L) of NaCl, all of the isolates were able to develop; however, at 8% (1.36 mol/L), none of the isolates expanded. The animal's digestive system could support the most resistant isolates to elevated NaCl concentrations. The gastrointestinal system has an osmolarity of 0.3 mol/L, thus probiotic bacteria must be able to withstand high salt concentrations in order to maintain their osmotic balance and proliferate (Kobierecka et al., 2017).

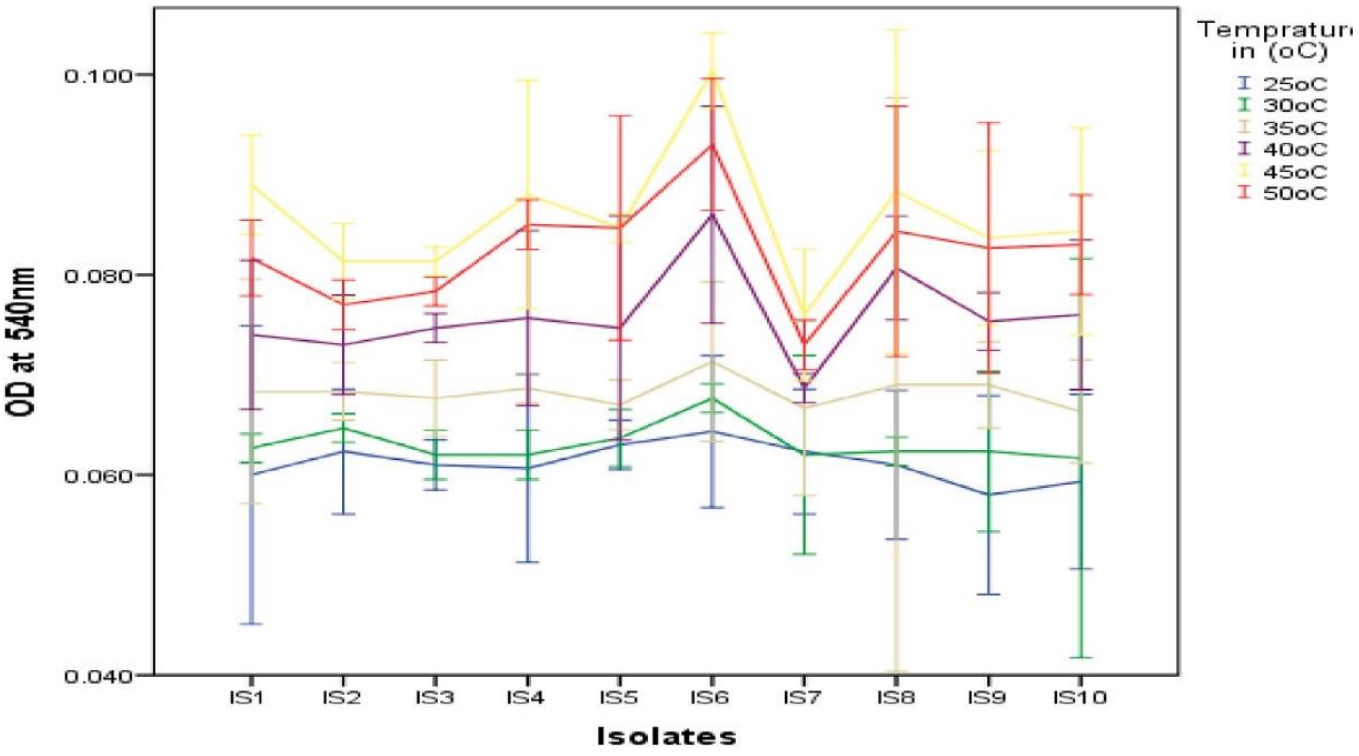

Fig. 6: Temperature tolerance of the isolated Lactobacillus bacteria. For every isolate, a temperature of 45 °C was ideal.

