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Screening and characterization of lactic acid bacteria from the gastrointestinal tract of a native chicken (*Gallus gallus* f. *domesticus*) as probiotics

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Abstract. *Anwar K, Putra RA, Karni I, Kurniawan A, Amalyadi R, Unsunnidhal L, Kisworo D, Ali M. 2025. Screening and characterization of lactic acid bacteria from the gastrointestinal tract of a native chicken (*Gallus gallus *f.* domesticus*) as probiotics. Biodiversitas 26: 2653-2661.* Probiotic bacteria have been reported to confer beneficial effects on poultry health and performance. Nevertheless, probiotic strains derived from Indonesian native chickens are not widely available, restricting the application of appropriate probiotic strains as an alternative substitution for antibiotic growth promoters in the poultry industry. In the present study, we aimed to screen, identify, and characterize Lactic Acid Bacteria (LAB) strains from the gastrointestinal tract of a native chicken (*Gallus gallus* f. *domesticus*). A native chicken was purchased from an officially approved slaughterhouse. The gastrointestinal tracts, including the small intestine and cecum, were gathered aseptically and used for bacterial isolation. A total of three LAB isolates were obtained and subjected to initial screening according to the morphological, biochemical, and molecular analyses. Two selected isolates (LC12 and LC16) demonstrating Gram-positive, rod-shaped, catalase-negative, and non-motile characteristics were tested under simulated gastrointestinal conditions to reveal potential probiotic characteristics, followed by amplifying and sequencing the 16S rRNA gene. The results demonstrated that two selected isolates had high survival rates, greater than 90% at pH 7.2 and 55% at pH 2.0 after 2-hour and 4-hour incubation, respectively, suggesting adaptability over a wide range of pH conditions. Both isolates indicated tolerance to 0.3% bile salt with a survival rate of more than 70%. Molecular identification using partial-length 16S rRNA sequences showed that the isolates LC12 and LC16 were genetically identified as *Lactobacillus johnsonii* strain 6-3 with a similarity of 100% for both isolates. The selected LAB isolates are promising probiotic candidates for favorable applications in the poultry industry.

Keywords: Gastrointestinal tract, lactic acid bacteria, *Lactobacillus johnsonii*, native chicken, probiotic

INTRODUCTION

Increasing demand for poultry products has led to intensified poultry production, which enhances the usage of antibiotics for growth promoters and disease prevention (Khurajog et al. 2023). Antibiotic Growth Promoters (AGPs) have broadly been utilized in poultry production during maintenance to modulate intestinal microbiota, increase nutrient digestibility, and promote poultry growth (Agustono et al. 2022). Nevertheless, the overuse of AGPs raises concerns about antibiotic residues, potentially causing antibiotic resistance in humans and animals. The overuse of antibiotics can trigger bacteria to acquire resistance, diminishing the efficacy of antibiotics. This contributes to transmitting antibiotic-resistant microorganisms from animals to humans, which potentially causes difficult-to-treat ailments (Agustono et al. 2022; de Mesquita Souza Saraiva et al. 2022; Khurajog et al. 2023). Given their adverse impacts, the government of Indonesia has taken precautionary measures by banning the use of AGPs in poultry feed additives (Agustono et al. 2022), resulting in decreased growth rates and increased feed conversion that affects high production expenses (Maria Cardinal et al. 2019). Addressing such problems, the development of alternative feed additives, such as probiotics, has been reported as a substitute for antibiotics in poultry production (Hossain et al. 2024).

Probiotics are recognized as live microorganisms, commonly bacteria and yeast, that confer positive effects on the health of the host when consumed in sufficient amounts (FAO/WHO 2001). Probiotics, given as Direct-Fed Microbials (DFMs), have been reported to improve poultry performance by balancing the normal microbiota (Heak et al. 2018). In addition, the advantageous effects of probiotics are linked to their ability to enhance growth performance, decrease feed conversion (Hossain et al. 2024), boost the immune system (Tomczyk et al. 2024), regulate the composition of intestinal microbiota (Wang et al. 2017), and inhibit the colonization of pathogenic bacteria, such as *Escherichia coli* E (Wu et al. 2021), *Salmonella pullorum* (Rettger, 1900) (Chen et al. 2020), and *Campylobacter jejuni* (Jones et al. 1931) Veron & Chatelain, 1973 (Saint-Cyr et al. 2017; Šikić Pogačar et al. 2020). Despite this, the efficacy of probiotics depends on

several factors, including strain, type, amount used, period of use, and administration procedure (Al-Fatah 2020; Khurajog et al. 2023; Hashemitabar and Hosseinian 2024). Furthermore, it is necessary to consider the importance of selecting suitable probiotic strains for particular applications related to host-originating sources (Kalia et al. 2017).

