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Identification of Probiotic Bacteria Isolated from Domestic Chickens (*Gallus domesticus*) Using the 16S rRNA Gene Method

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ABSTRACT

The intestines of domestic poultry (*Gallus domesticus*) are one of the potential sources of probiotic bacteria that can produce antibacterial agents. The objective of this study was to identify the types of probiotic bacteria obtained from the digestion of domestic poultry using the molecular analysis method of 16S rRNA gene sequencing. Observations were conducted on colony morphology, gram staining, biochemical tests, and antibacterial activity using the diffusion agar method. Molecular analysis of DNA extraction was carried out, followed by the amplification of samples using a 16S rRNA universal primer. Dielectrophoresis and sequencing were performed on the 16S rRNA gene. The identification of morphological observations, gram staining, and biochemical tests showed that probiotic bacteria isolates, including Gram-positive, rod-shaped, rounded colony form, flat elevation, entire nonmotile edge, and catalase-negative, could ferment all carbohydrate content in the TSIA medium. The antibacterial potential was also found in probiotic bacteria, as evidenced by the inhibition zone formed in the test. The results of the bacterial gene sequences of PaTa5 probiotic bacterial isolates had a similarity of 98.37% with *Lactobacillus plantarum.* These findings indicated the presence of some bacteria species that have antibacterial activity in the intestines of domestic chickens (*Gallus domesticus*).

Keywords: *Lactobacillus plantarum*, Native chicken, Probiotic, 16S rRNA

INTRODUCTION

Probiotics are live microorganisms that positively affect the health of organisms and can balance the bacteria in the digestive tract by suppressing the growth of harmful pathogenic bacteria (Anadón et al., 2016; Vahdatpour and Babazadeh, 2016). One of the potential sources of probiotic bacteria is found in domestic poultry (Husain et al., 2017; Husain et al., 2019). The habitat of domestic poultry in the open environment causes the composition of the population of microorganisms that live in the digestive tract to be suspected to be high. Microbiota in the digestive system can form colonies that protect the digestive tract by sticking to epithelial tissue on the enterocyte wall, thereby reducing colony formation from pathogenic bacteria (Shang et al., 2018).

The presence of bacteria in the digestive tract of chickens is partly due to the interaction of bacteria and the environment that contaminates the chicken's body through the feed. The difference in chickens' age will also influence the differences in the types of bacteria that exist (Talaiekhozani et al., 2015). The majority of bacteria found in adult chickens is dominated by the *Lactobacillus* genus, which is one of the variations in the composition of the bacterial population between 2-day-old chickens and adult chickens (Sugiharto et al., 2018).

In an effort to find potential bacterial strains as antibiotics, the identification of bacterial strains continues to develop from conventional physiological and biochemical characterizations. Identification by this method requires a long time, and identification results are less accurate. An efficient, highly sensitive, and specific

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molecular-based identification technique is currently being developed by analyzing 16S rRNA gene sequencing (16S ribosomal ribonucleic acid, S states Svedberg, the ribosome measurement unit) pioneered by Woese and Fox in 1977.

The objective of the current study was to identify potential types of probiotic bacteria originating from domestic chickens using the 16S rRNA method.

MATERIALS AND METHODS

Ethical approval

This research was conducted at the Microbiology Laboratory, Hasanuddin University, Indonesia. The procedure in this study followed the standards of the guidelines for the use of animals (Buchanan et al., 2012).

Sampling and isolation

Domestic chickens (*Gallus domesticus*) were obtained from Takalar, South Sulawesi, Indonesia (5°08.184'S,119°29.520'E). The inner walls of the chicken intestine were scraped and then inserted into a physiological sterile NaCl solution and diluted with graded dilution. A serial dilution was done at concentrations from 10^{-1} to 10^{-6}. 1 mL solution from the inoculated dilution in the De Mann–Rogosa–Sharpe agar (MRSA) medium (the pour plate method), then incubated at 37°C for 24-48 hours. The isolate obtained was coded PaTa5.