Lactobacillus isolates may survive at 1.5%–6% NaCl and 25–40 °C (Pundir et al., 2013). Kobierecka et al., 2017 reports that no L. reuteri strain survived on 0.69 mol/L NaCl, but other strains of L. salivarius exhibited varying resistance to 1 and 0.69 mol/L NaCl. Bacteria pump alkali outside and change the free acid to its salt form to stop the pH lactic acid from dropping too far. As a result, the bacterial cells' osmotic pressure is increased. As a result, the high osmotolerance characteristic is crucial for the separation of viable lactic acid bacteria strains, particularly for commercial production (Kobierecka et al., 2017).

Cell surface hydrophobicity and haemolytic activity

The isolates' hydrophobicity values varied, ranging from 26.4 to 79.3% (Fig. 7). The most hydrophobic activity ($P < 0.05$) against toluene was demonstrated by the IS2 isolate. Less than 30% hydrophobicity was demonstrated by IS1, IS8, and IS 10. When the isolates in this investigation were cultured on blood agar, none of them displayed any hemolysis. Probiotics must not injure the host in order to be safe (Demir et al., 2019), and one test used to determine this is the hemolytic activity test (Jose et al., 2015). When cultivated on blood agar, none of the isolates in our investigation displayed any symptoms of hemolysis. Comparable outcomes were noted (Balamurugan et al., 2014). As a result, the isolates pose no threat to their host.

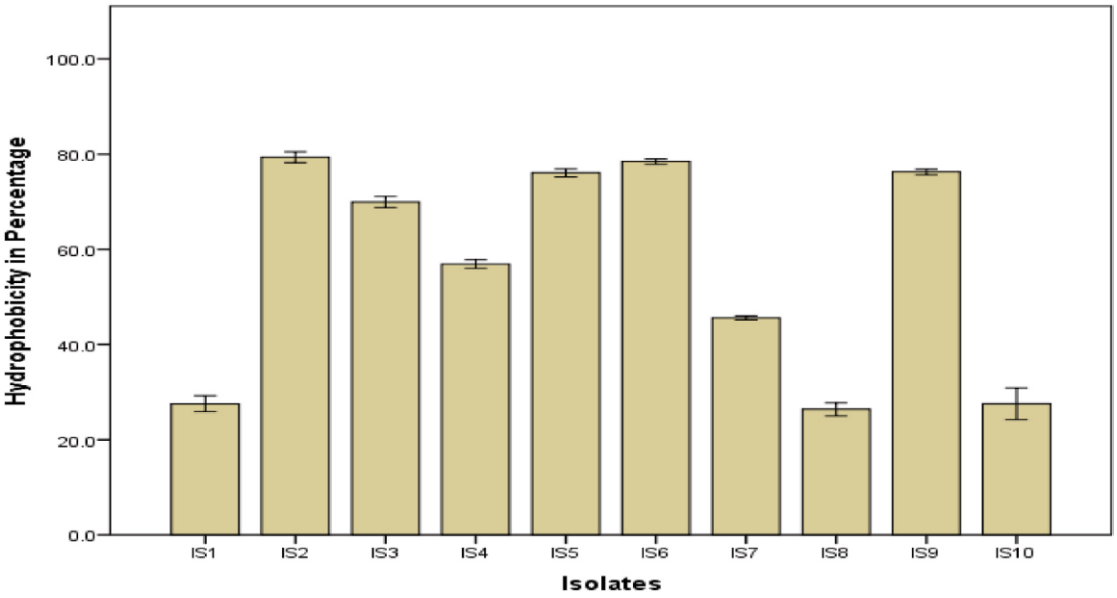

Fig. 7: Assay for cell surface hydrophobicity of isolated Lactobacillus bacteria

The hydrophobicity values of the isolates varied, ranging from 26.4 to 79.3%, as seen in Fig. 2. Adhesion to the intestinal mucosa is thought to be a necessity for colonization, making it one of the most significant selection criteria for probiotics (Demir et al., 2019). Lactobacillus may adhere to enterocytic cells lines; this is shown by its cellular hydrophobicity. Probiotics have the most advantageous impact on colonizing the host intestine when they have a high adhesiveness (Balamurugan et al., 2014).

