LACTIC ACID BACTERIAL SCREENING FROM GASTROINTESTINAL DIGESTIVE TRACT OF NATIVE AND BROILER CHICKEN FOR PROBIOTIC CANDIDATE PURPOSES

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ABSTRAK

Penelitian bertujuan untuk mendapatkan isolat bakteri asam laktat (BAL) dari saluran cerna unggas sebagai kandidat probiotik. BAL diisolasi dari saluran cerna ayam broiler dan ayam kampung menggunakan media selektif MRS+0,2% $CaCO_3$. Penapisan dilakukan berdasarkan karakteristik mikrobiologi, biokimia, daya antibakteri, dan kemampuan tumbuh pada berbagai suhu, aerobisitas dan agitasi, sensitifitas antibiotik dan viabilitas pada pH asam, garam empedu dan getah lambung. Tiga puluh sembilan isolat BAL didapatkan dari ayam kampung dan 18 isolat dari ayam broiler. BAL terpilih menghambat pertumbuhan *Escherichia coli* FNCC 0091, tumbuh pada suhu 30, 39 dan 45^oC, dan pada kondisi aerob, anaerob dan dengan agitasi. Identifikasi biomikia menggunakan API 50 CHL *kit* menunjukkan dua isolat terpilih yaitu I72 dari ileum ayam kampung sebagai *Lactobacillus salivarius* dan Db9 dari duodenum ayam broiler sebagai *Pediococcus pentosaceus*. Kedua BAL resisten terhadap antibiotik Erythromicin, Penicillin G dan Streptomycin. Keduanya menunjukkan viabilitas yang tidak berbeda nyata pada pH asam (1, 2 dan 3), getah lambung pH 2, dan garam empedu yaitu *L. salivarius* I72 sebesar 91,78% dan *P. pentosaceus* Db9 sebesar 94,48% ($P<0,05$). Berdasarkan karakteristik yang dimiliki, kedua BAL terpilih berpotensi untuk digunakan sebagai probiotik unggas.

Kata kunci: bakteri asam laktat, probiotik, ayam

ABSTRACT

The aim of his research was to obtain lactic acid bacteria (LAB) from gastrointestinal digestive tract (GIT) of chickens for probiotic candidate purposes. LAB was isolated from GIT of broiler and native chickens on selective medium (MRS+0.2% $CaCO_3$). Screening method based on microbiological and biochemical characteristics, antibacterial properties, growth on various temperature, aeration, and agitation, antibiotic sensitivity, and viability on acid pH, gastric juice and bile salt. Thirty nine of LAB isolates was selected from native chicken and 18 isolates from broiler chicken. The selected LAB inhibited *Escherichia coli* FNCC 0091 growth and grown on 30, 39 and 45^oC of temperature, aerobic, anaerobic and agitation conditions. Biochemical identification using API 50 CHL kit revealed that I72 from native chicken ileum as *Lactobacillus salivarius* and Db9 from broiler chicken duodenum as *Pediococcus pentosaceus*. All LAB were resistant to Erythromicin, Penicillin G and Streptomycin as tested antibiotics. Both of them have non significantly different of viability on acid pH (1, 2 and 3), gastric juice pH 2 and bile salt which were 91.78% for *L. salivarius* I72 and 94.48% for *P. pentosaceus* Db9 ($P<0.05$). Based on characteristics, both the selected LAB have potentiality as chicken probiotic candidates.

Keywords : chicken, lactic acid bacteria, probiotic

INTRODUCTION

The history of the Indonesian poultry industry illustrates significant contributions to the national meat supply. Indonesian consumers preference for chicken meat creates a large domestic market (Helinna, 2001). Indonesian people consumption rates only 6.1 kg per capita per year, it is still lower than others leading countries (Bond *et al.*, 2007). One of factor which

caused less production of chicken's meat is the diseases problem (Patterson and Burkholder, 2003). Animal enteric pathogens are a direct source for food contamination. The prohibition of antibiotics as growth promoters (AGPs) use has been a challenge for animal nutrition therefore need to find alternative methods to control and prevent pathogenic bacterial colonization. The modulation of the gut microbiota with new feed additives such as probiotics against host-protecting functions to support animal health, is a topical issue in animal breeding and creates fascinating possibilities (Gaggia *et al*., 2010).