Lactic Acid Bacteria (LAB) are distributed abundantly in nature and Generally Recognized as Safe (GRAS) as probiotics for humans and animals (Bin Masalam et al. 2018). These bacteria have been documented as the most widely used bacterial group as probiotics in poultry production owing to their tolerance to gastric acid and bile salt and their positive effects on production performance and inhibition activity against gastrointestinal pathogens (Khurajog et al. 2023). Among them, *Lactobacillus* species are predominantly utilized as probiotics in poultry and frequently found as a member of intestinal flora in poultry (Yan et al. 2017; Hashemitabar and Hosseinian 2024). *Lactobacillus* can be categorized into two groups based on their secondary metabolites: homofermentative, which turns glucose into lactic acid, and heterofermentative, which generates lactic acid, acetic acid, ethanol, and carbon dioxide from glucose (Axelsson 2004; Forte et al. 2018). Lactic acid and acetic acid are the primary metabolite compounds responsible for lowering pH conditions in the gastrointestinal tract, which contributes to creating undesirable circumstances for pathogenic bacteria, leading to the reduction of the colonization of potential pathogens. In addition, *Lactobacillus*-based probiotics demonstrate a competitive exclusion effect against pathogenic enterobacterial infections, including *Salmonella* spp. in broilers (Penha Filho et al. 2015).

Several studies have corroborated that the administration of probiotics can increase the status of health and productivity of poultry (Wang et al. 2017; Agustono et al. 2022; Hashemitabar and Hosseinian 2024; Hossain et al. 2024; Tomczyk et al. 2024). Risna et al. (2020) previously reported that several indigenous LAB isolates, including *Lactobacillus plantarum* (Orla-Jensen, 1919) Bergey et al. 1923, *Lactobacillus fermentum* Beijerinck, 1901, and *Pediococcus acidilactici* A, derived from a local Aceh duck (*Anas platyrhynchos* Linnaeus, 1758), show potential as probiotic feed supplements for poultry. Nonetheless, probiotic strains originating from Indonesian native chickens are not widely available, which limits the leveraging of appropriate probiotic strains for specific host-originating applications (Kalia et al. 2017; Khurajog et al. 2023). Probiotic bacteria isolated from the gastrointestinal tract of chickens are potentially more resilient to low pH and high bile salt concentration, allowing them to colonize and grow in the chicken intestine. Despite this potential, few studies have addressed the isolation, screening, identification, and characterization of indigenous probiotic strains from Indonesian native chickens, leaving a gap in the development of well-suited probiotic supplements as an alternative substitution for antibiotic growth promoters in the poultry industry. Therefore, the present study aims to screen, identify, and characterize lactic acid bacteria obtained from the gastrointestinal tract of a native chicken from Indonesia through morphological, biochemical, and molecular analyses, followed by evaluating bacterial tolerance to low pH and bile salt as promising probiotic candidates for poultry.

MATERIALS AND METHODS

Culture medium

The basic medium for the culture was de Man, Rogosa, and Sharpe (MRS), which served to isolate lactic acid bacteria. A total of 52.2 g of MRS powder (Central Drug House, India) was suspended in 1,000 mL of distilled water. A total of 20 g of agar bacteriological (Thermo Fisher Scientific, USA) was supplemented to prepare 1,000 mL of MRS-agar medium. The Sulfide-Indole-Motility (SIM) medium served as a semi-solid medium for testing hydrogen sulfide, indole production, and bacterial motility. A total of 36.23 g of SIM medium powder (Central Drug House, India) was dissolved in 1,000 mL of distilled water and dispensed into reaction tubes. Plate Count Agar (PCA) medium was used to count viable cells and determine survival rates under simulated gastrointestinal conditions. It contained 5.0 g/L of tryptone (Thermo Fisher Scientific, USA), 1.0 g/L of yeast extract (Thermo Fisher Scientific, USA), 1.0 g/L of glucose, and 15.0 g/L of agar bacteriological (Thermo Fisher Scientific, USA). All culture media used in this study were prepared as instructed by each manufacturer and kept at 4°C until use.

Sample collection

The samples were collected in 2022 from the gastrointestinal tract of a native chicken in Lombok, West Nusa Tenggara, Indonesia. The sample collection was carried out following the protocol reported by Risna et al. (2020). A two-month-old native chicken that was euthanized and humanely slaughtered by severing the jugular veins was purchased from an officially approved slaughterhouse for food consumption in compliance with animal welfare regulations and standards under the authorization of the local government within the jurisdiction where this study was performed. The gastrointestinal tract was harvested, and the samples were collected from the small intestine and cecum. The collected samples were aseptically transferred into 9 mL of Phosphate Buffer Saline (PBS) with pH 7.2 and preserved at 4°C for subsequent analyses.

