

# Identification of *Lactobacillus* spp. on Basis Morphological, Physiological, and Biochemical Characteristic from Jawa Super Chicken Excreta

Dini Dwi Ludfiani<sup>1</sup>, Widya Asmara<sup>2\*</sup>, Agnesia Endang Tri Hastuti Wahyuni<sup>2</sup>, and Pudji Astuti<sup>3</sup>

<sup>1</sup>Student in Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

<sup>2</sup>Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

<sup>3</sup>Department of Physiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

**Abstract.** The use of antibiotic growth promoter as a feed additive for animals in Indonesia has been prohibited and regulated in law, starting from January 1, 2018. Alternative replacement for antibiotic was investigated and introduced to farms such as probiotic. The commonly used probiotic was from the genus *Lactobacillus*, *Bifidobacterium*, and *Bacillus*. The aim of this study was to identify the *Lactobacillus* strains isolates from Jawa Super chicken excreta. Identification was based on the morphological, physiological, and biochemical characteristics. The sample was collected from Jawa Super chicken in Kalasan, Sleman, Yogyakarta. Each sample was diluted in 0.9% sterile saline and plated on MRS agar containing bromocresol purple. All plates were incubated at 37°C under anaerobic condition. Isolates were identified morphology, physiology, and biochemistry based on fermentation of carbohydrate by using Analytical Profile Index (API 50 CHL). This study showed that isolates belong to 2 categories of fermentation, facultatively heterofermentative and obligately heterofermentative. Not all isolates can grow at 10°C, but survive to low pH. Based on biochemical analysis isolates identify as *L. brevis* and *L. pentosus*. *L. brevis* and *L. pentosus* were strains of the *Lactobacillus* found in the excreta.

## 1 Introduction

Antibiotics in the use of non-therapeutic doses or growth promoters have been widely used as feed additives to increase livestock production and control infection in livestock, but now their use has been banned because of the impact of resistance and residues on livestock products [1-3]. Ban the use of antibiotic growth promoter (AGP) as a feed additive for animals in Indonesia has regulated in law and starting from January 1, 2018, and is written in the Law of the Republic of Indonesia Number 18 of 2009 Article 22 and in the Regulation of the Minister of Agriculture of the Republic of Indonesia Number 14 of 2017, part five.

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\* Corresponding author: [wied\\_as@ugm.ac.id](mailto:wied_as@ugm.ac.id)

One of the alternatives for AGP that can be chosen is probiotics. Probiotics are live microorganisms that can have a beneficial impact on the host by improving the microbiota environment in the digestive tract [4, 5]. Commonly, probiotics that use in animal feed was lactic acid bacteria (LAB) [6] such as *Lactobacillus*, *Bifidobacteria*, and *Bacillus* sp. [3].

All of LAB basically have the same characteristics, Gram-positive and do not form spores, do not produce catalase enzymes, facultative anaerobes, be able to ferment lactose with the main product being lactic acid, non-pathogenic, safe for consumption to improve human and livestock health [7]. *Lactobacillus* is acidotolerant, and has a mechanism to inhibit the growth of various pathogenic bacteria (Gram-positive and Gram-negative) and fungi through the production of lactic acid [8]. *Lactobacillus* probiotics are known several mechanisms to adhere to intestinal epithelial cells. This property is an important criterion for the colonization of *Lactobacillus* in the gastrointestinal tract [9].

*Lactobacillus* can be obtained from isolation from various sources such as processed food products (fermented meat and fish, fermented milk, kimchi, pickles), fermented feed such as silage, living organs (digestive tract), and feces or excreta. Isolation of *Lactobacillus* from feces or excreta can reflect the bacterial groups that are in the digestive tract. This study aimed to identify the *Lactobacillus* strains isolates from Jawa Super chicken excreta as probiotic candidates.

## 2 Materials and Methods

### 2.1 Sampling

A total of 12 samples of Jawa Super chicken excreta were collected from a farm in Kalasan, Yogyakarta. The samples were immediately brought to the laboratory and being analyzed.

