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Lactic Acid Bacteria Isolated from Digestive Tract of Broilers Treated with Fish Protein Hydrolysate

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Abstract. *The composition of the feed plays a role in stimulating the activity of the microbiota in the gastrointestinal tract; therefore, the addition of fish protein hydrolysate (FPH) is suspected to influence the activity of microbiota, especially probiotics. Therefore, the presence of probiotics in the gastrointestinal tract affects the weight and quality of the broiler. This research aims to investigate the effect of administering fish protein hydrolysate as a dietary supplement on the composition of lactic acid bacteria (LAB), a potential probiotic candidate. This research was conducted in several stages, including the diversification of feeding broilers, the isolation and purification of LAB from the caecum and small intestine, primary characteristics, pathogenicity tests, and biochemical identification of LAB. Feed diversification was conducted by supplementing diets with 1%, 1.5%, and 2% FPH in 20-day-old for 7 days. LAB from the small intestine and caecum samples were isolated on MRSA media by adding* $CaCO_3$*. The Gram test, catalase test, and endospore staining test were carried out to characterize the suspected LAB primarily. The pathogenicity test was conducted by inoculating LAB on blood agar medium. Furthermore, biochemical tests are carried out using the KB020 kit. The results showed that the highest population of LAB in the small intestine (*1.57×10^8 *CFU/mL) was observed with 2% FPH supplementation. In comparison, the caecum yielded the highest population (*1.22×10^8 *CFU/mL) under 1.5% FPH. Giving 2% FPH to broiler chicken feed provides a weight gain of 1.021 kg/head. The primary characteristics of the eight bacterial colony isolates suspected of being LAB were Gram-positive, catalase-negative, and did not form endospores. Eight LAB isolates of probiotic candidates were non-pathogenic as indicated by the occurrence of α-hemolysis and γ-hemolysis. Biochemical identification of probiotic candidates yielded four types of Lactobacillus, namely L. mucosae, L. frumenti, L. sanfranciscensis, and L. ferintoshensis. These LAB strains show promising probiotic potential for use as a feed additive in the broiler production system.*

Keywords: *fish protein hydrolysate, lactic acid bacteria*

Citation
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INTRODUCTION

Broiler chickens are a source of animal protein with a higher nutritional value compared to other poultry, such as free-range chickens and ducks. Broiler chickens contain 22% protein, while free-range chickens contain 13.4% and ducks 13.3% (Agromedia, 2011; Suwarta and Darmaji, 2022). The level of broiler chicken consumption in Indonesia has increased, necessitating an increase in the supply of broiler chickens to meet this demand (Qurniawan et al., 2016). Surahman et al. (2021) stated that feed is the primary factor in broiler chicken production, playing a crucial role in growth and energy supply. Broiler chicken feed must contain nutrients in accordance with the standards set by the National Standardization Agency (2015), which include a minimum crude protein of 19%, a minimum of 5% crude fat, a maximum of 6% crude fiber, and a maximum water content of 14%. One source of additional protein that can be added to broiler chicken feed is fish protein hydrolyzate (FPH), which is derived from the breakdown of protein in fish into peptides (Salamah et al., 2012).

Simple peptides that serve as additional nutrients support the growth of microorganisms and increase the feed absorption process (Salazar-Villanea et al., 2022). So, it is suspected that the addition of FPH in feed plays a role in determining the composition of the gastrointestinal tract microflora. The type of feed given to chickens can influence the number of lactic acid bacteria (LAB) naturally present in the chicken's digestive system (Widodo et al., 2015). The absorption of nutrients in feed is related to the composition of the feed, which influences the activity of microorganisms, such as LAB, in the digestive tract (Harumdewi et al., 2018).

Isolation of LAB from the intestines and caecum of broiler chickens was conducted because this region of the digestive tract serves as the natural habitat for LAB that have adapted to the host's physiological conditions. LAB isolates from the same host (host-specific) generally demonstrate superior colonization capability and probiotic efficacy compared to those from different sources, making them promising candidates for development as enhanced probiotics to support the natural and sustainable health and productivity of chickens (Halder et al., 2024).

LAB, which has the potential to act as a probiotic, can improve health in both humans and livestock by maintaining the balance of microflora in the digestive tract when consumed in sufficient quantities (Wahyuni, 2016). Probiotics will directly or indirectly influence the physiological function of the intestine by modulating the intestinal microbiota and the mucosal immune system, especially in the gastrointestinal mucosa. The presence of probiotics causes the villi in the intestine to become longer and denser, thereby increasing nutrient absorption activity by expanding the surface area available for absorption. Microorganisms in the chicken's digestive tract are found throughout the chicken's intestines (Jin et al., 2000). The main microorganisms found in the crop, small intestine, and ceca (also known as the appendix or caecum) belong to the *Lactobacillus* group, which produces lactic acid and acetic acid (Sari et al., 2013).