Molecular Identification Of Selected Isolates

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

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Table 3 illustrates the isolates' molecular identification by 16S rRNA gene sequencing. Using the data above as a guide, isolates IS3, IS4, IS6, and IS7 were chosen for molecular identification. Based on their antibacterial activity, resistance to 0.3% bile salt, survival at pH 2 ($P < 0.05$), and hydrophobicity of the cell surface, these isolates demonstrated the best probiotic qualities. The 16S rRNA gene sequence analysis was used to identify the genotypes of the four Lactobacillus isolates that were chosen, which are IS3, IS4, IS6, and IS7.

Aligning and comparing the 16S rRNA gene sequence data with known sequences from GenBank was accomplished with success. Lactobacillus salivarius NR_112759.1 and Lactobacillus reuteri NR_075036.1 were found to be 98.4% and 97% identical, respectively, among the isolates IS3 and IS4. lactobacillus reuteri NR_113820.1 and IS6 were comparable in 96.5% of the cases. GenBank was the source of all accession numbers.

Table 3 displays the accession numbers MK764683 to MK764686 for the 16S rRNA gene sequences of the four isolates (IS3, IS4, IS6, and IS7) that have been submitted in the GenBank database.

These isolates' sequences have been released in GenBank under the names Lactobacillus salivarius strain CEL1 (IS3), Lactobacillus reuteri strain CEC2 (IS4), Lactobacillus reuteri strain CEC3 (IS6), and Lactobacillus reuteri strain CEC4 (IS7).

The phylogenetic connections between the four Lactobacillus strains and the ten Lactobacillus-type strains that were retrieved from the GenBank are shown in Fig. 8, which is based on the 16S rRNA gene sequence analysis.

The outgroup utilized was Clostridium perfringens (M59103.1). Lactobacillus reuteri NR_075036.1 was most closely related to strains IS4 (MK764684), IS6 (MK764685), and IS7 (MK764686), with a bootstrap value of 96%. Nevertheless, a bootstrap score of 72% indicated that IS3 (MK764683) and Lactobacillus salivarius NR_112759.1 were grouped together.

Microorganisms with possible probiotic benefits typically have similar traits (Koenen et al., 2004). The main tests used to separate probiotic microorganisms from various sources are lower pH tolerance, salt tolerance, bile acid resistance, use of different carbon sources (oligosaccharide degradation), hemolytic properties, antibiotic sensitivity, antimicrobial activity, and in vitro adherence properties (Sanni et al., 2013).

Hens raised in tropical Africa are thought to have a diverse range of unidentified GIT microbiota, which makes them a potential source of probiotics. As a result, it is anticipated that isolating endogenous probiotic bacteria may be a viable probiotic source for addressing the fundamental difficulties related with chicken production (Lahtinen et al., 2011).

In earlier research, numerous types of probiotic bacteria were recovered from the chicken digestive system, including L. reuteri, L. salivarius (Kobierecka et al., 2017), Enterococcus faecium, and Enterococcus durans (Buahom et al., 2018).

CONCLUSION AND RECOMMENDATIONS

Ten putative probiotic Lactobacillus bacterial strains were identified from the GI tract of Rhode Island Red hens in Indonesia. All isolates showed tolerance to low pH and high bile salt levels, as well as strong hydrophobicity to hydrocarbons and antagonistic activity against E. coli, S. aureus, S. typhimurium, S. intermedius, and S. enteritidis. Based on the possible probiotic properties listed above, the isolates might be employed as probiotic candidates in chicken farms.

A recommendation that can be followed up is to use probiotics as an alternative to antibiotics in broiler chicken feed to improve health and productivity effectively and efficiently. policy on the use of probiotics in broiler chickens in Indonesia, including regulations and standards that must be met.