Morphological and biochemical characterization

Morphological observations were conducted by observing the characteristics revealed by the colonies formed using a stereo microscope. Gram staining technique was done by painting bacterial cells with a solution of violet crystals, lugol, alcohol-acetone, and safranin to determine the type of gram in bacteria. Biochemical characterization was done using several tests, including the Triple Sugar Iron Agar Test (TSIA), Motility Test, and catalase test (Husain et al., 2022).

Inhibition test

The test was carried out *in vitro* by the agar diffusion method using a 10 mm blank disk. The test media was incubated at 37 for 1×24 hours, and the inhibition area was measured using calipers. Incubation was continued for up to 2×24 hours to determine the properties of the active compounds in the culture (Husain and Wardhani, 2021).

DNA extraction

The Geneaid PrestoTM Mini gDNA Bacteria Kit's DNA (Geneid, Taiwan) extraction procedure was used to extract genomic DNA (Geneaid, 2017).

16S rRNA gene amplification

This technique was performed on isolated DNA samples using a previously prepared PCR reaction mixture consisting of 16 l 2 Mytaq HS Red Mix, 2 µl of each of the 16S rRNA Primary Forward gene 63F and the Primary Reversal gene 387R, and 4 μl of H2O. Tubes corresponding to amplifications of samples were filled with 22 µl PCR reaction mixture, then 5 μl of nuclease water and 5 µl of DNA extract were added. Amplification was carried out for 30 cycles, and each cycle consisted of denaturation at 95°C for 5 minutes, at 95°C for 1 m, annealing at 57°C for 1 minute, extending at 72°C for 1 m, and post-extending at 72°C for 10 m (Sune et al., 2020).

PCR products by electrophoresis detection

The amplified DNA was separated with 1% agarose gel electrophoresis. The ethidium bromide (UltraPure™, United States) dye was used to observe the results, and the UV-transilluminator was used to detect them. The results of the detection were recorded according to the method described by Marzuki et al. (2015).

DNA sequencing

The basic local alignment search tool (BLAST) program was used to compare the partial DNA sequences of 16S rRNA with the public database (NCBI) to find homologies. Query cover and E-value values could be seen in the blast analysis results. Query cover indicates the percentage of sample used in the BLAST analysis, and the E-value shows the level of probability statistically of an item. A low E value indicates a high level of homology (Madden et al., 2013). Clustal was used to perform multiple alignments, and a phylogenetic tree was constructed using MEGA version 6.0 utilizing the neighbor-joining technique and bootstrap values derived from 1,000 replications (Sune et al., 2020).

RESULTS AND DISCUSSION

Morphological and biochemical characterization

Based on the results of colony morphological tests, the characteristics of a colony were cream-colored, smooth surface of the colony, the entire edge, and the shape of flat elevation (Table 1). Morphological characterization was used to observe the morphology of bacterial colonies. Microorganisms grown on various media show different macroscopic appearances on growth (Wafula et al., 2015).

The results of gram staining on probiotic bacteria showed that the shape of the bacterial cell was in the form of a stem (bacilli). Probiotic bacterial isolates were also

classified as Gram-positive bacteria, where the results of staining indicated a purple color (Figure 1). Based on the gram staining results, the nature of bacterial cell walls against crystal violet dye stain (primary color) and safranin (opponent color) could be seen (Aisha et al., 2017).

