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Abstract. This study was performed to isolate lactic acid bacteria (LAB) from poultry feces, identify the lactic acid production capability and its inhibitory effect against pathogenes as a promising probiotic candidate for livestock. Lactic acid bacteria were isolated and cultured on De Man, Rogosa and Sharpe (MRS) media. The parameters of this study are bacterial characterization and antimicrobial activity of LAB Isolates against gram-positive and negative bacteria. LAB characterization was performed by Gram staining test, morphological test, catalase test, and fermentation type test. Microscopic observation of LAB isolated by feces showed that cocci and bacilli morphologies. The gram staining test showed Gram-positive and Gram-negative test results and the catalase test showed all LAB isolates obtained were facultative anaerobic. The results indicated that LAB M1 isolates could produce greater lactic acid with more effective inhibitory capability against *Staphylococcus aureus* ATCC 25293 and *Salmonella typhimurium* FNCC 0050 compared to other isolates.

1. Introduction

One widely used probiotics on poultry is lactic acid bacteria (LAB). LAB probiotics are more dominantly used as feed additives compared to other bacteria. A number of LAB strains had achieved GRAS (*Generally Recognized As Safe*) status considering its advantageous effects for poultry such as improving growth, feed efficiency without triggering the probiotic component translocation into the poultry body system, and to reduce the cholesterol content in meat [1,2]. The metabolites of LAB functioning as anti-microbial compounds (lactic and acetate acid), bacteriocin, hydrogen peroxide, diacetyl, and carbon dioxide.

The anti-microbial compounds could be employed to inhibit microbial growth or to destruct the pathogens through the mechanism of targetting the bacterial cell wall, causing lysis on the formed cell wall, and altering the cytoplasmic membrane permeability which subsequently causes nutrient leakage. This destruction of the cytoplasmic membrane will cause growth inhibition or cell death [3] LAB as probiotics should be capable of decreasing the colonies of pathogens in the intestinal tract since LAB possess the anti-microbial agents [4]. Drago et al [5] successfully tested the ability of a number of strains and isolates of clinical *Lactobacillus* to inhibit the pathogenic bacteria (*E. coli*, *S. enteridis* and *Vibrio cholerae*).

2. Material and method

2.1. Lactic acid bacteria (LAB) source

The LAB was obtained from poultry faeces considering that the LAB in the faeces were the bacteria with high survivability and adaptation to the digestive tract condition. One characteristic of a good probiotic is the survivability during the transit in the digestive tract.

2.2. Isolation, purification and identification of LAB

Microbial isolation is the separation of a strain from a natural, mixed population of other living microbes [6]. LAB Isolation from broiler faeces aimed to acquire LAB isolated from broiler faeces containing mixed bacterial colonies. LAB colony was indicated by the appearance of clear zone around the bacterial colony on MRSA + 1% $CaCO_3$ media. Purification on each colony with varying appearance was performed to acquire pure isolates.

The procedures of LAB Isolation from Broiler faeces are presented below:

25 g fresh poultry faeces were collected aseptically and poured with 225 ml sterilized *Buffer Pepton Water* diluent (Merck production) and homogenized with stomacher for 30 seconds at 230 rpm. After that, 10^{-1}-10^{-8} dilution was performed. Bacterial isolation was carried out through bacterial inoculation at 10^{-7} and 10^{-8} 1 ml dilution in a sterilized petri dish. The culture was mixed with MRSA (Oxoid) + 1% $CaC0_3$ for 15-20 ml at the temperature of 45^0C and homogenized. The mixture will be left to solidify and anaerobically incubated at 37^0C for 48 hours. The LAB colony on MRS + 1% $CaCO_3$ agar plate media was indicated by a clear zone around the bacterial colony. Colony selection with different morphologies from others will be performed. The observation on the colony morphology encompassed the colony colour, shape, margin, and elevation. The selected colony from the cultured bacteria will be isolated by applying quadrant streaking bacteria on a petri dish filled with MRSA + 1% $CaCO_3$. The colony was incubated at 37^0C for 48 hours. Isolation was performed several times to produce pure isolate. To identify the purity of an isolate, Gram staining method was applied. When there is no appearance of distinguished Gram-positive and negative, the isolate can be classified as pure isolate. From the pure isolates, working and stock culture were prepared. The pure isolates were identified according to the LAB Characterization

2.3. LAB characterization

Characterization of LAB Isolates aimed to identify the characteristics, morphology and the physiology of the isolates. The observed morphological characteristics included the cell shape and Gram stain. As for the physiological characteristics, catalase and fermentative test were performed.

