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Bile Salt and Acid Tolerant of Lactic Acid Bacteria Isolated from Proventriculus of Broiler Chicken

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ABSTRACT

The aim of this research was to obtain the lactic acid bacteria (LAB) as probiotic candidates which have resistance to bile salt and acid condition. LAB was obtained using isolation method from proventriculus of broiler chicken. Selective MRS media with 0.2% CaCO3 addition were used for LAB isolation using pour plate sampling method under anaerobic condition. The result showed that four selected isolates had morphological and biochemical characteristics as LAB. The selected LAB was characterized as follow: antibacterial activities, antibiotic sensitivity, resistance on bile salt, gastric juice and acid condition, and biochemical identification. Antibacterial activities assay of cell free supernatant was confirmed using disc paper diffusion method which was arranged on factorial design and each treatment consisted of three replications. The cell free supernatant of LAB isolates had antibacterial activities against *Escherichia coli*, *Pseudomonas aerugenosa*, and *Salmonella pullorum*. Molecular identification procedure using 16S rRNA sequence analysis showed that R01 and R02 as *Pediococcus acidilactici*. The viability of the two isolates were tested by acid pH (pH 1, 2, and 3), gastric juice pH 2, and bile salt condition for digestives tract simulation. The result showed that R01 and R02 had a high viability percentages at pH 1, 2, and 3 (95.45%, 99.49%, 104.01%, and 67.17%, 120.74%, 103.4%, respectively) and at bile salt simulation for 1-2 hours (100.35%-102.71% and 100.02%-102.65%, respectively), but at gastric juice simulation for 1-2 hours, the *P. acidilactici* R01 had higher viability than *P. acidilactici* R02 (59.69%-76.53% versus 43.57%-40.69%, respectively). In the antibiotic sensitivity test for three antibiotics (i.e. erythromicin 15 µg, penicillin G 10 µg, and streptomycin 10 µg), the *P. acidilactici* R02 showed resistance to Streptomycin and Penicillin. It is concluded that *P. acidilactici* R01 and *P. acidilactici* R02 isolated from proventriculus of broiler chicken potential as probiotic candidates for chicken.

Key words: lactic acid bacteria, probiotic, proventriculus, broiler chicken

ABSTRAK

Tujuan dari penelitian ini adalah mendapatkan bakteri asam laktat (BAL) kandidat probiotik yang memiliki ketahanan terhadap getah lambung dan pH asam. BAL didapatkan dengan cara mengisolasi dari proventrikulus ayam broiler. Media selektif MRS dengan penambahan 0,2% CaCO3 digunakan untuk media isolasi BAL menggunakan metode pour plate sampling secara anaerob. Hasil penelitian didapatkan 4 isolat terpilih yang memiliki karakteristik morfologi dan biokimia sebagai BAL. Keempat isolat BAL terpilih selanjutnya dikarakterisasi meliputi aktivitas antibakteri, sensitivitas terhadap antibiotic, ketahanan terhadap garam empedu, pH asam dan getah lambung serta identifikasi secara biomikimia. Uji aktivitas antibakteri dari supernatan bebas sel BAL dilakukan dengan menggunakan metode difusi kertas cakram yang disusun dalam rancangan faktorial dan setiap perlakuan terdiri atas 3 ulangan. Hasil pengujian menunjukkan bahwa supernatan bebas sel BAL mampu menghambat pertumbuhan bakteri *Escherichia coli, Pseudomonas aerugenosa,* dan *Salmonella pullorum*. Hasil identifikasi molekuler menggunakan analisis sekuens 16S rRNA menunjukkan bahwa isolat R01 dan R02 sebagai *Pediococcus acidilactici*. Dua isolat tersebut diuji viabilitasnya pada kondisi pH asam (pH 1, 2, dan 3), getah lambung pH 2, dan garam empedu. Hasil pengujian menunjukkan bahwa isolat R01 dan R02 memiliki persentase viabilitas yang tinggi dalam kondisi pH 1, 2, dan 3 (berturut-turut 95,45%; 99,49%; 104,01%; dan 67,17%; 120,74%; 103,4%) dan pada simulasi getah lambung selama 1-2 jam (bertu-

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rut-turut 100,35%-102,71% dan 100,02%-102,65%), tetapi pada simulasi garam empedu selama 1-2 jam, *P. acidilactici* R01 memiliki viabilitas lebih tinggi dibandingkan *P. acidilactici* R02 (berturut-turut 59,69%-76,53% versus 43,57%-40,69%). Uji sensitivitas antibiotik pada tiga antibiotik (eritromisin 15 μg, penisilin G 10 μg, dan streptomisin 10 μg) menunjukkan bahwa *P. acidilactici* R02 resisten terhadap streptomisin dan penisilin. Disimpulkan bahwa *P. acidilactici* R01 dan *P. acidilactici* R02 yang diisolasi dari proventrikulus ayam broiler berpotensi sebagai probiotik untuk unggas.

