ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 13 (1): 47-51, 2014
ISSN 1682-8356

Characteristic of *Lactobacillus* Isolated from Pengging Duck's Intestines as Probiotics

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Abstract: Characteristics properties of *Lactobacillus* such as their antagonistic against pathogenic bacteria, their survival at low pH and high concentration of bile salt are used *in vitro* to evaluate their potential as probiotic agent. Objectives of this research are to obtain lactic acid bacteria isolates from Pengging duck intestines and to screen their ability as a probiotic agent. The result showed that the Number of Isolate I11 showed its resistant to low acid (pH 1,5) until 120 min. Some of the strains showed their ability on pH 2,5 during 120 min (No. of isolate I1, I2, I4, I8, I9 and I10). The other isolates decreased their viability to survive at low pH, even they still able to survive at pH 3.5 during 120 min. The result based on the isolates resistance to bile salt showed that isolates could grow at media with 2% of bile salt. Their growth was inhibited with the increasing bile salt concentration. Number of Isolate I11 showed its survival in environment contain 6% bile salt until 120 min. Most isolates had antagonistic against pathogenic bacteria (Salmonella pullorum and Escherichia coli). Based on the characteristics properties. it can be conclude that the 12 isolates of lactic acid bacteria from Pengging duck's caecum were genus *Lactobacillus*. Isolate number I11 was potentially used as probiotic. Isolate number I11 was identified as *Lactobacillus* salivarius.

Key words: *Lactobacillus*, intestine of duck, probiotics

INTRODUCTION

Pengging duck (Anas javanica) is locally poultry in Indonesia that has potential as source of duck meat. Microorganism of gastro intestinal tract of poultry has potential probiotic. Probiotics are defined as microbial feed supplements that contain live (direct feed microbials) good bacteria, molds and yeasts that can be advantageous for its host by improving the microbial balance in the digestive tract (Fuller, 2001; Songisepp *et al*., 2005). They act as restrainer of the damage caused by pathogenic microorganisms, affirmative to balance digestive microflora and increase in antioxidation functioning (Shen *et al*., 2011). *Lactobacillus* is one of lactic acid bacteria that potentially as probiotics (Purwandhani and Rahayu, 2003) and its stability can be maintained during storage by dry cell preparation as a probiotic powder (Hartati and Harmayani, 2006). Acid-producing microbe in chicken manure is known comes from the caecum (Harimurti *et al*., 2005).

Beneficial flora including genera of *Bifidobacterium* and *Lactobacillus* promote the colonic environment by decreasing pathogenic bacteria, carcinogenic material and improving immunity (Chen *et al*., 2005). *Lactobacillus* entered gastro intestinal tract produce lactic acid that affected lower pH and inhibit the growth of pathogenic bacteria (Huang *et al*., 2004). This research focused on the Characteristics properties of *Lactobacillus* (isolated from Pengging duck intestines) such as their antagonistic against pathogenic bacteria, their survival at low pH and high concentration of bile salt are used in vitro to evaluate their potential as probiotic agent. Microbes which are preserved as probiotic should be able to survive against stomach acid and bile salt which are produced in human gastrointestinal tract (Saarela *et al*., 2000). Objectives of this research are to obtains *Lactobacillus* from Pengging duck intestines and to screen their ability as a probiotic agent.

MATERIALS AND METHODS

Materials: This investigation was conducted at Feed Technology Laboratory of Animal Agriculture Faculty, Diponegoro University. Materials were Pengging Duck's caecum and MRS medium (de-Mann Rogossa and Sharp). MRS agar consisted of 15 g agar, 10 g oxide peptone, 5 g yeast extract, 2 g K_2HPO_4, 2 g Diammonium Citrate, 20 g Glucose, 1 g Tween 80, 5 g natrium acetate, 0.58 g $MgSO_4.7\ H_2O$, 0.28 g $MnSO_4.H_2O$, 10 g meat extract and 1000 mL aquadest water.