2.4. Gram staining

Gram staining method aimed to identify the cell shape and Gram characteristics. Gram staining was performed according to the standard method [7].

2.5. Catalase test

Catalase test was performed to identify the presence of catalase enzyme in the examined bacterial isolates by using hydrogen peroxide (H_2O_2). Bacteria with catalase enzyme are able to degrade H_2O_2 into H_2O and O_2. Catalase test was conducted according to the following procedures: 1 inoculum of 24-hour LAB culture was collected aseptically from MRSA agar slant pure strain and transferred into microscopic slides. 3% H_2O_2 solution about 1-2 drops was added to the media and the following reaction was examined. Bubbling appearance indicated the catalase test was positive and no presence of bubbling indicated negative results of the catalase test [8].

2.6. Fermentative type test

Fermentative type test was performed by transferring 1 inoculum of LAB culture to Hungate anaerobic tube filled with 9 ml MRSB and Durham tube. The culture was anaerobically incubated for 24 hours at

37^0C. After 25 hours, an observation was carried out to investigate whether the bubbling appeared from Durham tube. Bubbling appearance indicated positive fermentation test result (heterofermentative). On the contrary, no appearance of bubbling indicated negative fermentative type (homofermentative).

2.7. The production of lactate acid

One metabolite of LAB functioning as an anti-microbial agent is the lactate acid. Lactic acid is able to inhibit various types of decomposing bacteria and pathogens [9,10].

The standard procedure for lactic acid production began with 2 samples of LAB inoculums from agar slant were inoculated into Hungate tube containing 18 ml MRSB and anaerobically incubated for 24 hours at 37^0C. After that, 10% of the culture from Hungate tube was inoculated into 100 ml synthetic medium. Synthetic medium contained 50 g glucose/ 1000 ml aquades, 0.4 urea, 0.1 % KH_2PO_4, 0,05% $MgSO_4$, 0,001% $FeSO_4.7H_2O$, 0,05% KCL, and 0.05 % yeast extract 0,05% Incubation was performed for 24 hours at 37 ^{0} C. Centrifugation of the samples was applied at 3000 rpm for 10 minutes to separate cell and insoluble solid matter. 10 ml sample results from centrifugation were pipetted and diluted 50 times. 100 ml was titrated to 0.009 NaOH N indicator phenolphthalein until pink color appeared. Acid total (mg/ml) was calculated by the following formula :

$$\text{Lactic acid} = \frac{\text{DF x V NaOH x N NaOH x EW}}{\text{sample ml}}$$

Description : DF = diluent factor (500/100); V NaOH= titration volume (ml)
EW = Equivalent weight of Lactic acid (90); N NaOH= 0,0093

2.8. Anti-microbial activity test

The anti-microbial activity aimed to identify the inhibition potency of LAB Isolate and select the isolate with the most effective inhibitory capability against pathogenes. This is in line with the LAB isolate capability in inhibiting pathogens to grow that enters the digestive tract.

In this study, 2 species of pathogens including *Salmonella typhimurium* FNCC 0050 and *Staphylococcus aureus* ATCC 25293 with the standard population of 10^5 CFU/ml The pathogens selection was based on the facts that these bacteria are commonly found in the digestive tract or contaminated environment.

3. Results and discussion

3.1. Lactic acid bacteria (LAB) characterization

Through the LAB isolation from broiler faeces, 19 isolates were successfully collected and characterized according to Gram staining, morphological shape, catalase test and fermentative test that can be seen from table 1.

Microscopic observation on LAB isolate cell indicated that LAB isolated from broiler faeces possess rod and spherical shape. Gram staining test demonstrated negative and positive Gram results. Gram-positive bacteria were characterized by the violet colour appearance of the microbial cell. In contrast, Gram-negative bacteria were characterized by the red colour appearance of the microbial cell.