Based on this description, this research aimed to determine the impact of administering FPH at various concentrations as a dietary supplement on the composition of LAB as probiotic candidates from the intestines and caeca of broiler chickens. Broiler feed mixed with FPH is expected to affect the number of probiotic populations and weight gain in broiler chickens. Alang et al. (2020) stated that bacteria considered candidates for probiotics must exhibit non-pathogenic properties, making pathogenicity testing necessary. Further identification is also required to determine the characteristics and species of LAB as probiotic candidates.

MATERIALS AND METHODS

This research was conducted in March-June 2023. The feeding treatment was conducted on March 6, 2023, when the chickens were 21 days old, with an average body weight of approximately 800 grams. Subsequently, sampling was conducted on March 13, 2023, seven days after the start of feeding. Broiler maintenance is carried out at the Subadri Farm broiler farm in Darsono Village, Jember, and analysis of broiler intestine samples at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, University of Jember.

Population and Research Sample

The total population of 40 chickens was divided into four groups, each consisting of 10 chickens, to facilitate replication. Maintenance of broiler chickens is carried out in vivo, while the observation of suspected LAB bacteria is conducted in vitro. The experiment was divided into four treatments based on feed diversification with different FPH concentrations. Treatment for giving FPH included C as control (basal feed without FPH addition); T1 (basal feed with the addition of 1% FPH); T2 (basal feed with the addition of 1.5% FPH); and T3 (basal feed with the addition of 2% FPH).

Feed Preparation and Feeding

The FPH used is based on the Lemuru fish, as developed by Ivana et al. (2019). Fish powder is obtained by drying in an oven at 105°C for 3 hours, then cooling and drying again for 15 minutes until it is stable. A total of 1 kg of fish powder was added to 1 L of distilled water or a ratio of 1:1. The suspension was incubated for 5 days at room temperature. The addition of 6M H_2SO_4 or 6M NaOH is performed to regulate the pH to 2.5. The resulting filtrate is then added to the new fish powder and incubated for an additional 5 days. The second filtrate is a fish protein hydrolysate. Feed preparation is carried out by mixing commercial feed "Pokphand", which contains 14% protein. We added an FPH of 0%, 1%, 1.5%, and 2% of the total amount of chicken feed. The prepared chicken feed was then given to broiler chickens in the morning for 7 days of treatment. Broiler chicken intestine samples were taken on the eighth day.

Broiler Growth Measurement

The growth of broilers is monitored through weight measurements, which were taken on day 0 and day 8 after the FPH-supplemented feeding treatment. The effect of adding FPH supplements is determined by weight gain, which is the difference between the final weight after treatment and the weight before treatment. Furthermore, LAB isolation was carried out on the eighth day after treatment with FPH-supplemented feeding.

Isolation of LAB from Broiler Intestine Samples

The isolation of LAB from the small intestine and caecum was carried out using a multilevel dilution method with a 0.85% NaCl solution (Kurnia et al., 2020) to a dilution of 10^{-10}. The two samples were then cut so that the liquid inside could be released when diluted. Dilution was performed using a 1:9 ratio for samples and 0.85% NaCl as a 10-fold dilution (Yang et al., 2012; Elisanti et al., 2020). The sample from each dilution series were inoculated on de Man Rogosa Sharpe Agar (MRSA) from Himedia with the addition of $CaCO_3$ media as an indicator of a clear zone formation on MRSA media (Yolanda & Meitiniarti, 2017). A total of 1 mL samples were inoculated in 10 mL MRSA media using the pour plate method and incubated for 48 hours in an anaerobic jar (Putri and Kusdiyantini, 2018).

Calculation of the LAB Population

The population of suspected LAB isolates was determined by counting colonies on MRSA media using a colony counter. The requirement for the number of bacterial colonies to be counted ranges from 30 to 300 colonies with units of CFU/mL (CFU = Colony Forming Units) (Ayun et al., 2023). The formula determines the calculation of the number of bacterial colonies:

Number of Bacterial Colonies = number of colonies × 1/dilution factor

Purification of Suspected LAB Bacterial Isolates

The primary characterization of suspected LAB bacterial isolates was carried out through microscopic observations, including Gram and endospore staining tests. The Gram staining test is carried out to determine the Gram type and cell shape of bacterial isolates (Putri and Kusdiyantini, 2018). The endospore staining test was performed to detect the presence of endospores in bacterial isolates (Bell et al., 2005). The bacterial isolate suspected to be LAB is negative because it does not form endospores, which are marked in red on the preparation, and is instead a vegetative cell (Zhi et al., 2021). The catalase test, as part of the primary characterization of LAB, is performed to detect the presence of the catalase enzyme produced by bacteria (Romadhon et al., 2012). Suspected LAB bacteria give a negative response or do not form air bubbles (Goyal, 2012).