Isolation and identification of lactic acid bacteria

Lactic acid bacteria were isolated from the small intestine and cecum of a native chicken following the protocol reported by Sumarsih et al. (2013) with a slight modification. The resuspended samples were transferred into 9 mL of PBS (pH 7.2) for serial dilutions that were prepared up to 10^{-5}. Each sample from the intestine and cecum was taken into serial dilution tubes separately. Subsequently, a 1 mL sample of the 10^{-4} and 10^{-5} dilution series was transferred into sterile plates, and MRS agar was gently poured. The plates were anaerobically incubated at 37°C for 48 hours in an anaerobic jar with the AnaeroGenTM 3.5 L (Thermo Fisher Scientific, USA). The grown colonies after the incubation were separated based on their size, shape, margin, and color. They were selected randomly and

streaked into MRS-agar medium to derive pure colonies, followed by morphological assays, including Gram staining and cell shape. Subsequently, a pure culture was transferred into 5 mL of MRS broth and incubated at 37°C with shaking at 120 rpm for 48 hours. Ultimately, the culture broth was added to sterile glycerol and stored at -80°C.

Biochemical test

The biochemical characteristics, such as catalase, sulfide, indole, motility, and carbohydrate fermentation tests, were examined to identify bacterial species by separating them according to their biochemical activities. The catalase activity of the obtained pure isolates was tested using 3% hydrogen peroxide (H_2O_2) following the procedure described by Saryono et al. (2023). The SIM test was conducted to distinguish three parameters, including hydrogen sulfide, indole, and motility. A single colony was inoculated by stabbing two-thirds into the SIM medium and incubated at 37°C for 24 hours. The extension of bacterial growth from the inoculation stab line indicated positive motility. The medium turning black showed hydrogen sulfide production due to the reaction between hydrogen sulfide and iron to form ferric sulfide. After adding Kovac's reagent to the medium, a pink color showed indole formation (Sutami et al. 2021). The carbohydrate fermentation using glucose, arabinose, sucrose, maltose, lactose, and rhamnose was tested following the protocol reported by Saryono et al. (2023). The phenol red was used as an indicator of a fermentative reaction. The color change to yellow in the medium test indicated a positive reaction, and the bubbles in the Durham tube represented the gas formed, suggesting that fermentation was heterofermentative.

Evaluation of survival rates under simulated gastrointestinal pH conditions

Survival rates under simulated gastrointestinal pH conditions were examined using the procedure described by Lin et al. (2006) with a slight modification. A colony of each isolate was cultured in MRS broth and incubated at 37°C with shaking at 120 rpm for 16 hours. 1 mL of bacterial culture was transferred into 5 mL of PBS (pH 7.2 and 2.0), followed by incubation at 37°C for 2 and 4 hours. The pH of PBS was adjusted with 2N HCl. After incubation, viable cells were determined by the pour plate method (with PBS pH 7.2). One milliliter of the serially diluted sample was transferred to sterile plates, and PCA medium was added gently. After solidifying, the plates were incubated anaerobically at 37°C for 24 hours in the anaerobic jar with the AnaeroGenTM 3.5L (Thermo Fisher Scientific, USA). The grown colonies were counted in the range of 30-300 colonies. For each isolate, the measurement was performed in triplicate. The percentage of survival rates was calculated by dividing the population after pH treatments by the initial population, and the result was multiplied by 100.

Evaluation of bile salt tolerance

The tolerance of bile salt was determined by treating 1 mL of a 16-hour bacterial culture with 5 mL of PBS (pH 2.0), followed by incubation at 37°C for 1 hour without shaking. After low pH treatment, the survival bacteria were collected by centrifugation (13,000 rpm for 5 minutes), resuspended in 10 mL of MRS broth with or without supplementing 0.3% (w/v) bile salt (Thermo Fisher Scientific, USA), and incubated at 37°C for 3 hours under anaerobic conditions as reported by Lin et al. (2006). The viable cells for each sample were assayed in triplicate by the pour plate method as described above. The bacterial tolerance toward bile salts was determined by comparing the viable cell number on PCA agar. The survival rates (%) were calculated as the bacterial counts in 0.3% bile salt divided by the control and then multiplied by 100.

Genomic DNA extraction and amplification of the 16S rRNA gene

The selected isolates were inoculated in 5 mL of MRS broth and subsequently incubated at 37°C with shaking at 120 rpm for 24 hours. The cell pellet was harvested by centrifugation at 13,000 rpm for 3 minutes. The bacterial cells were lysed using 200 µL of lysozyme reaction solution (20 mg/mL of lysozyme, 20 mM Tris-HCl (pH 8.0), 2.0 mM EDTA, and 1.2% Triton X-100 [Millipore, USA]), and then incubated at 37°C for 60 minutes. The genomic DNA of the selected isolates was extracted using the FavorPrepTM Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) following the manufacturer's protocol. The genomic DNA concentration was measured by NanoDrop and then stored at -20°C for further analysis.

The 16S rRNA gene was amplified from the genomic DNA by PCR using forward universal primer E8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse universal primer E939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3') as previously described by Baker et al. (2003). A total reaction volume of 25 µL contained 12.5 µL of Gotaq Green Master Mix 2× (Promega, USA), 0.8 µM of each primer, and 125 ng of genomic DNA as a template. The PCR program was run as follows: 2 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 58°C, and 60 s at 72°C; and 5 min at 72°C. The PCR products were visualized by a 0.8% agarose gel containing 0.5 µg/mL of ethidium bromide. The confirmed bands were subsequently analyzed by DNA sequencing.