Kata kunci: bakteri asam laktat, probiotik, proventrikulus, ayam broiler

INTRODUCTION

Lately, the demands for reducing bacterial pathogens in foods of animal origin is increasingly becoming a concern. A direct source of food contamination, animal enteric pathogens, was the cause of food-borne disease that has spread. Animal nutritionists are challenged to find alternative methods that can resolve the issue, in addition to the use of antibiotics as growth promoters that have been banned. Probiotics are the alternative solution in control and prevention of pathogenic bacterial colonization (Gaggia *et al*., 2010). The significant parameters for probiotic bacteria in delivering therapeutic actions are viability and survival. Low pH and bile salts are some factors that have been claimed to influence the viability of probiotic bacteria. LAB strains must be selected for their viability to simulate gastrointestinal tract conditions in order to be used as potential probiotics for broiler. Low pH (2.5-3.5) is a powerful barrier to the entrance of bacteria into the intestinal tract. The survival of bacteria weakened by bile released in the small intestine with crushing their cell membranes and the major constituent of which are lipids and fatty acids. These refinement may also influence the interactions between the membrane and the surroundings, in addition to the cell permeability and viability. Tolerance to bile salts is assessed as a significant parameter for determining probiotic strains. A level of 0.15%-0.3% bile salt has been referenced as an appropriate level for determining probiotic bacteria (Boke *et al*., 2010).

The upper segment of chicken gastrointestinal tract (GIT) comprises of the crop, proventriculus and gizzard. The crop is used for food storage and fermentation while digestion starts in the proventriculus, and the gizzard mechanically grinds food and acts as a microbial barrier due to its low pH (Stanley *et al*., 2014). The proventriculus (also known as the 'true stomach') is the glandular stomach where digestion begins. The term 'proventriculus' is used since it comes before the 'ventriculus' or gizzard, with 'pro' being a Latin term meaning before (Jacob *et al*., 2011).

Dominant and abundant normal micro biota in chicken GIT section such as the crop, gizzard, duodenum, ileum, caecum, and in feces have been studied. *Lactobacillus* is one of the dominant genus in all GIT section with a variety of population number (Stanley *et al.,* 2014). Normal micro biota (LAB) in proventriculus which has low pH condition as in gizzard is rarely studied. In order to select LAB isolates for probiotic candidate which have bile salt and acid tolerant characteristics, LAB is isolated from proventriculus of broiler chicken as an animal host. Corcionivoschi *et al.* (2010) stated that to ensure their survival during passage through the gastrointestinal tract, the probiotic strains were tested in terms of resistance to pH and bile acids.

The others expected characteristics and safety criteria of probiotic were nontoxic and nonpathogenic, accurate taxonomic identification, normal inhabitant of the targeted species, production of antimicrobial substances and antagonism towards pathogenic bacteria (Gaggia *et al*., 2010). Therefore, the aim of current research was to study the characteristics of LAB isolated from proventriculus of broiler chicken with safety criteria of probiotic candidates.

MATERIALS AND METHODS

Lactic Acid Bacteria Isolation and Identification

LAB was isolated from chicken's gastrointestinal tract (GIT) of 35 days old broiler chicken (Cobb strain) by using Torshizi *et al.* (2008) method. Proventriculus samples were cut and washed. The proventriculus mucus (surface) was scraped by using aseptic scraper and then diluted in steril peptone water (Oxoid) and made up to 10^5 dilutions. Each serial dilution was plated in de Mann Rogosa Sharpe (MRS) Agar media (Oxoid) with pH 6.2 and then 0.2% $CaCO_3$ (Merck) was added and incubated at 37 ^oC for 24 h. The LAB colonies were detected by clearing zone appearance. LAB identification procedures consisted 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 were grown on MRS Broth media at 37 ^oC for 24 h until the stationary phase (10^9 CFU/mL). Cell-free supernatant was obtained by centrifugation at 12,500 g for 20 min at 4 ^oC. Supernatant was neutralized by using 5 N NaOH (Merck), and sterilized by using miliphore filter 0.20 μ. Antibacterial activities against *Escherichia coli* FNCC 0091, *Salmonella pullorum* and *Pseudomonas aerogenosa* in nutrient agar (NA) (Merck) medium were observed by using diffusion methods with incubation time for 24 h at 37 ^oC as described by Bonev *et al.* (2008). The experiment was arranged on factorial design which consisted of treatment were seven antibacterial substances (bacteriocin from R01, R02, R03, R12, antiobiotic penicilin, streptomycin and erythromycin) and three bacterial test (*E. coli*, *S.*