Pathogenicity Test of Lactic Acid Bacteria Isolates

The pathogenicity test of purified LAB isolates was carried out using sheep blood agar media (Columbia agar plates) (Hawaz, 2014). One loopful of each LAB isolate was taken and placed on the surface of blood agar media in a petri dish. Incubation was carried out at 37°C for 24 hours (Chotiah and Damayanti, 2018). The results of the pathogenicity test were observed to see the presence of β-haemolysis (clear zone around the colony), α-haemolysis (green zone around the colony), or γ-haemolysis (no clear zone around the colony) (Jamaly et al, 2011).

Observation of Macroscopic Morphological Characteristics of Lactic Acid Bacteria

The growth of LAB colonies in MRSA media observed included colony shape, colony color, colony margins, and elevation. Lactic acid bacteria exhibit a macroscopic character of a rounded colony, characterized by a milky-white colony shape, a convex colony surface, and flat, smooth edges.

Identification of LAB using the HiLacto KB020 Kit

LAB inoculum was prepared by inoculating 5 mL of MRS Broth (MRSB) and incubating at room temperature or 35-37°C with 5-10% CO_2 for 6-18 hours, until the turbidity of the inoculum reached 0.1 OD at the 0.5 McFarland standard (HiMedia, 2022). Identification of LAB isolated from the intestine and caecum of broiler chickens was carried out using the HiLacto KB020 Kit. The inoculum was inoculated into 50 µl wells of the kit using the surface inoculation method. Kits that have been inoculated with LAB isolates are then incubated at 35-37°C with or without 5-10% CO_2 for 24-48 hours. Observations of identification results are adjusted to the results interpretation chart in the kit (HiMedia, 2022).

Data Analysis

The data obtained consisted of quantitative variables, including the population of suspected LAB bacteria and the weight gain of broiler chickens. These data were analyzed using One-Way ANOVA (Analysis of Variance) followed by the Least Significant Difference (LSD) post hoc test, using the R software. For data that did not meet parametric assumptions, the Kruskal-Wallis test was applied as a non-parametric alternative to ensure statistical robustness. Data from the primary characterization of suspected LAB bacterial isolates were analyzed descriptively in tabular form, and the identification of probiotic candidate LAB was carried out in the form of images and tables of research results.

RESULTS AND DISCUSSION

Population of Suspected LAB Bacterial Isolates

Suspected LAB bacteria grown on MRSA supplemented with $CaCO_3$ are characterized by the presence of a clear zone around the bacterial colony, which results from a chemical reaction. The alkaline nature of $CaCO_3$ neutralizes the lactic acid produced by LAB, leading to the formation of Ca-lactate, which appears as a clear zone surrounding the colony, as shown in Figure 1 (Hwanhlem et al., 2011). The colonies of suspected LAB are round, have flat edges, a slightly raised surface, and a milky white appearance (Pundir et al., 2013). The population of suspected LAB bacteria obtained *in vitro* from the intestines of broiler chickens fed FPH-based diversified feed is presented in Table 1.

Figure 1. Bacterial colonies suspected to be LAB on MRSA + $CaCO_3$ media sites

Based on the results of statistical analysis, feed diversification in broiler chickens has a significant effect on the population of suspected LAB bacteria in their intestines. The ANOVA test indicated that feed diversification had a statistically significant effect on the LAB bacterial population in both the small intestine and the caecum (p < 0.05), with F-value = 3.771, p-value = 0.032 for the small intestine, and F-value = 3.896, p-value = 0.029 for the caecum. Since the effect was statistically significant, the analysis was followed by an LSD post hoc test. Based on the LSD test results, feeding broiler chickens without FPH resulted in a significant difference compared to feeding with 1.5% and 2% FPH. However, there was no significant difference compared to the addition of 1% FPH. As presented in Table 1, the suspected bacterial population of LAB from the small intestine with 2% FPH feed exhibited the highest bacterial population, at 1.57 x 10^8 CFU/mL. Meanwhile, the highest population of suspected LAB bacteria from the caecum was found in the 1.5% FPH feeding treatment at 1.22 x 10^8 CFU/mL.