Currently, probiotics are used as health supplements in food and feeds and they are replacing the use of antibiotic growth promotors or chemical supplements (Kosin and Rakshit, 2006). Others, probiotic is a natural organic matter that could not leave the residual effect on animal product so it will not cause pathogen bacterial resistance effect. Fortunately, consumers are taking very serious attention on the food availability with the beneficial addition for their healthiness and also diseases prevention (de Lima and Filho, 2005).

Some of related research which contained lactic acid bacteria isolation and probiotic were *L. reuteri*, *L. salivarius*, or *Lactobacillus* spp use that could inhibited the pathogen bacterial such as *Enterococcus faecalis*, *Enterococcus faecium*, *Listeria monocytogenes*, and *Salmonella* spp. Some of *Lactobacillus* isolates could produce anti microbe's peptide or bacteriosin (Lima *et al*., 2007; Pilasombut *et al*., 2006). *L. salivarius* CTC2197 already know had inhibitory effect on *S. enteritidis* C-114 colonization by *in vivo* on GIT tract of chicken after single doses addition on feed mixture (Pascual *et al*., 1999).

The ideal requirements for probiotic agent purposes of microbes are animal host origin, non-pathogenic, withstand processing and storage, resist on gastric acid and bile, adhere to epithelium or mucus, persist in the intestial tract, produce inhibitory compounds and modulate immune respons (Pattershon and Burkholder, 2003). The objectives of this research was to select LAB from native and broiler chicken which have ideal probiotic characteristics.

MATERIALS AND METHODS

Lactic Acid Bacteria Isolation and Identification

LAB isolated from chicken's GIT tract of 5 month old of native chicken and 35 days old of broiler chicken (*Cobb* strain) using Torshizi *et al*. (2008) method. GIT sampling location was gizzard, duodenum, jejunum, ileum, and caecum. Samples were cutted, washed, and diluted in steril peptone water (Oxoid) and made up to 10^5 dilution. Each serials dilution was plated in de Man Rogosa Sharpe (MRS) Agar media (Oxoid) pH 6.2 then was added by 0.2% $CaCO_3$ (Merck) and incubated at 37°C for 24 h. The LAB colonies was detected by clearing zone appearance. LAB identification procedures consist of morphology, catalase, gas production, Gram staining, and motility tests. LAB isolates were maintained on microbank (Pro-lab) containing 15% glycerol.

Antibacterial Activity Assay

The selected LAB isolates grown on MRS Broth media at 37°C for 24 h. Cell free supernatant was obtained by centrifugation at 12,500 g for 20 min at 4°C. Supernatant were neutralized using 5 N NaOH (Merck), and sterilized using miliphore filter 0.20 µ. Antibacterial activities against *E. coli* FNCC 0091 in Nutrient Broth (NB) (Merck) medium were observed using turbidimetric method with incubation time for 48 h at 37°C. Sterile supernatants were mixed with double strength of NB about 1:1 (v/v) comparison and inoculated with 2% (v/v) of bacterial test. NB media without supernatant which had been inoculated with *E. coli* was used as a control. The optical density (OD) were observed at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h incubation time using a spectrophotometer at $\lambda_{600\ nm}$ (Seeley *et al*., 2001).

Optimization of the Growth Temperature and Condition

A total of 1% (v/v) new cultures of the selected LAB inoculated on MRSB in Hungate tube then incubated at 30, 49 and 45°C, anerobic with CO_2 addition, aerobic without and aerobic with 100 rpm agitation. The control tube contained MRSB without LAB culture addition. The OD were measured using a spectrophotometer at $\lambda_{600\ nm}$ at 12 and 24 h incubation time.