DNA sequencing and phylogenetic tree analysis

The sequence analyses of the amplified 16S rRNA gene were performed using ABI PRISM 3730xl Genetic Analyzer (Applied Biosystem, USA) through the 1st Base DNA Sequencing Service. The analyses employed the same primer sets for the 16S rRNA gene amplification. The resulting sequences were edited using BioEdit software, and the consensus sequences were generated by aligning the forward and reverse sequences. Subsequently, the bacterial species were identified by comparing the obtained sequences with the database in the NCBI (https:// blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST-searched program. Subsequently, MEGA11 software was used to build a phylogenetic tree following the maximum likelihood with Kimura-2 parameter models.

Data analysis

Data were statistically analyzed using Student's t-test to determine the differences in incubation periods and bacterial isolates. A paired t-test was conducted to differentiate the values of the same isolates under different incubation times, while an independent t-test was used to compare the values between different isolates. A *p*-value less than 0.05 was considered a statistically significant difference. The mean and standard deviation were calculated to determine the essential features of the acquired data. Statistical analyses were performed using JASP 0.19.3 software.

RESULTS AND DISCUSSION

Isolation, identification, and screening of lactic acid bacteria

Lactic Acid Bacteria (LAB) were isolated from the small intestine and cecum of a native chicken using MRS agar media. A single colony was randomly selected and purified to obtain pure isolates. The initial identification of LAB isolates was conducted by morphological and biochemical analyses as suggested by Bergey's Manual of Determinative Bacteriology (Bergey and Holt 1994), resulting in three isolates with different characteristics, as shown in Table 1.

Survival rates under simulated gastrointestinal pH conditions

The colonies indicating the LAB characteristics based on morphological and biochemical analyses were tested in correspondingly to gastrointestinal pH conditions (pH 7.2 and 2.0) with different incubation periods. The results showed no significant decreases in survival rates between 2-hour and 4-hour incubations at pH 7.2 for both isolates LC12 and LC16 ($p > 0.05$). When comparing both isolates, isolate LC12 demonstrated significantly higher survival rates than isolate LC16 ($p < 0.05$) after 2 and 4 hours at pH 7.2, as shown in Figure 1.A. The isolate LC12 had survival rates of 99.62% after 2 hours of incubation and 98.85% after 4 hours at pH 7.2, while the isolate LC16 indicated survival rates of 93.61% after 2 hours and 93.27% after 4 hours at the same pH (Figure 1.A). In contrast, a significant decline in survival rates of both isolates was documented between 2-hour and 4-hour incubations after exposure to pH 2.0 ($p < 0.001$), as depicted in Figure 1.B. The isolate LC12 reduced to 77.85% (after 2 hours) and 62.10% (after 4 hours), while the isolate LC16 decreased to 77.92% (after 2 hours) and 55.52% (after 4 hours). In addition, the isolate LC12 demonstrated a significantly greater survivability after 4-hour incubation at pH 2.0 compared to isolate LC16 ($p < 0.001$), as shown in Figure 1.B. These data collectively suggested that the survival rates under simulated gastrointestinal pH conditions gradually declined while maintaining a relatively high tolerance.

Table 1. Morphological and biochemical characteristics of bacterial isolates obtained from the gastrointestinal tract of the native chicken

Parameters	Isolate characteristics		
	LC12	**LC16**	**LC18**
Isolation spot	Ileum	Cecum	Ileum
Shape	Rod	Rod	Rod
Gram staining	Positive	Positive	Positive
Catalase	Negative	Negative	Positive
H_2S production	+	+	-
Indole	+	+	-
Motility	-	-	-
Glucose	+	+	+
Arabinose	+	+	+
Sucrose	-	-	+
Maltose	+	+	+
Lactose	-	-	+
Rhamnose	-	-	-
Gas from glucose fermentation	-	-	-