The LAB population in the digestive tract is influenced by nutrients such as protein contained in feed (Millah et al., 2016), which serves as a source for cell multiplication. The greater the protein availability, the probiotic population will also increase (Sahara et al., 2008). The population of suspected LAB bacteria from the small intestine was higher compared to that from the caecum. This is related to the nutrients contained in the two digestive tracts. According to Widodo et al. (2015), LAB growth is influenced by the nutritional content in the digestive tract. The small intestine contains a higher concentration of nutrients, which are absorbed by the villi, whereas the remaining unabsorbed nutrients are passed on to the caecum. As a result, nutrient availability in the caecum is significantly reduced, since most proteins and carbohydrates have already been absorbed in

Table 1. Population of suspected LAB bacteria from the intestines of broiler chickens resulting from feed diversification treatment using FPH

Treatment	Population of suspected LAB bacteria from the intestines of broiler chickens (CFU/mL)	
	Small intestine	Caecum
T_3	1.57 x 10^{8a}	9.93 x 10^{7a}
T_2	1.16 x 10^{8a}	1.22 x 10^{8a}
T_1	6.92 x 10^{7ab}	6.26 x 10^{7ab}
C	1.04 x 10^{6b}	7.40x 10^{5b}

Note: Different notations indicate different meanings, Treatment (T) was basal feed by addition FPH (1%, 1.5%, and 2%), and Control (C)

Growth of Broiler Chickens

Feed diversification treatment using FPH influences the growth of broiler chickens, as indicated by an increase in body weight. The average weight gain for broiler chickens from diversification feed treatments with FPH concentrations of 0%, 1%, 1.5% and 2% is presented in Table 2. The addition of 2% FPH resulted in the highest average weight gain for broiler chickens of 1.021 kg/head. The average weight gain of broiler chickens in the feeding treatment with the addition of 2% FPH was significantly different from that of the treatment with 1% FPH and without FPH, but not significantly different from that of the treatment with 1.5% FPH. This increase in body weight indicates the absorption of nutrients from feed, which plays a key role in the growth process. Anatomical changes in the intestine, such as increased and higher villi density, contribute to more efficient nutrient absorption (Opheim et al., 2016).

The ANOVA test was conducted to assess whether feed diversification affected the growth of broiler chickens. The analysis yielded an F-value of 1.781 with a p-value of 0.170. Since the p-value exceeded the 5% significance level ($\alpha = 0.05$), the differences among treatments were not statistically significant. To validate this interpretation, a nonparametric Kruskal–Wallis test was performed as a sensitivity check. The Kruskal–Wallis results (H = 4.41, p = 0.22) were consistent with those of ANOVA, indicating that the differences among treatments were not statistically significant at the 5% significance level. This consistency further supports the robustness of the statistical findings.

The weight gain of 796 grams/head in the feeding treatment with 1.5% FPH was not significantly different from the treatment with 1% FPH and without FPH, although it was significantly different from the 2% FPH treatment. Chaudary and Pati (2016) stated that the protein content in protein hydrolysates is more straightforward, consisting of amino acids produced through the hydrolysis process, which makes it easier for broilers to digest. The feeding treatment with 1% FPH and without FPH showed no significant effect on broiler weight gain. This may be due to the low concentration of FDH (1%) and the absence of FPH, which did not produce a meaningful impact on weight gain.

Table 2. Average weight gain for broiler chickens resulting from diversification treatment feed using FPH

Treatment	Average Weight Gain for Broiler Chickens (kg/head)
T_3	1.021^{a}
T_2	0.796^{ab}
T_1	0.675^{b}
C	0.542^{b}

Note: Different notations indicate different meanings, treatment (T) was basal feed by addition FPH (1, 1.5 and 2%) and C (control)

LAB can effectively increase the growth of broiler chickens by producing short-chain fatty acids (SCFAs). SCFA will neutralize toxins produced by pathogenic bacteria. SCFA is also able to release butyric acid, which stimulates the expression of tight junction protein and stimulates Goblet cells to produce mucin. The SCFAs released by LAB will also be absorbed by the intestines as a source of energy, allowing intestinal cells to a Table 2. Average weight gain for broiler chickens resulting from diversification treatment feed using FPH ctively divide and lengthen the villi (Tang et al., 2023). The longer the villi, the higher the absorption of nutrients in the chicken, which can accelerate the weight gain of broilers. According to research by Wang et al. (2023), LABs such as Lactobacillus plantarum and Pediococcus pentosaceus have a significant influence on the weight gain of Tibetan chickens. Additionally, the study reported that LAB can increase the length of villi in the intestines of Tibetan chickens. This suggests that the weight increase of Tibetan chickens is proportional to the enlargement of the villi in their intestines.