Table 1. Morphological characteristics of *Lactobacillus plantarum*

Color	Colony Surface	Shape	Edge	Elevation
Cream	Smooth	Circle	Entire	Flat

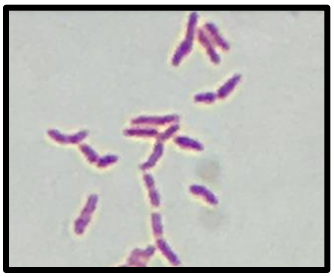

Figure 1. Gram staining of *Lactobacillus plantarum*

The morphological character of probiotic bacterial cells was obtained following the characteristics of probiotic bacteria classified as *Lactobacillus* gram-positive bacteria (Mannan et al., 2017). Gram-positive bacteria have a thick cell wall and a cell membrane layer so that when the bacteria become dehydrated by giving alcohol, 96% of the pores will shrink, which causes the main color (crystal violet) not to get out (Peristiawati et al., 2019).

The results obtained from the TSIA Test revealed that the PaTa5 probiotic isolate was not formed by gas and black sediment (H_2S), and the yellow Slant and Butt parts indicated that the PaTa5 isolate was acidic. Therefore, it could ferment the three types of sugar, namely glucose, lactose, and sucrose. TSIA media contain sugar, glucose, and lactose/sucrose (Dalyn, 2014). Probiotic bacteria obtain energy, while it only depends on fermentative metabolism. There are types of lactic acid bacteria that can ferment the three types of sugar found in the TSIA medium, namely glucose, lactose, and sucrose. It is marked in yellow on the slant (slanted agar) and yellow on the Butt (Murugan, 2017).

Motility test observation results showed that the isolate of PaTa5 probiotic bacteria was nonmotile since there was no propagation around the inoculation area. The PaTa5 isolate did not have a movement tool (flagella). Probiotic bacteria were nonmotile due to their minimal capacity for biosynthetic activity. Energy acquisition is totally dependent on the fermentative metabolism that takes place in its place (Wu et al., 2017).

The catalase test results were negative, indicating that air bubbles (O_2) did not form when the probiotic bacteria isolate was dripped with H_2O_2 solution. Isolates showed negative results on the catalase test. It can be concluded that the isolate of Lactic acid bacteria was homofermentative (Mannan et al., 2017).

Inhibition test

The inhibition test on pathogenic bacteria aims to determine the ability of isolates of probiotic bacteria obtained from domestic poultry *Gallus domesticus* to inhibit pathogenic bacteria, especially on the test bacteria used, namely *Escherichia coli* (*E. coli*, negative gram) as enteric bacteria and *Staphylococcus aureus* (*S. aureus*, positive gram) as pathogenic bacteria (Rouger et al., 2017). *E. coli* and *S. aureus* are bacteria often found in the digestive tract of small poultry intestines and are pathogenic.

The PaTa5 probiotic bacterial isolate had a clear zone diameter in the *E. coli* test bacteria of 17.5 mm, an incubation period of 1×24 hours, and 18 mm during the incubation period of 2×24 hours (Figure 2). Meanwhile, the *S. aureus* test bacteria obtained a diameter of 12.5 mm during the incubation period of 1×24 hours and 13 mm during the incubation period of 2×24 hours (Li et al., 2016). The inhibition zone of 10-15 mm was classified as weak, 15-20 as moderate, and strong if> 20 mm. Therefore, the isolate of the PaTa5 probiotic bacteria had a moderate inhibitory effect on *E. coli*, while it was classified as having a weak inhibitory effect on the *S. aureus* bacteria. Probiotic bacteria are a type of bacteria that occupy the gastrointestinal tract. They can inhibit the growth of some bacteria by producing various antibacterial components (organic acids, hydrogen peroxide, and bacteriocin) that can suppress the growth of pathogenic bacteria (Husain et al., 2019; Weerapong et al., 2016).