Biochemical Identification

Biochemical identification of the selected LAB were observed by API 50 CHL kit (bioMērieux). The test procedure using the manual standard of API 50 CH kit. The

observation data was analyzed by API web software (bioMērieux).

Antibiotic Sensitivity Test

The antibiotic sensitivity test were measured using Kirby Bouer method (Cappuccino and Natalie, 1986) with Erithromycin 15 µg, Penicillin G 10 µg and Streptomycin 10 µg as antibiotics. The 100 µL of LAB isolates were inoculated on MRSA plate. The antibiotic paper discs were put on MRSA surface and then incubated at 37ºC for 24 h. Diameter of clear zone (mm) around paper disc was observed using calipers.

Acid Tolerance

Acid tolerance test refers to modified methods of Torshizi *et al*. (2008). LAB cultures on MRS Broth were centrifuged at 5000 rpm for 10 min at 4ºC. Pellet were washed two times by sterile phosphat buffered saline (PBS) and diluted in sterile PBS before inoculated on MRS Broth (pH 2, with 1 M HCl addition). Cell viability were calculated by the total plate count (TPC) method on MRS Agar media.

Gastric Juice Tolerance

Gastric juice tolerance were observed according to modified gastric juice simulation (Thorsizhi *et al*., 2008). The selected LAB isolates were incubated on MRS Broth at 37 ºC for 18 h. A total of 1 ml culture was centrifuged at 5000 g, 10 min, 4ºC then it washed in two times using steril PBS and diluted on 0.3 ml steril PBS. A total of 0.2 ml dilution was taken and then mixed with 1 ml of artificial gastric juice. The mixture liquid was homogenized and incubated at 37ºC for 2 h and then sampled after 0, 1 and 2 h. Serial dilutions of samples was made on sterile PBS and then inoculated on MRS Agar media for cell viability observation. The artificial gastric juice was made from pepsin (Sigma) (3 g/l) dilution at pH 2.

Bile Salt Tolerance

Bile salt tolerance was determined by modified method of Torshizi *et al*. (2008). A total of 1 ml LAB culture was centrifuged at 5000 g for 10 min at 4ºC and washed in two times by using strile PBS. The cells were diluted in 0.3 ml of PBS then mixed 0.2 ml of dilution and 1 ml PBS containing 0.3% (w/v) bile salt (Merck). The mixture was incubated at 37ºC for 3 h and sampled after 0, 1 and 3 h. The cell viability was calculated using serial dilution and plated on MRS Agar media. Three replicates were used for each treatment.

Data Analysis

The quantitative data was analyzed by using One-way analysis of variance (ANOVA) with post hoc test (Duncan multiple F-test ($P<0.05$)) to distinguish the treatments means. The total of bacteria cell (cfu/ml) from viability test was converted to the logatimic value before statistically analysis.

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) which obtained from gastrointestinal digestive tract (GIT) were 39 isolates from native chicken and 18 isolates from broiler chicken. All LAB isolates had negative catalase, non gas production, non motile, Gram positive, rod and coccus shape characteristics. The LAB isolates characteristic and the optical density (OD) of *E. coli* on NB media containing extracelluler metabolite of LAB isolates are shown in Table 1. The *E. coli* growth on media with extracelluler metabolite of LAB isolates was lower than media without extracelluler metabolite and they were significantly different ($P<0.05$) from control. Antibacterial activities of LAB isolates against *E. coli* was came from bacteriocins compound of metabolite extracelluler during grow in media. Extracelluler metabolite which have antibacterial activities such as antimicrobial peptide or bacteriosin of *L. reuteri*, *L. salivarius*, and *Lactobacillus* spp. were isolated from gizzard and caecum of poultry and inhibitted *Enterococcus*, *Listeria*, and *Salmonella* (Lima *et al*., 2007). Alpha and beta bacteriosin Abp 118 produced by *L. salivarius* isolated from digestive tract of poultry and showed inhibition activities against *B. coagulans* JCM 2257T (Pilasombut *et al*., 2006). Bacteriocin from *L. salivarius* NRRL B-30514 could reduced *Campilobacter jejuni* population in digestive tracts of poultry (Stern *et al*., 2006). *Lactobacillus* sp. isolated from silage feed had antibacterial activities against *E. coli* and *S. aureus* (Damayanti *et al*., 2009). *L. plantarum* fed to broiler chicken showed terapeutic effect of bacteriocin against *E. coli* infection in broiler chickens (Ogunbanwo *et al*., 2004).