According to the catalase test, all LAB isolates collected in this study were facultative anaerobic. The fermentation test on 19 isolates detected 16 isolates as homofermentative and 3 isolates as heterofermentative. According to Surono [11] the final product of homofermentative LAB metabolism process is dominantly lactic acid [12]. On the contrary, heterofermentative LAB metabolism process produces various final products including lactic acid, CO_2, alcohol and lactic acid. Moreover, a study performed by Mujnisa et al [13] found that the lactic acid in determined dosage can affect the percentage of carcass and cholesterol level in broilers.

Table 1. LAB characteristics isolated from poultry faeces.

LAB Isolate Code	Gram Staining	Morphology	Catalase Test	Fermentative Test
M 1	+	Cocci	-	Homofermentative
M 2	+	Cocci	-	Homofermentative
M 3	+	Cocci	-	Homofermentative
M 4	+	Cocci	-	Homofermentative
M 5	+	Cocci	-	Homofermentative
M 7	+	Bacilli	-	Homofermentative
M 8	+	Cocci	-	Homofermentative
M 14	-	Cocci	-	Homofermentative
M 15	-	Cocci	-	Homofermentative
M 16	-	Bacilli	-	Heterofermentative
M 17	-	Bacilli	-	Heterofermentative
M 23	+	Bacilli	-	Homofermentative
M 24	+	Cocci	-	Heterofermentative
M 25	-	Bacilli	-	Homofermentative
M 26	+	Bacilli	-	Homofermentative
M 27	-	Cocci	-	Homofermentative
M 28	+	Cocci	-	Homofermentative
M 29	-	Cocci	-	Homofermentative
M 30	-	Bacilli	-	Homofermentative

Description : M = Isolate Code

3.2. Anti-microbial activity test

From 19 identified isolates of LAB isolated from broiler faeces, only 10 isolates with isolate code of M1, M2, M3, M4, M5, M7, M8, M23, M26 and M28 were tested for anti-microbial activity. The anti-microbial activity of 10 LAB isolates against two species of pathogens, *Staphylococcus aureus* ATCC 25293 and *Salmonella typhimurium* FNCC 0050 can be seen from figure 1 and figure 2.

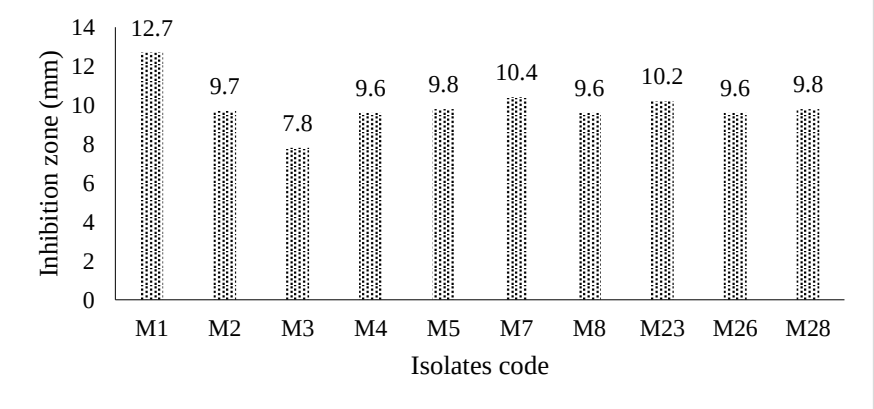

Figure 1. Anti-microbial activity of LAB Isolates against *Staphylococcus aureus* (Gram-positive).

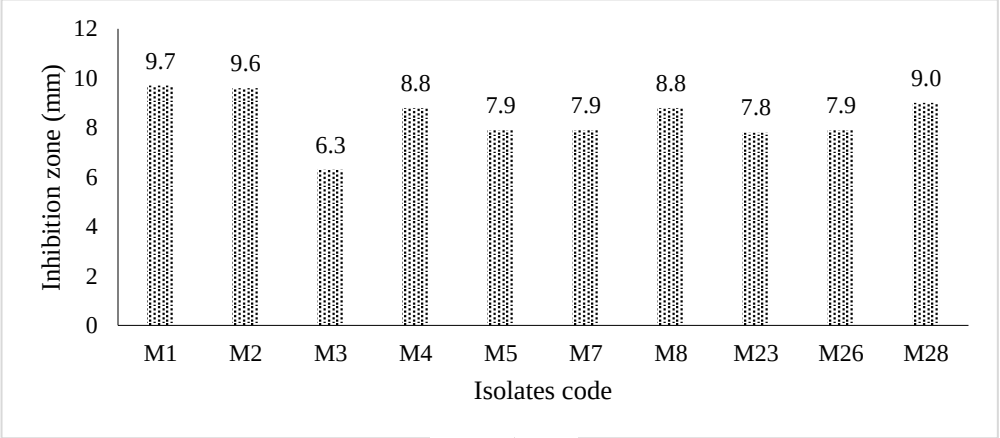

Figure 2. Anti-microbial activity of LAB Isolates against *Salmonella typhimurium* (Gram-negative).