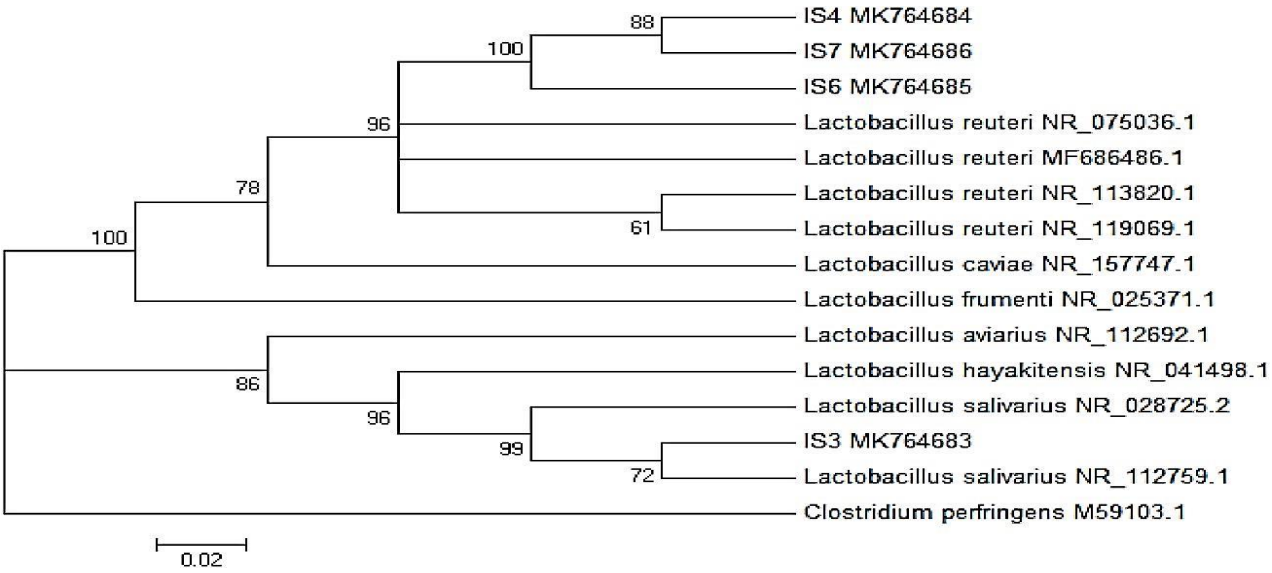

Fig. 8: phylogenetic tree constructed using Lactobacillus isolates' 16S rDNA gene sequencing data.

The tree was created using the neighbor-joining technique. C. perfringens was the outgroup member. At the nodes of the tree, bootstrap values based on 1000 replications are displayed. 0.02 substitutions were shown for each nucleotide position on the scale bar. Sequence accession numbers derived from the NCBI database.

REFERENCES

1. Anjana, & Tiwari, SK (2022). Bacteriocin-Producing Probiotic Lactic Acid Bacteria in Controlling Dysbiosis of the Gut Microbiota. In Frontiers in Cellular and Infection Microbiology (Vol. 12, Issue May, pp. 1–11). https://doi.org/10.3389/fcimb.2022.851140
2. Ada´mkova A, Mleck J, Kourimska L, Borkovcova M, Busina T, Adamek M, Bednarova M, Krajsa J (2017). Nutritional Potential Of Selected Insect Species Reared On The Island Of Sumatra, 14(5): 521. https://doi.org/10.3390/ ijerph14050521.
3. Balamurugan R, Chandragunasekaran AS, Chellappan G, Rajaram K, Ramamoorthi G, Ramakhrisna BS (2014). Probiotic potential of lactic acid bacteria present in home made curd in southern India, Indian J. Med. Res. 140(3): 345–355.
4. Berg C, Raj M (2015). A review of different stunning methods for poultry—animal welfare aspects (stunning methods for poultry), Animals 5(4):1207–1219. https://doi.org/10.3390/ani5040407.
5. Bernardeau M, VernouX JP (2013). 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):321–330. https://doi.org/ 10.1111/1469-0691.12130.
6. Buahom J, Siripornadulsil S, Siripornadulsil W (2018). 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. 43:3417–3427. https://doi.org/10.1007/s13369-017-3045-6.
7. Olmo MC, Oneca M, Torre P, Diaz JV, Encio IJ, Barajas M, Arana M (2022). Influence of storage temperature and packaging on bacteria and yeast viability in a plant-based fermented food, Foods 9(3). https://doi.org/10.3390/foods9030302.
8. Carvalho IT, Santos L. Antibiotics in the aquatic environments: A review of the European scenario. Environ Int. 2016;94:736–757.
9. Cisek AA, Bak I, Stefanska I, Binek M (2022). Selection and optimization of high-yielding DNA isolation protocol for quantitative analyses of methanogenic archaea, Microorganisms 10(3):523. https://doi.org/10.3390/microorganisms10030523.
10. Cummings, J. H., McFarlane, G. T., & Macfarlane, S. (2018). The control and consequences of antibiotic resistance in the human gut microbiome. Journal of Clinical Gastroenterology, 52(9), 759-