Isolation Methods: *Lactobacillus* sp., isolated from Pengging duck's caecum with MRS medium-$CaCO_3$ 1% of added 10 ppm Syclo-hexamide to suppress the growth of yeast and 10 ppm of Na Azida to suppress

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microaerobic (Rahayu, 2003). Duck's caecum 10 g were taken aseptically and homogenized in 90 ml of NaCl solution. Serial dilution up to 10^{-7} were prepared and appropriate dilutions were plated onto MRS medium-$CaCO_3$ 1% of added 10 ppm syclo-hexamide. All plates were incubated at 37°C for 48 h. LAB can be observed from clear zones around the colonies which indicated the dissolving of $CaCO_3$ by an acid.

Each single colony was purified with streaking method and identify based on morphological characters, biochemical and physiological. Morphological parameters including cell shape and coloring grams. Biochemical parameters including catalase test. Physiological parameters including clear zone at colony of lactic acid bacteria. Cells form colonies of bacteria were observed in lactic acid bacteria isolates grown on MRS at room temperature for 2-3 days (Harimurti *et al*., 2005).

Gram staining method was conducted by methods coloring (Seely *et al*., 2001). Catalase test performed by dripping a solution of hydrogen peroxide on microbe culture, a positive reaction if the CO_2 bubbles appear. Clear zone was observed in colonies of lactic acid bacteria isolates grown on MRS-1% $CaCO_3$.

Assay of probiotic potency: Assay of probiotic potency was carried out at various pH (1.5, 2.5 and 3.5) and bile salts (2, 4 and 6%) at 60, 90 and 120 min. MRS Broth was adjusted to pH 1.5; 2.5; and 3.5 and containing 2, 4 and 6% bilesalt (oxgall), then each isolates (10^7 cfu/mL) was inoculated into 20 mL of MRS broth. Incubation was done at 37°C for 60, 90 and 120 min. *Lactobacillus* sp., growth expressed as OD (optical density) at λ = 660 nm. The antimicrobial activity of LAB against Escherichia coli and Salmonella sp was performed by the wall diffusion assay (Lade *et al*., 2006). *Lactobacillus* sp., culture were grown in MRS broth at 37°C for 24 h. Escherichia coli and Salmonella sp., were grown in nutrient broth at 37°C for 24 h. 10 mL of nutrient soft agar inoculated with 50 µL broth culture of Escherichia coli and Salmonella sp. MRS agar poured on petri dish and allow to solidity. Then overlaid with nutrient broth and in placed at a temperature of 4°C for 1 h. 50 µL *Lactobacillus* sp., culture filled and incubated at 37°C for 24 h. *Lactobacillus* sp., which gave clear zones that have antimicrobial activity against Escherichia coli and Salmonella sp. The diameter of the inhibition zone was measured. The data obtained were analyzed descriptive statistics.

Identification of lactic acid bacteria using api 50 CHL system: Lactic acid bacteria which have the best probiotic properties were identified using API 50 CHL test kit (bioMerieux, France). The strains were identified using APILAB software from bioMerieux.

RESULTS AND DISCUSSION

Characteristics of *Lactobacillus* isolated from duck's caecum is presented in Fig. 1 and Table 1. This 12 isolates were bacilli in pair or short chain (Fig. 1). Table 1 shown morphological, biochemical and physiologically performances of *Lactobacillus* sp., isolate that is taken from duck's caecum. The isolates showed that are 12 isolates have a rod shape and positive reaction to gram staining. Among the isolates, there are not positively produce catalase and all isolates presented clearly zone around its colony. LABs, in general has been characterized by gram positive, Catalase negative and positively provide Clearly Zone around the colony in the medium of MRS-$CaCO_3$ 1% (Rahayu, 2003; Seely *et al*., 2001).

Probiotic assay of *Lactobacillus* sp., were carried out at MRS broth pH 1.5, 2.5 and 3.5 showed in Table 2. Table 2 showed the ability of *Lactobacillus* to survive at acid environment. Number of Isolate I11 showed its resistance to low acid (pH 1,5) until 120 min. Some of the strains showed their ability on pH 2,5 during 120 min (number of isolate I1, I2, I4, I8, I9 and I10). The other isolates decreased their viability to survive at low pH, even they still able to survive at pH 3.5 during 120 min. This Condition showed Isolate number I11 had the best ability to survive at low pH. Resistance to low pH is one of the major characteristic for probiotic bacteria (Saarela *et al*., 2000).