Primary Characterization of Bacterial Isolates Suspected to be LAB

Isolation of samples from the small intestine and caecum on MRSA resulted in 8 bacterial isolates suspected to be LAB. Microscopic morphological observations were conducted using three tests, including the Gram staining test and endospore staining. The results of the primary characterization of suspected LAB bacterial isolates are shown in Table 3.

The catalase test on bacterial isolates suspected to be LAB showed an adverse reaction, indicated by the absence of air bubbles forming when the isolates were treated with 3% H_2O_2. Detha et al. (2019) stated that the characteristic of LAB is that it does not produce the enzyme catalase, which functions to break down hydrogen peroxide into water and oxygen. Endospore staining also yielded a negative result, indicating that the bacterial isolate did not form endospores (Figure 2a). Vegetative cells appeared red because of the safranin stain, whereas endospores, if present, would appear green because of the malachite green dye (Nurhayati et al., 2017). In general, LAB do not form endospores, which are used for self-defense when environmental conditions are unfavorable (Zhi et al., 2021). The Gram staining test showed that the bacterial isolates suspected to be LAB are Gram-positive, appearing purplish-blue with bacilli cells, as in Figure 2b. Gram-positive bacteria will appear purplish because they retain the crystal violet dye (Nurhayati et al., 2017). The peptidoglycan structure in the cell walls of Gram-positive bacteria is thicker compared to that of Gram-negative bacteria, allowing the violet crystals to remain bound and purple after decolorization (Rahayu and Qurbaniah, 2019).

Table 3. Primary characterization of suspected LAB bacterial isolates from the intestines of broiler chickens feed diversification treatment using FPH

Isolate Code	Gram	Catalase	Endospores
SUA_1	+, Bacilli	-	-
SUA_2	+, Bacilli	-	-
SUA_3	+, Bacilli	-	-
SUA_4	+, Bacilli	-	-
SUA_5	+, Bacilli	-	-
SSA_1	+, Bacilli	-	-
SSA_2	+, Bacilli	-	-
SSA_3	+, Bacilli	-	-

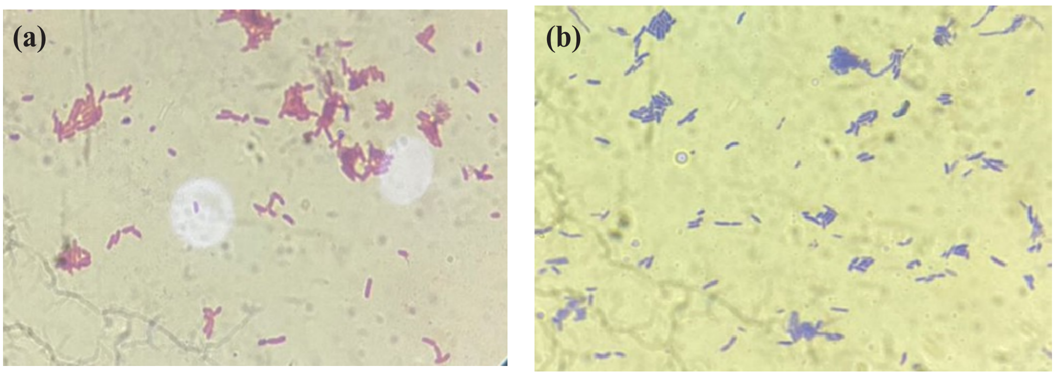

Figure 2. Results of endospore staining of bacterial SUA_5 isolate suspected to be LAB with 1000x magnification (a) and Gram staining of suspected bacterial SUA_5 isolate from LAB at 400x magnification

Lactic Acid Bacteria Pathogenicity Test

One of the characteristics of LAB as a probiotic candidate is that it must be non-pathogenic and non-toxicogenic (Sgouras, 2004). Pathogenicity testing was conducted by inoculating probiotic candidates onto blood agar media (Chotiah and Damayanti, 2018). The hemolytic activity of LAB isolates suspected to be probiotics is presented in Table 4. Based on the results of the pathogenicity test, seven isolates were α-hemolytic and one was γ-hemolytic (Figure 3).