Inhibition mechanism of bacteriocin occured in two phases. First phase was bacteriocin absorption on specific and nonspesific receptor on target bacterial membrane cells. During this

Table 1. Identification and Antibacterial Test Results of LAB Isolated from Gastrointestinal Digestive Tracts of Native and Broiler Chickens

Isolates Code	Location	Catalase Test	Motility	Gas Production	Gram Staining	Morphology	OD of *E. coli*
D1	Duodenum of NC		-	-	+	Coccus	0.508^{ab}
D2	Duodenum of NC	-	-	-	+	Coccus	0.553^{b}
D23	Duodenum of NC	-	-	-	+	Coccus	0.642^{c}
I72	Ileium of NC	-	-	-	+	Rod	0.546^{b}
T4	Crop of NC	-	-	-	+	Rod	0.624^{c}
Db1	Duodenum of BC	-	-	-	+	Rod	0.550^{b}
Db2	Duodenum of BC	-	-	-	+	Rod	0.536^{b}
Db5	Duodenum of BC	-	-	-	+	Rod	0.546^{b}
Db9	Duodenum of BC	-	-	-	+	Coccus	0.465^{a}
Ib1	Ileum of BC	-	-	-	+	Rod	0.556^{b}
K	Control						0.694^{d}

NC : Native chicken, BC : broiler chicken, OD : optical density at $\lambda_{660\ nm}$

Means in the same column with different superscript indicates differ significantly ($P<0.05$)

phase, the bacteriocine became sensitive especially to proteolitic enzyme. Second phase was irreversibel and involves lethal changes in the sensitive strains. The idea that bacteriocins act on the cell membrane has been well accepted (de Lima and Filho, 2005).

Based on data in Table 1, LAB isolates which had the highest inhibition against *E. coli* were Db9, D1, I72, Db2, Db5, Db1, D2, Ib1, T4 and D23, respectively. The differences of antibacterial activities in each isolates were based on the differences of LAB species and the ability to produce antibacterial compounds. Previous result from Torshizi *et al.* (2008) showed that *P. pentosaceus* TMU457 significantly had higher inhibition activity than *L. fermentum* TMU121 and *L. rhamnosus* TMU094 against *E. coli* and *S. pullorum*. Tatsadjieu *et al*. (2009) had reported that free cell supernatant from several strains of *Lactobacillus* had clear zone difference against *E. coli*.

One of expected characteristics of probiotic LAB was the stability during industrial processing, storage and delivery and had viability at high population (Gaggia *et al*., 2010). Beside its able to produce the antibacterial compounds, LAB as probiotic agent must be able to survive in host intestinal (Patterson and Burkholder, 2003). Probiotic survival in agitation condition became essensial factor because after entering the gastrointestine of the host, the probiotic strains have to attach to the brush border of microvilli or adhere to the mucus layer to prevent sweep from the colon by peristalsis (Kim *et al*., 2007). All of LAB isolates had ability to growth at anaerobic and aerobic conditions whereas at aerobic condition with 100 rpm agitation, both I72 and Db9 isolates had higher growing ability than D1 and Db2 isolates. Differences in species level were effect on physiology and biochemical characteristic especially in growth optimum temperature which shown in Table 2. According to the growth curves of LAB during 24 h, the fourth of LAB isolates had the best growth at 39ºC. The lowest growth for I72, Db9 and D1 occured at 45ºC, except for Db1 which occured at 30ºC.