Note: (-): negative; (+): positive

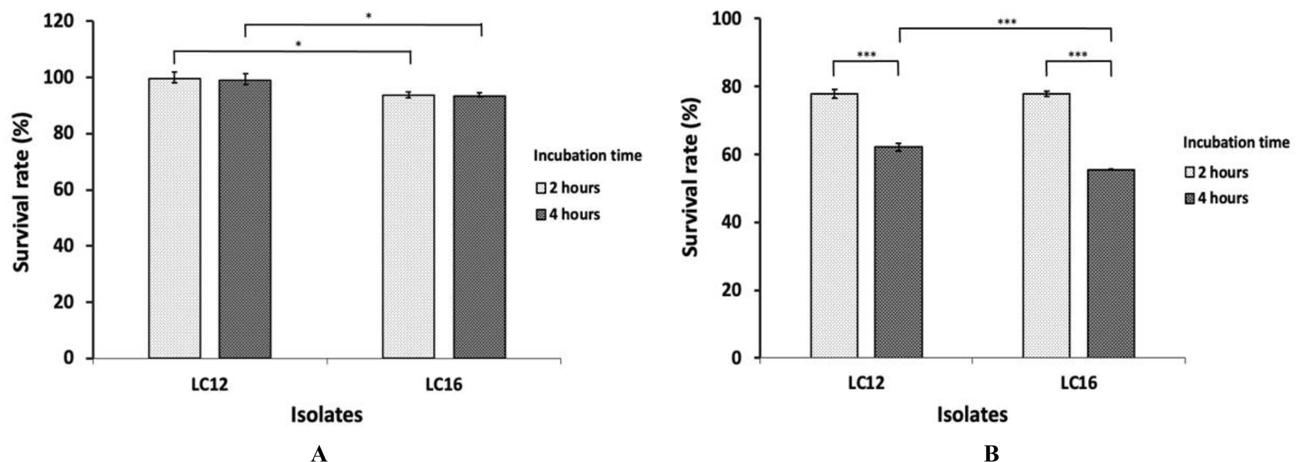

Figure 1. Survival rates (%) of the selected isolates under simulated gastrointestinal pH conditions with different incubation times (2 and 4 hours). A. pH 7.2, B. pH 2.0. Each value indicates the mean value ± standard deviation (SD) from three replications. The *p*-values are <0.05 (*) and <0.001 (***)

Tolerance to bile salt

The tolerance to bile salt was tested to determine the capability of the selected isolate to survive in the presence of bile salts in the gastrointestinal tract of poultry. In the present study, the selected isolates that survived acid (pH 2.0) treatment were in vitro tested using MRS broth with or without 0.3% bile salt for 3 hours. Our results demonstrated that the bacterial counts in MRS broth containing 0.3% bile salt after 3 hours for both isolates were significantly lower than those in the control group ($p < 0.01$), as shown in Figure 2.A. The bacterial count of LC12 was 6.41 Log CFU/mL in the control group and 4.62 Log CFU/mL (27.8% reduction) in the 0.3% bile salt treatment. Similarly, LC16 had a bacterial count of 6.40 Log CFU/mL in the control group and 4.59 Log CFU/mL (28.26% reduction) in the 0.3% bile salt treatment, respectively. Furthermore, Figure 2.B showed that the survival rates of both isolates were higher than 70%, suggesting tolerance to 0.3% bile salt.

Molecular identification using the 16S rRNA gene

The selected isolates were genetically identified using the amplification and sequencing of the 16S rRNA gene. Figure 3 demonstrated that a single band was confirmed with a size of around 932 bp, which covers variable regions V1-V5 of the 16S rRNA gene. The amplified bands presented in Figure 3 were sequenced to uncover bacterial identity. The sequences were compared with a genetic database available in GenBank by using the BLAST-searched program, demonstrating that two selected isolates, LC12 and LC16, were genetically identified as *Lactobacillus johnsonii* Fujisawa et al. 1992 strain 6-3 with 100% sequence similarity for both isolates. A phylogenetic tree was successfully constructed from the multiple sequence alignment of partial-length 16S rRNA sequences of two selected isolates, as depicted in Figure 4.

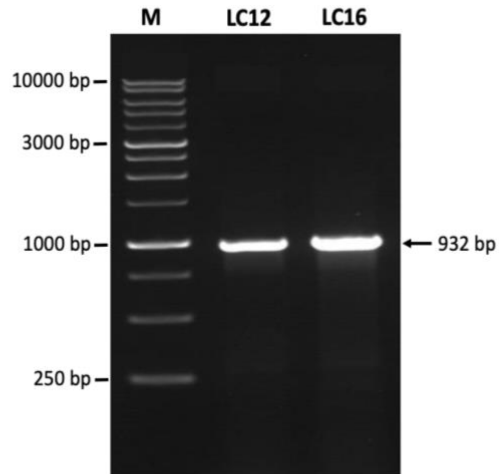

Figure 3. Amplification of the 16S rRNA gene from the selected isolates on a 0.8% agarose gel. M indicates a 1 kb DNA marker. The arrow showed the amplified bands