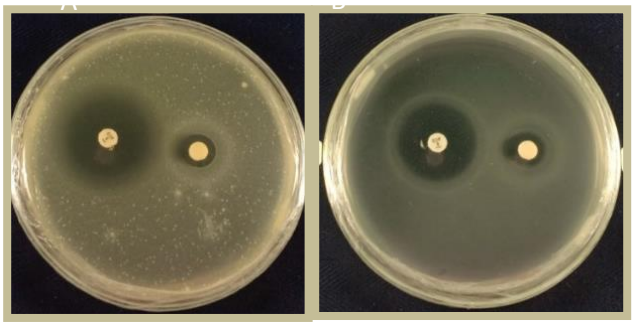

Figure 2. Inhibition test results for *Escherichia coli* (A) and *Staphylococcus aureus* (B)

16S rRNA gene amplification

Genomic DNA isolated and extracted from probiotic bacterial isolates was then analyzed by PCR to amplify the 16S rRNA gene using universal primers for 16S rRNA genes, namely forward primer 63F and reserve primer 1387R.

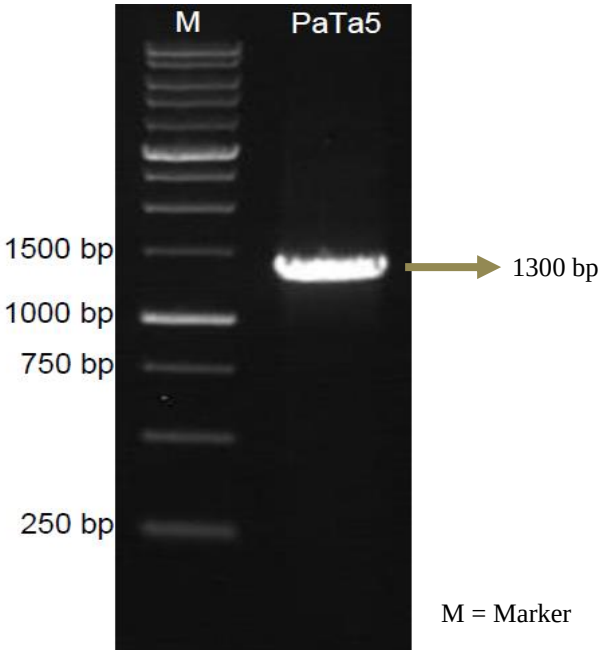

Figure 3. Results of 16S rRNA gene amplification with 63F and 1387R primers

The pairing of the 63F and 1387R PCR primary designs for 16S rRNA gene amplification of bacteria were systematically evaluated and tested in terms of the specificity and efficiency of various types of bacteria and samples in the environment. Primer pairs are more useful for 16S rRNA gene amplification in ecology and systematic studies. This primer could identify organism coryneform and genus *Micrococcus* (Gram-positive, high guanine (G) and cytosine (C), *Eubacterium*, and *Proteobacteria*. In addition, 63F was found to have a greater hybridization potential, compared to other primers (27F and 1392R), where the primer cannot amplify the conserved area optimally compared to the primary pair 63F and 1387R (Marchesi et al., 1998). The amplification results were electrophoretic with agarose 1.5% and visualized by UV light.

Based on the results of electrophoresis visualization, the primer used could be amplified well in PaTa5 bacteria probiotic isolates and is at a size of about 1300 bp (Figure 3). As can be seen in Figure 3, the pattern of DNA bands resulting from PCR amplification was formed using primers in the form of a single band. The DNA bands of PaTa5 probiotic bacteria isolates appear thick. This shows the DNA of the amplified PaTa 5 sample had a high concentration, indicating that the PaTa5 sample was well amplified using 63F and 1387R primers.

The suitability of the primers greatly affected the identification results. Misuse of primers could lead to the amplification of other regions in the genome that are not targeted or otherwise. No genomic regions were amplified. Optimization of annealing temperature was also a factor that affected the success of identification (Rychlik et al., 1990; Guillen et al., 2016).

The PaTa5 sample was amplified using a universal primer pair 63F and 1387R, which showed a clear and thick DNA band, then proceeded to the next stage of DNA sequencing. The intensity of the fragment during visualization was feasible to be used at a later stage with a clearly visible band, and no smears were found. The difference in band thickness indicated different DNA concentrations (Kuhn et al., 2018).