Identification result by API 50 CHL kit are presented in Table 3. Two selected strains were identified as *L. salivarius* I72 and *P. pentosaceus* Db9. Both of LAB isolates had different ability to ferment carbohydrate, but they had similar ability to ferment monosacharide such as glucose and fructose, and the other carbohydrate like N-acetylglucosamine and D-Trehalose. Several studies had found LAB isolate from digestive

Table 2. The Growth Parameter of Selected LAB in Different Temperature and Condition

Isolate	Hours	Optical density (OD) $\lambda_{600\ nm}$					
		Temperature (^oC)			Condition		
		30	39	45	Agitation	Anaerobic	Aerobic
I72	12	+ +	+ + +	+ +	+ + +	+ + +	+ + +
	24	+ + +	+ + +	+ +	+ + +	+ + +	+ + +
D1	12	+ +	+ +	+	+ +	+ + +	+ + +
	24	+ + +	+ + +	+ +	+ + +	+ + +	+ +
DB1	12	+	+ + +	+ +	+ +	+ + +	+ +
	24	+ +	+ + + +	+ + +	+ + +	+ + +	+ + +
DB9	12	+ +	+ + +	+ +	+ + +	+ + +	+ + +
	24	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

OD λ_{600nm} = + : OD 0.5 – 1.0; + + : 1.0 – 1.5; + + + : 1.5 – 2.0; + + + + : >2

Table 3. Identification of LAB Isolates using API 50 CHL Kit

No	Type of test	Db9	I72	No	Type of test	Db9	I72
1	Temoin	-	-	26	Salicin	+	-
2	Glycerol	-	-	27	D-Cellibiose	-	-
3	Erythritol	-	-	28	D-Maltose	-	+
4	D-arabinose	+	-	29	D-Lasctose	+	-
5	L-arabinose	+	-	30	D-Melibiose	-	+
6	D-ribose	+	-	31	D-Sacharose	-	+
7	D-xylose	-	-	32	D-Trehalose	+	+
8	L-xylose	-	-	33	Inulin	-	-
9	D-adonitel	-	-	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	Gentibiose	+	-
15	L-rhamnose	-	+	40	D-Turanose	-	-
16	Dulcitol	-	-	41	D-Lyxose	-	-
17	Inositol	-	-	42	D-Tagatose	+	-
18	D-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	Potasium gluconate	-	-
23	Amygdaline	-	-	48	Pottasium 2 ketogluconate	-	-
24	Arbutine	+	-	49	Pottasium 5 ketogluconate	-	-
25	Esculine	+	-	0	Control	-	-

I72 : *Lactobacillus salivarius* (99.9%); Db9:*Pediococcus pentosaceus* (85.1%)

Table 4. Antibiotic Sensitivity Test of LAB Isolates

LAB isolates	clear zone (mm)			Average (B)
	Erythromycin 15 µg	Penicilin G 10µg	Streptomycin 10 µg	
P. pentosaceus Db9	9.82 (R)	3.50 (R)	3.77 (R)	5.69[a] (R)
L. salivarius I72	11.83 (R)	3.30 (R)	4.15 (R)	6.43[a] (R)
Average (A)	10.83[a] (R)	3.40[b] (R)	3.96[b] (R)	

R = resistance; Means in the same rows (A) and column (B) with different superscript indicate differ significantly (P<0.05)

tract of poultry such as *L. salivarius* from gizard and caecum of broiler breeder (Cobb strain) in 56 weeks of age (Lima *et al*., 2007), *L. salivarius* TMU121 and *P. pentosaceus* TMU457 from digestive tract of broiler in 42-50 days of age (Thorshizi *et al*., 2008), *L. salivarius* K7 from poultry intestine (Pilasombut *et al*., 2006), *L. salivarius* NRRL B-30514 from feces of intestine broiler (Stern *et al*., 2006), *L. acidophilus*, *L. salivarius*, and *L. brevis* from broiler feces (Kizerwetter-Świda and Binek, 2006).