Probiotic assay of *Lactobacillus* were carried out at MRS broth with concentration of 2, 4 and 6% bile salt showed in Table 3. The result based on the isolates resistance to bile salt showed that isolates could grow at media with 2% bile salt. Their growth was inhibited with the increasing bile salt concentration. Number of Isolate I11 showed its survival in environment contain 6% bile salt until 120 min. The other isolates decreased their viability and inhibited with the increasing bile salt concentration. Bile salt was secreted in small intestine. This bile salt presence creates more stress full condition for bacteria. Bile salt reduce the survival of bacteria because impairment with bacteria cell membrane in which its major component is lipid and fatty acid. Resistance to bile salt is one of the major characteristic for probiotic bacteria (Praasad *et al*., 1998).

Table 1: Phenotypic characters of isolates of *Lactobacillus*

	Code of Isolate											
Characteristics	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12
Rod shape	+	+	+	+	+	+	+	+	+	+	+	+
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-
Clear Zone+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2: *Lactobacillus* sp growth at various pH

Isolates		1.5			pH 2.5			3.5	
Number	60'	90'	120'	60'	90'	120'	60'	90'	120'
I1	++	+	+	++	++	++	++	++	++
I2	++	+	+	++	++	++	++	++	++
I3	+	+	+	++	+	+	++	++	++
I4	++	+	+	++	++	++	++	++	++
I5	+	+	+	++	++	+	++	++	++
I6	+	+	+	++	+	+	++	++	++
I7	+	+	+	+	+	+	+	+	++
I8	++	+	+	++	++	++	+++	++	++
I9	++	+	+	++	++	++	++	++	++
I10	++	+	+	+++	++	++	+++	+++	+++
I11	+++	++	++	+++	+++	+++	+++	+++	+++
I12	+	+	+	++	++	+	+++	++	++

Initial OD : 0.1-0.2 +++: when growth reached OD>2 ++: OD 1-2 +: an increase in OD, but OD<1

Table 3: *Lactobacillus* growth at various concentration of bile salt

Isolates		2		Bile	Salt 4	(%)		6	
Number	60'	90'	120'	60'	90'	120'	60'	90'	120'
I1	++	++	++	++	++	+	+	+	+
I2	++	+	+	+	+	+	+	+	+
I3	+	+	+	+	+	+	+	+	+
I4	+++	++	++	++	++	++	+	+	+
I5	++	++	+	+	+	+	+	+	+
I6	++	++	++	++	++	+	+	+	+
I7	+++	++	++	++	++	+	+	+	+
I8	++	++	++	++	++	++	+	+	+
I9	++	++	++	++	++	++	++	++	+
I10	+++	++	++	++	++	++	++	++	++
I11	+++	+++	+++	+++	+++	+++	+++	+++	+++
I12	++	++	++	++	++	++	++	+	+

Initial OD: 0.1-0.2 +++: When growth reached OD>2 ++ : OD 1-2 +: an increase in OD, but OD<1

Table 4: Antimicrobial Activity of *Lactobacillus* sp.

Isolates	Clear Zone (mm)	
Numbers	*E. coli*	*S. pullorum*
I1	9.3	12.7
I2	9.8	13.1
I3	7.1	9.6
I4	10.8	13.9
I5	9.2	12.9
I6	8.6	9.9
I7	12.2	14.8
I8	15.4	16.2
I9	8.5	9.7
I10	11.5	13.7
I11	19.6	22.4
I12	9.4	10.2

E: Escherichia *S: Salmonella*

Test of inhibition of *Lactobacillus* against the bacteria Escherichia coli showed that all isolates tested could inhibit the growth of Escherichia coli and Salmonella (Table 4). Lade *et al.* (2006) classifies bacterial isolates inhibitory zone on the growth of LAB in 3 criteria. Covering in area 6-9 mm, namely are moderate inhibition. Strong criteria (strong inhibition width of 10-14 mm and very strong inhibition width of 15-18 mm. Lactic acid bacteria on fermentation of carbohydrates produce lactic acid which can lower the pH. The decrease of pH can inhibit the growth of other microorganisms, particularly bacterial pathogens (Huang *et al.*, 2004).