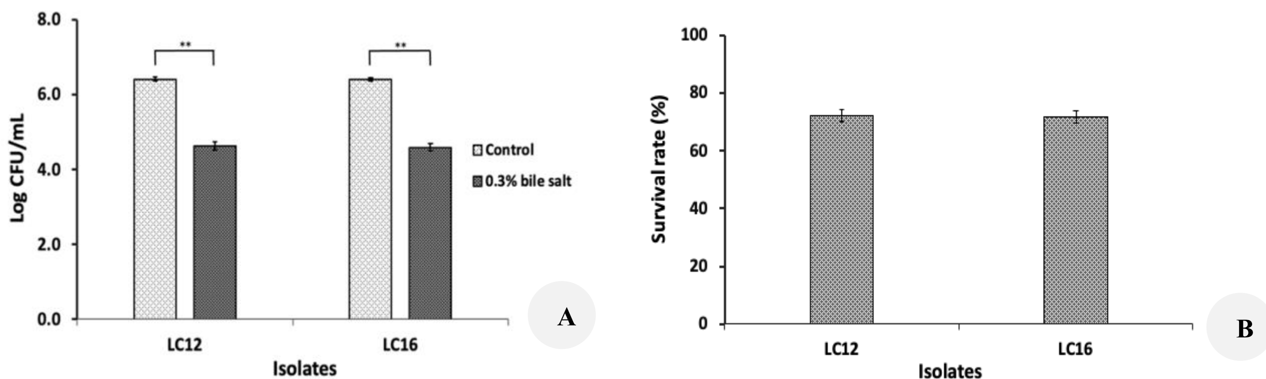

Figure 2. Bile salt tolerance of the selected isolates. A. A comparison of bacterial counts (Log CFU/mL) in two different conditions: control (without bile salt) and 0.3% bile salt, B. survival rates at 0.3% bile salt. Each value represents the mean value ± standard deviation (SD) from three trials. The *p*-value is <0.01 (**)

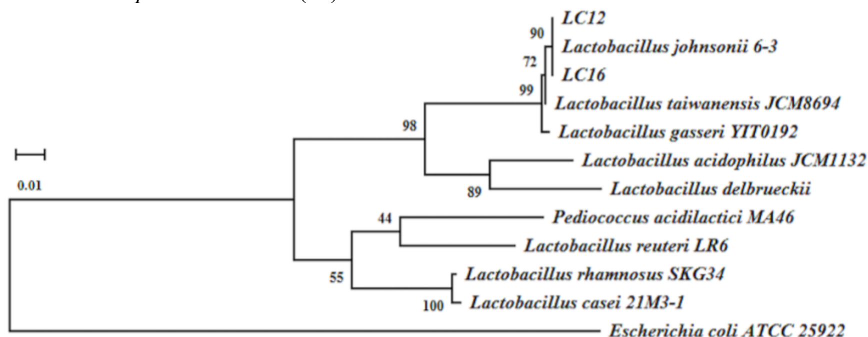

Figure 4. The phylogenetic tree demonstrated the genetic similarities of the isolates LC12 and LC16 with other species based on their 16S rRNA sequences. It was generated by the Maximum likelihood with the Kimura-2 parameter models. The phylogenetic distance of nucleotide substitutions per site is denoted by a scale bar of 0.01. The bootstrap percentage is represented by the values on the branches after 1,000 repetitions

Discussion

The isolation and screening of bacteria from the gastrointestinal tract of a native chicken demonstrated that three isolates were obtained. The preliminary identification was conducted by morphological and biochemical analyses, as shown in Table 1. The results revealed that all the isolates were Gram-positive and rod-shaped. The cell wall of Gram-positive bacteria is made of a thick layer of peptidoglycan, accounting for 23-33% of the cell wall's dry weight (Wallinder and Neujahr 1971), retaining the crystal violet color after staining (Risna et al. 2020). The acquired isolates were subsequently tested for their capability of producing catalase by dripping 3% hydrogen peroxide on the isolates, suggesting that only LC18 produced catalase enzymes, and two other isolates (LC12 and LC16) were found to be catalase-negative. The catalase enzyme converts hydrogen peroxide into water and oxygen (Watanabe et al. 2023). Sorescu et al. (2021) found that *Lactobacillus* strains isolated from the gut content of broiler chickens were biochemically tested as catalase-negative.

The SIM test indicated variations in bacterial motility, indole formation, and hydrogen sulfide production, as shown in Table 1. All the isolates were non-motile, suggesting the absence of flagellar activity. Two isolates (LC12 and LC16) tested indole-positive, demonstrating bacterial tryptophanase activity, which synthesizes indole from tryptophan in agreement with a previous study (Ferrer et al. 2022), while the other lacked this ability. Additionally, those two isolates (LC12 and LC16) could produce hydrogen sulfide, indicating the presence of sulfur reduction, whereas the other did not. These biochemical properties assist in the preliminary identification of isolates; for example, *L. plantarum* is typically non-motile, indole-negative, and does not produce hydrogen sulfide (Adikari et al. 2021; Ngouénam et al. 2024). Conversely, *L. rhamnosus* MN-431 can generate indole derivatives (Niu et al. 2022). Furthermore, *W. confusa* isolated from fermented sour rice has been identified among LAB isolates to produce indole (Nath et al. 2021).