### 2.2 Isolation and identification of LAB

The collected samples were serially diluted in 0.9% sterile saline and plated on de Man, Rogosa and Sharpe (MRS) agar containing bromocresol purple. Plates were incubated under anaerobic conditions at 37°C for 48 h. Individual colonies were randomly selected to purified by streaking on MRS agar, and stored at -30°C for stock in cryo tube containing 10% (v/v) skim milk and 20% (v/v) glycerol. Isolates were subcultured in MRS broth at 37°C for 24 h for identified morphology, physiology, and biochemical test. Biochemical identification based on fermentation of carbohydrates by using Analytical Profile Index (API 50CHL) [10-12].

The effect of temperature on the growth of bacterial was carried out by inoculating bacterial in MRS Broth at 10°C, 35°C, 40°C. Growth at different pH was evaluated by inoculating the bacterial strains in MRS broth at pH 2, 3, 4, and 5. After 24h incubation at 37°C the growth of bacterial was expressed as a value of optical density at 620 nm (O.D.620). The same way is done for the bile salt test 0%, 0.05%, 0.15%, and 0.3%. Data of pH and bile salt tests were analyzed using RStudio. Analysis of variance (ANOVA) with the Tukey test was done to verify if there is a significant difference  $P < 0.05$  in isolates growth at the different conditions tested.

### 2.3 Hemolytic activity

The hemolytic activity was determined by inoculating the culture on blood agar plates and the plates were observed for the formation of any clean ( $\beta$ -hemolysis) or greenish ( $\alpha$ -

hemolysis) hemolytic zones, or no such zone ( $\gamma$ -hemolysis) around the *Lactobacillus* colonies [13]

### 3 Results and Discussion

Identification of phenotypic and genotypic are part of the first step in the selection of potential probiotic bacteria. In this study, the results presented were phenotypic identification. From the 12 samples analyzed, only 4 samples were able to be isolated and identified. They are J1, J5, J10, and J12. Based on morphologically test, all bacteria isolates were Gram-positive, catalase-negative, homo fermentative, non-motile, and rod shape. The colonies were circle, slightly raised, and white. All bacteria isolates were found to be good acid producers by changing the color of the medium completely from purple to yellow within 48h incubation. Similar study was reported by Tilahun [14] group of *Lactobacillus* and *Enterococcus* were found to be good acid producers by changing the color of the medium completely from green to yellow within 12h of incubation. LAB which produces acid belongs to genera *Pediococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Bacillus* species [15]. The general characteristics of LAB are Gram-positive and do not form spores, almost all strains are unable to produce the enzyme catalase, most isolates are facultatively anaerobic and able to ferment lactose with lactic acid as the main product [16]. The reaction of catalase-negative shows that there is no degradation of  $H_2O_2$  to  $O_2$  by the catalase enzyme [17].

**Table 1.** Physiological characterization of four isolates

Isolate name	Resistance to acid			
	pH 2	pH 3	pH 4	pH 5
J1	0.122 ± 0.012 <sup>a</sup>	0.104 ± 0.020 <sup>b</sup>	0.105 ± 0.019 <sup>c</sup>	0.624 ± 0.045 <sup>d</sup>
J5	0.135 ± 0.000 <sup>b</sup>	0.110 ± 0.010 <sup>b</sup>	0.111 ± 0.021 <sup>c</sup>	0.341 ± 0.021 <sup>d</sup>
J10	0.111 ± 0.001 <sup>c</sup>	0.094 ± 0.008 <sup>b</sup>	0.112 ± 0.021 <sup>c</sup>	0.599 ± 0.008 <sup>d</sup>
J12	0.764 ± 0.005 <sup>d</sup>	0.102 ± 0.037 <sup>b</sup>	0.103 ± 0.034 <sup>c</sup>	0.296 ± 0.034 <sup>d</sup>
	Bile tolerance			
	0%	0.05%	0.15%	0.3%
J1	2.137 ± 0.002 <sup>a</sup>	0.402 ± 0.086 <sup>b</sup>	0.882 ± 0.065 <sup>c</sup>	0.648 ± 0.078 <sup>d</sup>
J5	1.862 ± 0.053 <sup>b</sup>	0.553 ± 0.066 <sup>b</sup>	1.165 ± 0.028 <sup>c</sup>	1.330 ± 0.044 <sup>d</sup>
J10	2.117 ± 0.084 <sup>c</sup>	0.525 ± 0.067 <sup>b</sup>	0.827 ± 0.048 <sup>c</sup>	1.020 ± 0.030 <sup>d</sup>
J12	1.466 ± 0.058 <sup>d</sup>	0.565 ± 0.054 <sup>b</sup>	1.084 ± 0.071 <sup>c</sup>	1.253 ± 0.088 <sup>d</sup>

Results were expressed as a value of O.D. 620. Each value represents the mean value ± standar deviation (SD) (n=4). For each isolate, average values (±SD) within the same row followed by common superscript letters do not differ significantly (P < 0.05).