765.
11. Demir E, Kılıç GB, Zbalcı DO (2019). Biosafety assessment of probiotics "Probiotics", Turk. J. Agr. Food Sci. Tech. 7(4):639–645. https://doi.org/10.24925/
12. Donaldson EE, Stanley D, Hughes R, Moore RJ (2017). The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs, PeerJ 5: e3587. https://doi.org/10.7717/peerj.3587.
13. Doron S, Snydman DR (2015). Risk and safety of probiotics, Clin. Infect. Dis. 60 (suppl_2):S129–S134. https://doi.org/10.1093/cid/civ085.
14. Dowarah R, Verma AK, Agarwal N, Singh P, Singh BR (2018). 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):e0192978. https://doi.org/10.1371/journal.pone.0192978.
15. Ehrmann MA, Kurzak P, Bauer J, Vogel rf (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry, J. Appl. Microbiol. 92(5):966–975. https://doi. org/10.1046/j.1365-2672.2002.01608.x.
16. Fijan S (2014). Microorganisms with claimed probiotic properties: an overview of recent literature, Int. J. Environ. Res. Publ. Health 11(5):4745–4767. https:// doi.org/10.3390/ijerph110504745.
17. Fuller R (1989). Probiotics in man and animals, J. Appl. Bacteriol. 66(5): 365–378. https://pubmed.ncbi.nlm.nih.gov/2666378
18. Ganzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Current Opinion in Food Science, 1, 16-22.
19. Gonzalez, GN., Battista, N., Prete, R., & Corsetti, A. (2021). Health-promoting role of lactiplantibacillus plantarum isolated from fermented foods. Microorganisms, 9(2), 1–30. https://doi.org/10.3390/microorganisms9020349
20. Gupta R, Jeevaratnam K, Fatima A (2018). Lactic acid bacteria: probiotic characteristic, selection criteria, and its role in human health (A review), J. Emerg Tech. Innov. Res.(JETIR) 5(10):411–424. https://ssrn.com/abstract 3462244.
21. Hall BG(2013). Building phylogenetic trees from molecular data with MEGA, Mol. Biol. Evol. 30(5):1229–1235. https://doi.org/10.1093/molbev/mst012.
22. Hammer, J. A., et al. (2019). Probiotics and antibiotic resistance: a review of the evidence. International Journal of Food Science and Technology, 54(7), 2177-2190
23. Irawan MI, Amiroch S (2015). 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):424–429.
24. Jannah SN, Dinoto A, Wiryawan KG, Rusmana I (2014). Characteristics of lactic acid bacteria isolated from gastrointestinal tract of Cemani chicken and their potential use as probiotics, Media Peternakan 37(3):182–189. https://doi.org/10.5398/medpet.2014.37.3.182.
25. Jha R, Das R, Oak S. Mishra P (2020). 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): 1863. https://doi.org/10.3390/ani10101863.
26. Jose NM, Bunt CR, Hussain MA (2015). Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and Microorganisms 3(2076–2607):198–212. https://doi.org/10.3390/microorganisms3020198.
27. Torshizi MAK, Rahimi S, Mojgani N, Esmaeilkhanian S (2008). Screening of indigenous strains of lactic acid bacteria for development of a probiotic for poultry, Asian-Australas. J. Anim. Sci. 21. https://doi.org/10.5713/ajas.2008.80081.
28. Khalil ES, Manap MY, Mustafa S, Amid M, Alhelli AM, Aljoubori A (2018). Probiotic characteristics of exopolysaccharides-producing Lactobacillus isolated from some traditional Malaysian fermented foods, CyTA - J. Food 16(1):287–298. https://doi.org/10.1080/19476337.2017.1401007.
29. Kobierecka PA, Wyszynska AK, Piekarczyk TA, Kuczkowski M, Tuzimek A, Piotrowska W, Gorcki A, Adamska I, Wieliczko A, Bardowski J, Krynicka EKJ (2017). 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): e00512. https://doi.org/10.1002/mbo3.512.
30. Koenen ME, Hulst RVD, Leering M, Jeurissen SHM, Boersma WJA (2004). 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):119–127.