We subsequently tested the carbohydrate fermentation to evaluate the ability of isolated bacteria to metabolize several selected sugars, such as glucose, arabinose, sucrose, maltose, lactose, and rhamnose. The results showed the differences in fermentation activity among the isolates (Table 1). Two isolates (LC12 and LC16) revealed a similar capacity to ferment glucose, arabinose, and maltose, but did not ferment sucrose, lactose, and rhamnose. In contrast, LC18 metabolized all tested sugars, except rhamnose. Interestingly, none of the isolates produced gases, demonstrating that they belong to a homofermentative group (Saryono et al. 2023). The observed variations in sugar fermentation profiles possibly suggest the presence of diverse carbohydrate metabolism-related genes among the isolates. These genes are commonly found within the bacterial chromosome and plasmids, and their presence and expression may vary. For instance, the inability to ferment rhamnose could indicate the absence or inactivation of the *rha* operon, which contains diverse enzymes required in rhamnose metabolism. These enzymes, including L-rhamnose mutarotase (*rha*M), L-rhamnose isomerase (*rha*A), rhamnulokinase (*rha*B), and rhamnulose-1-phosphate aldolase (*rha*D), are essential factors for converting rhamnose into intermediate molecules that enter central metabolic pathways (Elvan Gezer et al. 2025). The rhamnose metabolism-related gene clusters are present in a few strains of *Lactobacillus sakei* Katagiri et al. 1934, *Lactobacillus salivarius* Rogosa et al., 1953, *L. pentosus*, *P. acidilactici*, and *Pediococcus pentosaceus* Mees, 1934 (Cui et al. 2021). Similarly, the utilization of lactose and sucrose may be impeded by the absence of specific permeases or hydrolases (Wang et al. 2021). Different LAB strains exhibit various carbohydrate utilization profiles, with *L. plantarum* strains demonstrating a stronger carbohydrate utilization capacity. The number of sugar metabolism-related genes in *L. plantarum* strains is far more than those found in other lactic acid bacterial genomes (Cui et al. 2021; Takenaka et al. 2021; Elvan Gezer et al. 2025). Yulianto and Lokapirnasari (2018) previously reported that three *Lactobacillus* strains obtained from the digestive tract of chickens exhibited differences in their ability to utilize various sugars as a carbon source: *L. plantarum* fermented mannitol, sorbitol, glucose, xylose, rhamnose, sucrose, lactose, arabinose, and raffinose, while *L. casei* could ferment glucose, sucrose, lactose, arabinose, and raffinose. In contrast, *Lactobacillus acidophilus* (Moro, 1900) Hansen & Mocquot, 1970 demonstrated a more restricted sugar fermentation capacity, utilizing only glucose, rhamnose, sucrose, and lactose. In addition, Wang et al. (2024) reported that *L. johnsonii* showed the capability of fermenting several sugars, including glucose, galactose, cellobiose, maltose, melibiose, sucrose, and melezitose.

The obtained bacterial isolates, which indicated similar characteristics to LAB, were selected for further identification and characterization. The LAB strains are typically non-motile, catalase-negative, unsporulated, Gram-positive, spherical or rod-shaped, and acid-tolerant (Mokoena 2017; Amelia et al. 2021). Therefore, the isolates LC12 and LC16 were presumed to be LAB isolates since they demonstrated similar characteristics to LAB strains based on the results of the morphological and biochemical assays. Two selected isolates were tested under simulated gastrointestinal conditions owing to the importance of low pH and bile salt resilience in probiotic candidates.

The essential criterion for screening probiotic candidates for poultry relates to the functional features, including viability and transit tolerance under gastrointestinal conditions and the capability of adhering to the intestinal mucosa (Nemska et al. 2019). Probiotic bacteria orally administered to the host must remain alive under undesirable circumstances during digestion and their transit before reaching the intestines. Furthermore, to exert positive effects on the host, they need to survive against acidic conditions and high bile salt concentrations (Gharbi et al. 2019). Both isolates tested demonstrated high survival rates, greater than 90% at pH 7.2 (Figure 1.A) and 55% at pH 2.0 (Figure 1.B) after 2-hour and 4-hour incubation, suggesting adaptability over a wide range of pH conditions. The selected isolates showed excellent acid tolerance, which is an important key for selecting probiotic candidates. The survival rates of several *Lactobacillus* strains varied from 4.25% to 45.5% at pH 2.0 after 4 hours, with *L. acidophilus*

indicating the highest survival rate among the tested strains (Sirisopapong et al. 2023). Wang et al. (2024) reported that *L. jonhsonii* N5 could survive under pH 2.5 for 4 hours. A survival rate exceeding 50% in acidic conditions is considered high acid tolerance, in agreement with a previous study by Gharbi et al. (2019). Furthermore, previous studies demonstrate that a bacterial isolate, tolerating pH 3.0 for 3 hours, can be regarded as a high-acid-resistant strain with promising probiotic characteristics (Aiba et al. 2015; Mulaw et al. 2019; Mishra et al. 2023).

The LAB isolates obtained from the digestive tract of poultry are known to possess great adaptability to low pH conditions, thus improving their efficacy as probiotics (Noohi et al. 2016; Sirisopapong et al. 2023; Kassa et al. 2024). They likely employ one of three key mechanisms for their survival under acidic stress: cell membrane adaptations, proton pumps, and alkaline compound production. Moreover, several LAB strains belonging to the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Oenococcus*, and *Leuconostoc* Leverage Malolactic Fermentation (MLF) pathways to improve survival under stressful environments, such as low pH conditions, starvation, and excessive carbohydrates (Papadimitriou et al. 2016). This pathway ultimately allows for enhancing the cytoplasmic pH and Proton Motive Force (PMF), thereby increasing bacterial resistance (Papadimitriou et al. 2016). More importantly, *Lactobacillus casei* (Orla-Jensen, 1916) Hansen & Lessel, 1971 and *L. plantarum* were reported to employ proton pumps, F-ATP-ase, to maintain intracellular pH homeostasis that prevents acidification of their cytoplasm. Trip et al. (2012) reported that the elevated resilience of *Lactococcus lactis* NZ9000 (formerly *Streptococcus lactis* Pasteur 1857) and *Streptococcus thermophilus* Orla-Jensen 1919 in acidic environments was associated with the activity of histidine decarboxylase (HDC), which can be activated at low pH. Therefore, the tolerance of LAB strains to acidic conditions supports their broad application as probiotics for enhancing gut health in poultry.