The result of antibiotic sensitivity test showed that both of the selected strains had the same tolerance and not significantly different (P>0.05) to the other antibiotics (Table 4). Several poultry feeds contained some antibiotic in certain amount. The resistance characteristic of two LAB isolates to the broad spectrum of antibiotic (Erithromycin 15 µg), as well as Gram negative specify antibiotic (Penicillin G 10 µg) and Gram positive specify antibiotic (Streptomycin 10 µg) caused both of LAB isolates had possibility to survive in digestive tract of poultry which have exposed antibiotics. In a previous report, Torshizi *et al*. (2008) reported that all three selected LAB isolated from broiler chicken had some degree of antibiotic resistance againts several of the tested antibiotic.

One of the essential characteristic of probiotic in order to give beneficial health for individual host was resistance to the effect of gastrointestinal enviroment such as acid and bile salt in digestive tract (Kosin and Rakshit, 2006). The result of pH acid, gastric juice and bile salt tolerance test of the two selected LAB isolates were shown on Table 5. This study showed that both of LAB isolates had ability to survive on pH 1, 2 and 3 after 1 hour incubation. Based on cell viability percentage, *L. salivarius* I72 and *P. pentosaceus* Db9 had the higher viability on the higher pH but not significantly different (P<0.05) to others. In the gastric juice tolerance test, both of LAB isolates also showed had viability after 1–2 hours of incubation. The cell viability of two LAB isolates were decreased at second hour of incubation but not significantly different to others (P<0.05). Similar to the result in gastric juice tolerance test, both of LAB had higher cell viability on 0.3% (b/v) bile salt at one hour of incubation than three hours of incubation. However, both of LAB isolates were categorized had ability to survive in bile salt condition after 3 hours of incubation with 102.43-105.62% of cell viability.

LAB as an intestinal bacteria could experience a wide number of stresses in the intestinal tract including those caused by low pH and presence of bile. In this case, bile salt tolerances was though to be an important aspect of survival for bacteria which inhabit the intestinal tract. Bile salt tolerance in intestinal lactobacilli assosiated with bile salt hydrolase (BSH) activity (O'Sullivan *et al*., 2009). BSH split the peptide linkage of bile acids, which results in removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low pH (Begley *et al*., 2006). On the basis of the results of molecular screening, both of selected LAB *L. salivarius* and *P. pentosaceus* had a genetic equipment for their survival at low pH (such as *groEL* gene for heat shock protein 60) and in the presence of bile salt (such as *bsh* gene for conjugated bile salt acid hydrolase) (Turpin *et al*., 2011). Based on the average of cell viability, both of the selected LAB had an equal viability on all treatments and had characteristic as probiotic bacteria.

Table 5. The Cell Viability of Two LAB Isolates on Acid pH, Gastric Acid and Bile Salt

LAB isolates	Cell Viabilty (%)							Average (B)
	Acid pH after 1 h			3g/L Gastric Juice		0.3% Bile Salt		
	pH 1	pH 2	pH 3	1 h	2 h	1 h	3 h	
P. pentosaceus Db9	92.91	100.5	106.13	86.62	81.76	99.12	94.37	94.48[a]
L. salivarius I72	69.93	85.97	98.74	89.81	75.66	112.12	110.24	91.78[a]
Average (A)	81.42[ab]	93.23[ab]	102.43[ab]	88.21[ab]	78.71[b]	105.62[a]	102.43[ab]	

Means in the same rows (A) and column (B) with different superscript differ significantly (P<0.05)

CONCLUSION

The selected LAB were *Lactobacillus salivarius* I72 found in ileum of native chicken and *Pediococcus pentosaceus* Db9 found in duodenum of broiler chicken. Both LAB isolates have antibacterial activities to *E. coli* FNCC 0091 and survive in anaerobic, aerobic and agitation conditions. They were also resistant to Erithromycin, Penicillin G and Streptomycin antibiotics. In generally, both of LAB isolates had tolerance in low pH (1,2, and 3), gastric juice pH 2 and bile salt 0.3%. Based on the essential characteristics, it was concluded that *L. salivarius* I72 and *P. pentosaceus* Db9 were potential as chicken probiotic chandidates.

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