https://doi.org/10.1016/s0928-8244(03)00306-7.
31. Kosin B, Rakshit SK (2006). Microbial and processing criteria for production of probiotics, Rev. Food Tech. Biotech. 44:371–379.
32. Lahtinen S, Ouwehand AC, Salminen S, Wright AV (2011). Lactic Acid Bacteria: Microbiological and Functional Aspects. CRC Press.
33. Lee HK, Choi SH, Lee CR, Lee SH, Park MR, Kim Y, Lee MK, Kim GB (2015). 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):91–100. https://doi.org/10.5851/kosfa.2015.35.1.91.
34. Mahesh M, Mohanta RK, Patra AK (2021). Probiotics in livestock and poultry nutrition and health, Adv. Prob. Sustain. Food Med, 149–179. https://doi.org/ 10.1007/978-981-15-6795-7_7.
35. Manshur, DN., Hidayat, MN (2019). Resistance of Lactic Acid Bacteria of Broiler Gastrointestinal Origin to pH and Bile Salts. JIIP. (1): 27-37. https://journal.uin-alauddin.ac.id/index.php/jiip/article/view/11101
36. Masood MI, Qadir MI, Shirazi JH, Khan IU (2011). Beneficial effects of lactic acid bacteria on human beings, Crit. Rev. Microbiol. 37(1):91–98. https://doi.org/10.3109/ 1040841X.2010.536522.
37. Mbye M, Baig MA, Abuqamar SF, Tarabily KAE, Obaid RS, Osailli TM, Nabulsi AAA, Turner MS, Shah NP, Ayyash MM (2020). 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):1110–1124. https://doi.org/10.1111/1541-4337.12554.
38. Mekuria SA, Teshager AA, Endeshaw AG, Atinaw MB, Sendeku AT (2018). Small ruminant fattening practices in Amhara region, Indonesia, Agric. Food Secur. 7(1):1-9. https://doi.org/10.1186/s40066-018- 0218-9.
39. Milkias M (2016). Review on exotic chicken status, production performance and constraints in Indonesia, J. Biol. Agr. Heal. 6 (15).
40. Moniño, A.M., Ibañez, R.Y., & Moniño, P.D. (2023). Effects of Locally-Made Probiotics in Drinking Water on the Performance of Chickens. Biosciences, 15(2), 262–269. https://doi.org/10.15294/biosaintifika.v15i2.45217
41. Nakphaichit M, Thanomwongwattana S, Phraephaisarn C, Sakamoto N, Keawsompong S, Nakayama J, Nitisinprasert S (2011). 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):2753–2765. https://doi.org/10.3382/ps.2011-01637.
42. Negm GES (2018). Classification, antimicrobial potential, industrial applications and probiotic capability of lactic acid bacteria: a review article, Res. J. Appl. Sci. 13, (12):742–757. https://www.researchgate.net/publication/330006181
43. Nourizadeh R, Sepehri B, Abbasi A, Sayyed RZ (2022). Impact of Probiotics in Modulation of Gut Microbiome. Microbiome-Gut-Brain Axis: Implications on Health, Springer Nature Singapore, pp. 401–409. https://doi.org/10.1007/978-981-16-1626-6_20.
44. O'Connor, M., et al. (2006). Risk assessment of probiotic strains with antibiotic resistance traits. Journal of Applied Microbiology, 101(1), 148-156.
45. Pan D, Z. Yu (2014). Intestinal microbiome of poultry and its interaction with host and diet, Gut Microb. 5(1):108–119. https://doi.org/10.4161/gmic.26945.
46. Peng Q, Zeng XF, Zhu JL, Wang S, Liu XT, Hou CL, Thacke PA, Qiao SY (2016). Effects of dietary Lactobacillus plantarum B1 on growth performance, intestinal microbiota, and short chain fatty acid profiles in broiler chickens, Poultry Sci. 95(4):893–900. https://doi.org/10.3382/ps/pev435.
47. Pundir RK, Kashyap SRN, Kaur A (2013). Probiotic potential of lactic acid bacteria isolated from food samples: an in vitro study, J. Appl. Pharmaceut. Sci. 3(3):85–93. https://doi.org/10.7324/JAPS.2013.30317.
48. Sa´nchez B, Delgado S, Miguez AB, Lourenco A, Gueimonde M, Margolles A (2017). Probiotics, gut microbiota, and their influence on host health and disease, Mol. Nutr. Food Res. 61 (1), 1600240. https://doi.org/ 10.1002/mnfr.201600240.
49. Sanni A (2013). Characterization and technological properties of lactic acid bacteria in the production of "Sorghurt," a cereal-based product, Food Biotechnol. 27(2):178–198. https://doi.org/10.1080/08905436.2013.781949.