Hemolytic activity occurs due to extracellular enzymes produced by bacteria that can lyse red blood cells on agar media (hemolysis) (Sanatang and Lio, 2021). Seven LAB isolates suspected of being probiotics exhibited α-hemolysis, as indicated by a greenish circle around the isolate, due to partial lysis of blood cells. The LAB SSA$_2$ isolate showed no visible changes around the colony, with no lysis of blood cells. The results of the pathogenicity test on eight isolates showed that the isolates belonged to the group of non-pathogenic bacteria, characterized by the absence of a clear zone around the colony. In contrast, pathogenic bacteria are characterized by the formation of a clear zone (hemolysis zone) around the colony (Hajar et al., 2018). Cappucino and Welsh (2019) stated that α-hemolysis is an incomplete lysis of red blood cells that occurs when hemoglobin is converted to methemoglobin, resulting in a greenish zone around the colony. Type β-hemolysis is a total hemolysis that occurs, and there is damage to hemoglobin so that a clear zone is formed around the colony. Type γ-hemolysis occurs when there are no red blood cells that undergo lysis, so that they do not produce discoloration in the media around the colony.

Table 4. Hemolytic type of suspected LAB bacterial isolates from the intestines of broiler chickens feed diversification treatment using FPH

Isolate	Color change around the isolate	Hemolysis type	Result
SUA$_1$	Green	α-hemolysis	Non-pathogen
SUA$_2$	Green	α-hemolysis	Non-pathogen
SUA$_3$	Green	α-hemolysis	Non-pathogen
SUA$_4$	Green	α-hemolysis	Non-pathogen
SUA$_5$	Green	α-hemolysis	Non-pathogen
SSA$_1$	Green	α-hemolysis	Non-pathogen
SSA$_2$	Not changing	γ-hemolysis	Non-pathogen
SSA$_3$	Green	α-hemolysis	Non-pathogen

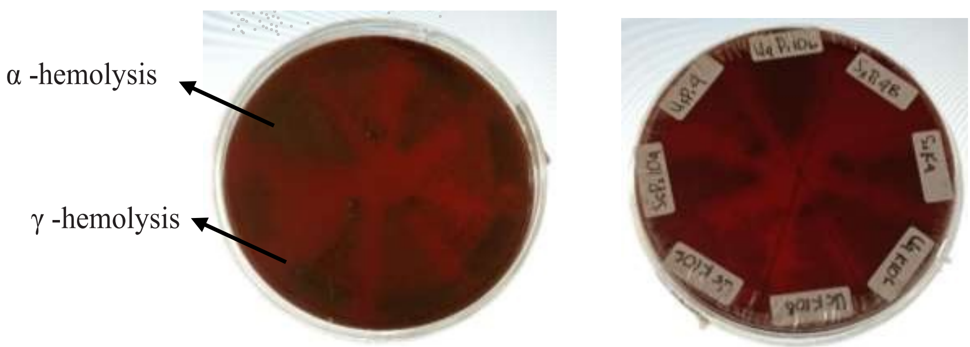

Figure 3. Pathogenicity test results of LAB isolates on blood agar media: A. Top view, B. Bottom view

Macroscopic Identification of Lactic Acid Bacteria

Single isolates obtained from purification can be morphologically characterized by observing colony characteristics and bacterial cell characteristics through Gram staining (Fibriana et al., 2017). Bacterial colony morphology on growth media includes color, shape, and colony size (Cappuccino et al., 2013). The isolates selected for morphological observation were those suspected to be probiotic LAB, based on their Gram-positive nature, lack of catalase activity, and absence of endospore formation. The morphological characteristics of LAB colonies suspected to be probiotics are presented in Table 5.

Macroscopic observations of the colony morphology of LAB isolates suspected of being probiotics on MRSA showed that most isolates exhibited circular colonies with convex elevations. The colony edges were entire, and the colonies appeared white and cream-colored. Slight morphological differences were observed in intestinal isolates with the names SUA_4 and SUA_1, which exhibited elevated type colony morphology. Differences were also observed in intestinal isolates, with isolates named SUA_2, which had undulating colony edges, and SUA_3, characterized by cream-colored colonies, lobate colony edges, and raised elevations. Among the caecum isolates, SSA_3 displayed a distinct, cream-colored colony. Clear zones were observed around all bacterial colonies on MRSA + $CaCO_3$. The morphological characteristics of the bacteria found were consistent with those of LAB, as reported by Putri et al. (2018), who stated that LAB isolates typically exhibit circular and punctiform macroscopic characteristics. Colony elevations are convex, flat, and raised. Bacterial colonies are white or cream-colored, with entire and undulating edges, and clear zones surrounding the colonies.