The tolerance of isolated bacteria to bile salt is considered a subsequent critical probiotic criterion for colonization and metabolic activity in the host's gastrointestinal tract. The results revealed that both isolates (LC12 and LC16) tolerated 0.3% bile salt (survival rate >70%), as shown in Figure 2.B. These findings correspond with those obtained by Zommara et al. (2023), who reported that the survival rate of *Lactobacillus* strains at 0.3% bile salt varied from 42.25% to 85.25%. Consistently, Gharbi et al. (2019) found that multiple *Lactobacillus* strains, excluding *L. fermentum* 10, indicated a survival rate varying from 53% to 108% after a 3-hour exposure to 0.3% bile salt. Moreover, Yadav et al. (2016) suggested that several strains of *L. plantarum* could survive under 0.3% bile salt for 3 hours, ranging from 68.31% to 80.49%. The LAB isolates with a survival rate greater than 50% at a concentration of 0.3% ox-bile salt are classified as bile salt-resistant strains, allowing their function as probiotics (Zommara et al. 2023).

The differences in probiotic tolerance to bile salts depend on the strain, bile salt concentration, and exposure period. Some probiotic strains from the genera *Lactobacillus* have frequently been reported to deconjugate bile salts found in the gastrointestinal tract through the activity of Bile Salt Hydrolase (BSH) enzymes, which are considered to aid in the colonization and survival of bacteria in the gastrointestinal tract (O'Flaherty et al. 2018; Risna et al. 2020). Besides this, the polysaccharides found on the outer cell membrane may also contribute to bacterial resistance to bile salts (Tarique et al. 2022; Zommara et al. 2023).

We confirmed the data obtained from morphological and biochemical assays by amplifying and sequencing the 16S rRNA gene to identify the selected isolates further. A single band with a size of approximately 932 bp was successfully amplified from the genomic DNA of the isolates LC12 and LC16, as shown in Figure 3. The amplification and sequencing of the 16S rRNA gene around 900 bp, covering variable regions V1-V5, were sufficient to identify LAB isolates at the species level (Jomehzadeh et al. 2020). The 16S rRNA sequences of the isolates LC12 and LC16 demonstrated a similarity (100%) with *L. johnsonii* strain 6-3 (Figure 4). Two or more sequences with greater than 95% identity are categorized into the same genus, whereas those sequences with greater than 99% identity represent the same species (Schloss and Handelsman 2005). Our findings demonstrated that the sequences containing V1-V5 regions fully classified the isolated bacteria in agreement with Balcázar (2007), who revealed that the V1 and V2 regions completely distinguished among the LAB strains. Another study showed that the V1, V3, V5, V6, and V7 regions of the 16S rRNA gene are conserved, whereas the V2, V4, V8, and V9 regions are hypervariable in the family (Chakravorty et al. 2015). We consider that a combined approach between morphological, biochemical, and molecular analyses would ensure a more accurate identity of LAB strains since molecular approaches can confirm conventional identification methods by providing appropriate genetic distinction.

L. johnsonii is a member of lactic acid bacteria, which is widely distributed and commonly discovered in the gastrointestinal tract of poultry with a long history of applications in the food and fermented feed industries (Johnson et al. 2023; Zhang et al. 2023). The probiotic *L. johnsonii* strain isolated from the digestive tract of poultry is recognized as a well-adapted microbe in the host for probiotic application, increasing poultry performance through beneficial modulations of the gut microbiota (Ward et al. 2019; Johnson et al. 2023). More importantly, *L. johnsonii* No. 1088 was observed to have high acid resistance and strong anti-*H. pylori* activity, as reported by Aiba et al. (2015). These merits indicate its promising role in enhancing gut health and productivity in poultry.

In conclusion, the LAB strains isolated from the gastrointestinal tract of a native chicken were successfully identified as *L. johnsonii* strain 6-3 based on the result of molecular analyses. These isolates, with their remarkable high survival rates against acidic conditions and bile salts, indicate their robustness and potential as probiotic candidates for beneficial applications in poultry. The findings in this study contribute to the development of probiotic feed supplements as alternatives to antibiotic growth promoters in poultry. Future research is expected to focus on safety evaluations, including antibiotic resistance characteristics

and non-pathogenicity, followed by in vivo trials to examine the impact of probiotics on poultry health and performance.

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