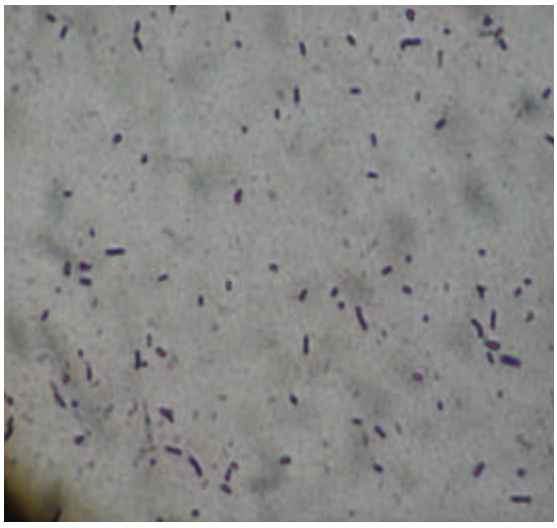

Fig. 1: Genus *Lactobacillus* was isolated from Pengging duck's *caecum*

Murry *et al.* (2004) observed in vitro that media with culture of *Lactobacillus* salivarius and *Lactobacillus*

Table 5: Identification of Lactic Acid Bacteria using API 50 CHL

Tube	Test	Isolate I 11	Tube	Test	Isolate I 11
1	Temoin	-	26	Salicin	-
2	Glycerol	-	27	D-Cellibiose	-
3	Erythritol	-	28	D-Maltose	+
4	D-arabinose	-	29	D-Lactose	+
5	L-arabinose	-	30	D-Melibiose	+
6	D-ribose	-	31	D-Saccharose	+
7	D-Xylose	-	32	D-Trehalose	+
8	L-Xylose	-	33	Inulin	-
9	D-adonitol	-	34	D-Melezitose	-
10	Methyl-βD-Xylopyranoside	+	35	D-Raffinose	+
11	D-Galactose	+	36	AmiDon	-
12	D-Glucose	+	37	Glycogen	-
13	D-Fructose	+	38	Xylitol	-
14	D-Mannose	-	39	Gentiobiose	-
15	L-rhamnose	-	40	D-Turanose	-
16	Dulcitol	-	41	D-Lyxose	-
17	Inositol	-	42	D-Tagatose	-
18	-Mannitol	+	43	D-Fucose	-
19	D-Sorbitol	+	44	L-Fucose	-
20	Methyl-αD-Mannopyranoside	-	45	D-Arabitol	-
21	Methyl-αD-Glucopyranoside	-	46	L-Arabitol	-
22	N-acetylglucosamine	+	47	Potassium gluconate	-
23	Amygdaline	-	48	Potassium 2 ketogluconate	-
24	Arbutin	-	49	Potassium 5 ketogluconate	-
25	Esculin	-	50	control	-

I11: *Lactobacillus* salivarius (99,99%)

plantarum produce more acetic and lactic acid and the pH was lower. *Lactobacillus* salivarius and *Lactobacillus* plantarum can ferment carbohydrates in poultry feed to produce pH levels and concentrations of lactic and acetic acid that inhibit the growth of pathogenic bacteria. The greater population on lactic acid bacteria with application *Lactobacillus* might inhibit harmful bacteria in the intestinal tract by blocking possible intestinal receptors of these pathogens or by secreting toxic metabolites against gram negative bacteria (Choi *et al*., 2009). Lactic acid bacteria produce various antimicrobial compounds, such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3 butanedione) and bacteriocin. All of these can antagonize the growth of some spoilage and pathogenic bacteria (Erdogrul and Erbilir, 2006).

Identification lactic acid bacteria using API 50 CHL (Table 5) showed that isolate I11 were identified using API 50 CHL. The testing is done based on ability of each isolates to degrade different carbon sources. The results were observed, the tube that changes color from purple to yellow showed positive results. Isolates I11 can degrade carbon source no : 10, 11, 12, 13, 18, 19, 22, 28, 29, 30, 31, 32 and 35. Isolates I11 was identified as *Lactobacillus* salivarius (Biomeriueux, Marcy l'Etoile, 2006).

Conclusions: Based on the characteristics properties, it can be conclude that the 12 isolates of lactic acid bacteria from Pengging duck's caecum were genus *Lactobacillus*. Isolate number I11 was potentially used as probiotic. Isolate number I11 was identified as *Lactobacillus* salivarius.

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