Table 5. Macroscopic identification of Lactic Acid Bacteria.

Colony Name	Shape	Color	Edge	Elevation
SUA_1	Circular	White	Entire	Raised
SUA_2	Circular	White	Undulate	Convex
SUA_3	Circular	Beige	Lobate	Raised
SUA_4	Circular	White	Entire	Raised
SUA_5	Circular	White	Entire	Convex
SSA_1	Circular	White	Entire	Convex
SSA_2	Circular	White	Entire	Convex
SSA_3	Circular	Beige	Entire	Convex

Identification of Lactic Acid Bacteria using the KB020 Kit

Biochemical identification of LAB was performed using a carbohydrate fermentation test, as LAB are bacteria capable of fermenting carbohydrates to produce lactic acid (Bintsis, 2018). Tests with various types of sugar were conducted to identify bacteria capable of fermenting carbohydrates. Fermentative activity was indicated by a color change from purple to yellow (Widiastutik et al., 2014). Carbohydrate fermentation testing was carried out using the HiLacto Kit (HiMedia). Isolates inoculated on the

Kit were used to identify members of the Lactobacillaceae family (Basumatry et al., 2022). The HiLacto kit (HiMedia) was used to isolate bacterial isolates by determining the carbohydrate fermentation profile of LAB isolates. Several types of carbohydrates contained in the strip kit are arabinose, cellobiose, galactose, maltose, mannose, melibiose, raffinose, sucrose, trehalose, and xylose. A positive esculin hydrolysis test is indicated by a change in the kit's color from cream to black (Singh et al., 2021). The cell density (OD) of each isolate was measured using a spectrophotometer and adjusted to 0.1 OD before identification using the kit.

The test results were obtained by observing sugar fermentation after 24-48 hours of incubation and were interpreted according to the kit instructions (Alonso et al., 2018). Probiotic candidate LAB isolates were tested biochemically using a kit, totaling eight isolates, including chicken intestine sample isolates (SUA_1, SUA_2, SUA_3, SUA_4, and SUA_5) and chicken caecum sample isolates (SSA_1, SSA_2, and SSA_3). The identification results of LAB isolates suspected to be probiotics, originating from the intestine and caecum of broiler chickens, are presented in Table 6a-b.

The identification results of eight isolates revealed four types of bacteria with distinct carbohydrate fermentation capabilities. The fermentation test of the SUA1 isolate identified the *Lactobacillus mucosae*. The color of the kit changes from purple to yellow during the fermentation of cellobiose, arabinose, maltose, galactose, mannose, melibiose, raffinose, and sucrose, indicating the presence of carbohydrate fermentation by *L. mucosae*. The ability of LAB to ferment types of carbohydrates is characteristic of metabolism for LAB species. Negative carbohydrate fermentation occurs in xylose and trehalose fermentation. The esculin hydrolysis test on the SUA_1 isolate showed positive results, as indicated by a color change from cream to black. Catalase activity was tested using 3% H_2O_2 and showed negative results (no bubbles formed). *L. mucosae* can attach to intestinal mucus and inhibit the growth of pathogens in the gastrointestinal tract, both of which are associated with potential probiotic bacteria. The characteristics of *L. mucosae* as a LAB include a rod-shaped morphology, Gram-positive status, catalase-negative properties, non-spore-forming, non-motile nature, and the presence of heterofermentative bacteria (De Moraes et al., 2016). Strains of *L. mucosae* are capable of fermenting D-galactose, D-lactose, maltose, fucose, D-ribose, sucrose, D-xylose, raffinose, α-lactose, FOS (Fructo-oligosaccharide), and XOS (Xylo-oligosaccharide). However, they are unable to utilize gum arabic, D-mannitol, sorbitol, trehalose, rhamnose, pinotriose, and salicin (Jia et al., 2020).