16S rRNA fragments by sequencing analysis

DNA sequencing is the process of sequencing a DNA base. This process uses the principle of enzymatically polymerizing DNA reactions (Kchouk et al., 2017). In order to determine the identity of the bacteria, BLAST first determines the base type.

Several parameters, including Query cover, E-value, and ident, can be seen in Table 2. The query cover indicated the percentage of samples used in the BLAST analysis, and the E-value showed the level of probability statistically of an item. Of the four parameters, the most accurate was the E-value. A high percentage of homology was indicated by a smaller e-value (Madden et al., 2013).

Identification results were 98% since differences in several pairs of nucleotide bases aligned. This can be caused due to mutation. Mutations are defined as changes in the DNA base. Mutations can occur spontaneously, where one of the bases is lost from the nucleotides through hydrolysis (Beyene et al., 2010).

Analysis of the 16S rRNA gene sequence shows that the isolate of the PaTa5 probiotic bacteria obtained from the intestine of domestic poultry *Gallus domesticus* had the closest resemblance to *Lactobacillus plantarum* (*L. plantarum*). The *L. plantarum* bacteria can be classified into the domain of Bacteria, phylum of *Firmicutes*, class of *Bacilli*, order of *Lactobacillales*, family of *Lactobacillaceae*, genus of *Lactobacillus*, and plantarum species of *Lactobacillus* (Zheng et al., 2020).

Table 2. Sequencing analysis result of PaTa5 isolates

Homologous Lactic acid bacteria species	Query coverage (%)	E Value	Identities
Lactobacillus plantarum strain CAU:227	99 %	0.0	98.37%
Lactobacillus plantarum strain CAU:222	99 %	0.0	98.37%
Lactobacillus plantarum subsp. strain Ni997	99 %	0.0	98.37%
Lactobacillus plantarum strain MMB03	99 %	0.0	98.36%
Lactobacillus plantarum strain KLDS 1.0344	99 %	0.0	98.36%
Lactobacillus plantarum strain YLL-03	99 %	0.0	98.36%

CONCLUSION

Morphological characterization shows that PaTa5 isolate is a gram-positive with bacillus (rods) form. PaTa5 isolates could ferment all carbohydrate content in the TSIA medium, nonmotile in the SIM medium, and negative results at the catalase test. The inhibition test for pathogen bacteria shows a clear zone (inhibition zone) around the PaTa5 isolate. Metabolite was produced by this isolate from the inhibition zone in the *E. coli* test bacteria of 17.5 mm during the incubation period of 1×24 hours, and 18 mm during the incubation period of 2×24 hours. Meanwhile, the *S. aureus* test bacteria obtained a diameter of 12.5 mm during the incubation period of 1×24 hours and 13 mm during the incubation period of 2×24 hours. The results of the bacterial gene sequences of PaTa5 probiotic bacterial isolates have a similarity of 98.37% with *L. plantarum*. Probiotic bacteria are often used as a food fermentation agent since they can improve food flavor. Considering the obtained results of the current study, probiotic bacteria have biological activity, such as antibacterial, making them suitable as fermentation agents for the production of functional foods and beverages.

DECLARATIONS

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Authors' contribution

Dirayah Rauf Husain and Riuh Wardhani conceptualized. Dirayah Rauf Husain, Riuh Wardhani, Fiqha Septia Ningsih, and Fuad Gani implemented the research. Dirayah Rauf Husain, Riuh Wardhani, and Fiqha Septia Ningsih wrote the manuscript. Riuh Wardhani and Fuad Gani commented on research improvement. All authors have read and agreed to the last version of the manuscript.

Competing interests

There is no competing interest exists in this research.

Ethical consideration

All authors have checked statistical analysis as well as the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

Availability of data and materials

The data of the article will be provided by the corresponding author according to reasonable requests.

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