The results of anti-microbial activity test on 10 LAB Isolates against pathogens indicated that all LAB Isolates possess the various inhibitory activity against pathogens. The results of the study generally indicated that *Salmonella typhimurium* FNCC 0050 as Gram-negative bacteria had a better resistance compared to *Staphylococcus aureus* ATCC 25293 as positive gram bacteria. Diameter range of LAB Isolate inhibition against *Staphylococcus aureus* and *Salmonella typhimurium* was 7.8 mm-12,7 mm (Figure 1) and 6.3 mm to 9.7 mm (figure 2) respectively.

Inhibitory activity from 10 LAB isolates demonstrated that M1 isolate produces the most effective inhibition against both species of tested bacteria compared to other isolates. The diameter of LAB Isolate inhibition against *Staphylococcus aureus* and *Salmonella typhimurium* was 12.77 mm and 9.7 mm respectively. This was supported by evidence of the highest lactic acid production in M1 isolate compared to other isolates and the lower final incubation pH (Table 2).

Table 2. The production of lactic acid and final incubation pH of LAB isolate.

LAB Isolate Code	The Production of Lactic acid (mg/ml)	Final incubation pH
M1	4.06	4.02
M2	1.26	4.50
M3	1.17	4.60
M4	1.51	4.30
M5	1.13	4.66
M7	1.30	4.59
M8	0.52	4.78
M23	1.34	4.40
M26	1.32	4.40
M28	1.05	4.70

Description : M = Isolates code

Diameter of Inhibition by M1 isolate against both pathogens was more effective compared to the study results by Wirawati [14] on the inhibitory capabilities of LAB isolated from tempoyak with the inhibition diameter against *Staphylococcus aureus* and *Salmonella typhimurium* ranging from 5.4-10.7mm and 4.3-6.1mm respectively. LAB different activities in inhibiting *Staphylococcus aureus* ATCC 25293 and *Salmonella typhimurium* FNCC 0050 were caused by the difference in lactic acid production as anti-microbial compound generated during the fermentation process (table 2) and the difference in the pathogen cell wall. Lactic acid produced by LAB isolated during the incubation caused

the accumulation of end products and pH decrease triggering the inhibition of gram-positive good bacteria and gram-negative bacteria that were vulnerable to low pH condition [11]

The average production of lactic acid produced by 10 LAB isolates ranged from 0.5 mg/ml to 4.1 mg/ml. The isolate producing the highest quantity of lactic acid was M1 isolate. The average production of lactic acid produced by LAB in this study could be categorized as high compared to the LAB production isolated from fish digestive tract production from a study performed by Pinuji [13] ranging from 0.35 mg/ml to 0.65 mg/ml.

In addition, the difference of LAB activity in inhibiting *Salmonella typhimurium* FNCC and *Staphylococcus aureus* ATCC 25293 was caused by the difference in microbial cell wall of *Staphylococcus aureus* ATCC 25293 as gram-positive bacteria and *Salmonella typhimurium* FNCC 0050 as gram-negative bacteria.

The cell wall structure of negative gram bacteria is more complex and consists of lipoprotein as the outer wall, polysaccharide as the middle wall and peptidoglycan as the inner wall [16]. The cell wall of gram-positive bacteria is simpler and therefore, it allows the anti-microbial agent to diffuse through the cell. The anti-microbial compounds, in turn, inhibit the microbial growth by destructing the wall, cause lysis on the cell wall, induce changes in the permeability of the cytoplasmic membrane which lead to nutrient leakage from the cell. Damage to the cytoplasmic membrane will lead to the inhibition of cell growth or cell death [17].

4. Conclusion

M1 LAB isolate produced greater lactic acid and possess the most effetive inhibitory capability against *Staphylococcus aureus* ATCC 25293 and *Salmonella typhimurium* FNCC 0050 compared to the other isolates.

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