The carbohydrate fermentation test of SUA_2 and SUA_5 isolates identified *Lactobacillus frumenti* bacteria. Positive fermentation was observed for cellobiose, arabinose, maltose, galactose, mannose, melibiose, raffinose, sucrose, and trehalose. No color change was observed for xylose fermentation, indicating a negative result. The catalase test for both isolates showed negative results, evidenced by the absence of bubble formation. *L. frumenti* is a bacterium that can be an alternative to antibiotics to prevent damage to the epithelial barrier function in the intestine (Jun Hu et al., 2018). *L. frumenti* produces acid from the fermentation of ribose, galactose, fructose, mannose, mannitol, sorbitol, N-Acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, lactose, melibiose, trehalose, melezitose, raffinose, and gluconate. However, negative fermentation was observed in the xylose sugar group, and variable in the DL-arabinose sugar group (Muller et al., 2000). *Lactobacillus sanfranciscensis* was identified in isolates SUA_3, SSA_1, and SSA_2. The fermentation test yielded negative results in all nine tests, and the hydrolysis test also returned negative results. The change in color of the kit to yellow occurs during maltose fermentation, which is indicated by the color change. A negative test result is indicated by no color change on the kit, which remains its original purple color. Catalase-negative isolate with no bubbles when the isolate was placed in 3% H_2O_2. *L. sanfranciscensis* is one of the good lactic acid bacteria that comes from the intestines. *L. sanfranciscensis* bacteria can produce sucrose and cannot use fructose as an energy source, but can reduce fructose to mannitol. *L. sanfranciscensis* can hydrolyze sucrose into glucose and fructose (Korakli et al., 2003). These bacteria are capable of fermenting maltose and establishing a non-competitive relationship with yeast (Zhang et al., 2022).

Table 6a. Table of identification results using the HiLacto KB020 Kit

Isolate Code	Carbohydrate Fermentation						
	Esculin	Catalase	Xylose	Cellobiose	Arabinose	Maltose	Galactose
SUA_1	+	-	-	+	+	+	+
SUA_2	+	-	-	+	+	+	+
SUA_3	-	-	-	-	-	+	-
SUA_4	+	-	+	+	+	+	+
SUA_5	+	-	-	+	+	+	+
SSA_1	-	-	-	-	-	+	-
SSA_2	-	-	-	-	-	+	-
SSA_3	+	-	+	+	+	+	+

Table 6b. Table of identification results using the HiLacto KB020 Kit

Isolate Code	Carbohydrate Fermentation					
	Mannose	Melibiose	Raffinose	Sucrose	Trehalose	Closest Taxon
SUA_1	+	+	+	+	-	*L. mucosae*
SUA_2	+	+	+	+	+	*L. frumenti*
SUA_3	-	-	-	-	-	*L.sanfranciscensis*
SUA_4	+	+	+	+	+	*L. ferintoshensis*
SUA_5	+	+	+	+	+	*L. frumenti*
SSA_1	-	-	-	-	-	*L.sanfranciscensis*
SSA_2	-	-	-	-	-	*L.sanfranciscensis*
SSA_3	+	+	+	+	+	*L. ferintoshensis*

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The eighth identified isolates were SUA_4 and SSA_3, both of which were identified as *Lactobacillus fermentum*. The fermentation test yielded positive results in eleven wells, specifically for the fermentation of xylose, cellobiose, arabinose, maltose, galactose, mannose, melibiose, raffinose, and sucrose. Catalase-negative isolate with no bubbles when the isolate was placed in 3% H_2O_2. The esculin hydrolysis test on SUA_4 and SSA_3 isolates yielded positive results, as indicated by a change in the kit from a cream color to black. *L. ferintoshensis* strains are generally cultivated on MRS medium with 0.5% maltose. Vancanneyt et al. (2005) reported that *L. ferinoshensis* was able to ferment L-arabinose, D-fructose, galactose, gluconate, D-glucose, maltose, melezitose, melibiose, ribose, and sucrose.

CONCLUSION

The study aimed to determine the impact of giving fish protein hydrolysate (FPH) at various concentrations as a dietary supplement on the composition of lactic acid bacteria (LAB) as probiotic candidates from the intestines and caecum of broiler chickens. This study investigated the increase in the LAB population in the broiler chicken intestine and caecum following the addition of FPH to feed. The highest BAL population in the small intestine was obtained with the addition of 2% FPH, while in the caecum, it was achieved at a concentration of 1.5%. The macroscopic characteristics of the LAB isolate found were circular or round colonies of white and beige colors, with entirely undulate edges and a convex elevation. Its microscopic character is Gram-positive and does not form endospores. The bacteria do not produce catalase. This study obtained eight LAB isolates of probiotic candidates that are non-pathogenic, namely α-hemolysis and γ-hemolysis. Based on biochemical identification, four types of *Lactobacillus* were obtained: *L. mucosae, L. frumenti, L. sanfranciscensis*, and *L. ferintoshensis*. These are considered probiotic candidates for addition to broiler chicken feed.

AUTHOR CONTRIBUTION

E.U designed the research and supervised all the processes. **E.T.U** revised the manuscript. **A.S.W.** prepared the Fish Protein Hydrolysate. **M.B.** and **R.D.M**. collected and analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest on this research.

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