50. Sebho HK (2016). Exotic chicken status, production performance and constraints in Indonesia: a review, Asian J. Poultry Sci. 10(1):30–39. https://doi.org/ 10.3923/ajpsaj.2016.30.39.
51. Shakoor G, Akbar A, Samad A, Khan SA, Rehman FU, Shakoor M, Sunniya H, Ahmad D, Rafique A (2017). Isolation of lactic acid bacteria from chicken gut and its probiotic potential characterization, Int. J. Biosci. 11(3):1–9. https://doi.org/ 10.12692/ijb/11.3.1-9.
52. Shin MS, Han SK, Ji AR, Kim KS, Lee WK (2008). Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use, J. Appl. Microbiol. 105(6): 2203–2212. https://doi.org/10.1111/j.1365-2672.2008.03935.x.
53. Shokryazdan P, Jahromi MF, Liang JB, Ho YW (2017). Probiotics: from isolation to application,J.Am.Coll.Nutr.36(8):666–676. https://doi.org/10.1080/07315724.2017.1337529.
54. Silva DR, Sardi JCO, Pitangui NZ, Roque SM, Silva ACB, Rosalen PL (2020). Probiotics as an alternative antimicrobial therapy: current reality and future directions, J. Funct.Foods 73, 104080. https://doi.org/ 10.1016/j.jff.2020.104080.
55. Singh TP, Malik RK, Kaur G (2014). Safety assessment and evaluation of probiotic potential of Lactobacillus reuteri strains under in vitro conditions, Int. J. Curr. Microb. Appl. Sci. 3 (2):335–348. https://www.ijcmas.com
56. Sisay T, K. Alemayehu, Zeleke G (2017). Trends of exotic chicken dissemination in north western Amhara, Indonesia: challenges and opportunities, J. Biol. Agr. Heal. 7 (24):41–51.
57. Szydłowska A, B. Sionek (2022). Probiotics and postbiotics as the functional food components affecting the immune response, Microorganisms 11(1):104. https://doi.org/10.3390/microorganisms11010104. turjaf.v7i4.639-645.2327.
58. Vinderola, G., et al. (2019). Probiotics in health and disease. Microbial Cell Factories, 18(1), 24.
59. Yadav S, Jha R (2019). Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry, J. Anim. Sci. Biotechnol. 10(1): 1–11. https://doi.org/10.1186/s40104-018-0310-9.