From the physiology test, all bacteria isolates were not able to grow at temperatures of 10°C, and survive under low pH and bile salt conditions after 24h incubation (Table 1). The bacteria to be used as probiotics should be able to resist inhibitory factors in the gastrointestinal tract such as gastric juice and bile salts. For this purpose, the effect of different concentrations of acid and bile salts on the growth of bacteria isolates was investigated. Based on Table 1, the growth of the bacteria decreased at pH 3, and the maximum growth was observed at pH 5, and J5 showed the best tolerance to bile salts.

Thus far, researches have been made to identify potential probiotic LAB isolated from any resources because of their benefits. Stringent selection criteria for selecting highly potent probiotic strain and safety are highly important and should be studied using in vitro and/or in vivo screening methods [9]. Tolerance to acids and bile salts is an important criterion in the selection of potential probiotic. The digestive tract has high acidity in the proventriculus and ventriculus, while the intestine has a high concentration of bile salts, therefore probiotic

bacteria are expected to be able to maintain their viability and functionality while in the digestive tract [18]. Acid tolerance of bacteria is important for withstanding gastric stresses [9]. Various hydrogen ion concentrations affect the growth of bacteria and suppressed growth. This is because the overall proton permeability of the plasma membrane and mechanism in fermentative bacteria appears to be the proton-translocating ATPase [19]. The lipid components that dominate the membrane of Gram-positive bacteria play an important role in maintaining the membrane structure in reducing cell leakage caused by bile salts [20]. Bile acts an important role in specific and nonspecific defense mechanism of the gut and the effect of inhibitory is determined by the bile salts concentrations. The resistance of bile salt is an important characteristic of LAB to know them to survive, grow and their action in gastrointestinal [21].

**Table 2.** Identification by API 50CHL

Isolates	Identification by API 50CHL	%
J1	<i>Lactobacillus pentosus</i>	88,7
J5	<i>Lactobacillus brevis</i>	99,1
J10	<i>Lactobacillus raffinolactis</i>	54,8
J12	<i>Lactobacillus pentosus</i>	99,7

Based on the biochemical test by API 50CHL (Table 2), all isolates were identified as *Lactobacillus*. The similarity rate above 90% with *Lactobacillus* are J5 (99.1%) and J12 (99.7%). All isolates could ferment 20 types of carbohydrate, including L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, D-raffinose, and B-gentibiose.

Hemolytic activity was used to evaluate the safety of probiotic strains [22]. The absence of hemolytic activity is considered safe for the selection of probiotic strains. Hemolysis is a known virulence factor among pathogenic microorganisms [21]. Based on hemolytic activity, all bacteria isolates showed  $\gamma$ -hemolysis. A similar study was reported by Tallapragada et al. [21] and Maragkoudakis et al. [23] *Lactobacillus* sp. G3\_4\_1TO2, *L. fermentum*, and *L. paracasei* were isolated from dairy products which showed  $\gamma$ -hemolysis. WHO and FAO recommended the other evaluation for the safety of probiotic strain by antibiotic susceptibility testing. Previous studies [22] reported *L. brevis* and *L. pentosus* isolated from duck and Jawa Super chicken excreta showed resistance to Streptomycin.

## 4 Conclusion

The bacteria were successfully isolated from Jawa Super chicken excreta and identified as *Lactobacillus* using the morphological, physiological, and biochemical tests. The bacteria identity as *L. brevis* and *L. pentosus* were confirmed using API 50CHL with a similarity level of 99%. Further studies are needed to adhesion assay, antimicrobial activity, and microbial resistance to complement property for probiotics.

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