pullorum and *P. aerogenosa*) and each treatment replied three times. Twenty five milliliters of sterile supernatant (crude bacteriocin) was embedded in blank paper disc and placed in plate containing nutrient agar inoculated with tested bacteria (*S. pullorum* and *P. aerogenosa*). The tested bacteria were isolated from digestive tract of broiler chicken and were maintained in Laboratory of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta.

Antibiotic Sensitivity Assay

The antibiotic sensitivity test was measured by using Kirby Bouer method (Cappuccino & Natalie, 1986) with 15 μg erythromycin, 10 μg penicillin G and 10 μg streptomycin 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 37 ºC for 24 h. Three replicates were used for each treatment. Diameter of clear zone (mm) around paper disc was observed by using calipers.

Biochemical Identification

Biochemical identification of the selected LAB was observed by API 50 CHL kit (bioMērieux). The test procedure used the manual standard of API 50 CH kit. The observation data were analyzed by API web software (bioMērieux).

Bile Salt Tolerant Assay

Bile salt tolerance was determined by modified method of Torshizi *et al.* (2008). A total of 1 mL LAB culture was centrifuged at 4137 *x g* for 10 min at 4 °C and washed twice by using sterile PBS. The cells were diluted in 0.3 mL of PBS then mixed with 0.2 mL of dilution and 1 mL PBS containing 0.3% (w/v) bile salt (Merck). The mixture was incubated at 37 °C for 3 h and then was sampled after 0, 1, and 3 h. The cell viability was calculated by using serial dilution and plated on MRS agar media. Three replications were used for each treatment.

Acid Tolerant Assay

Acid tolerance test referred to modified method of Torshizi *et al*. (2008). LAB cultures on MRS Broth were centrifuged at 4137 x g for 10 min at 4 °C. Pellets were washed two times by sterile phosphate buffered saline (PBS) and diluted in sterile PBS before being inoculated on MRS Broth (pH 2, with 1 M HCl addition). Cell viability was calculated by the total plate count (TPC) method on MRS Agar media.

Gastric Juice Tolerant Assay

Gastric juice tolerance was observed according to modified gastric juice simulation (Thorsizhi *et al*., 2008). The selected LAB isolates were incubated on MRS Broth at 37 °C for 18 h. A total of 1 mL culture was centrifuged at 5000 g, 10 min, 4 °C then it was washed two times by using sterile PBS and diluted on 0.3 mL sterile 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 37 °C 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.

Data Analysis

The quantitative data were analyzed by using One-way analysis of variance (ANOVA) with post hoc test (Duncan multiple range test ($P<0.05$)) to distinguish the treatments means using CoSTAT statistical software (Cohort, 2008). The total of bacteria cell (cfu/mL) from viability test was converted to the logarithmic value before statistical analysis. Viability percentages was calculated by dividing a total of colonies in final incubation (log10 cfu/mL) with a total of colonies in initial incubation (log10 cfu/mL) and multiplied by 100%.

RESULTS AND DISCUSSION

Lactic acid bacteria isolation from proventriculus of broiler chicken was successful to select 4 LAB isolates i.e. R01, R02, R03, and R12. The selected LAB had single colony and clear zone appearance on MRSA+0.2% CaCO3. All of isolates had negative catalase, non-gas production on Glucose Yeast Peptone (GYP) medium, non-motile, Gram-positive and had coccus and rod shape of morphology. Antibacterial assay of crude bacteriocin against bacterial pathogens using paper disc method was shown in Table 1. In antibacterial assay, paper disc which contained antibiotic was used as a positive control.

Based on Table 1, all of LAB isolates had antibacterial properties against all pathogenic bacteria. The highest antibacterial activity was showed by R01 isolate with 15.21 mm of clear zone diameter and it has no significant difference with two antibiotics (Streptomycin and erythromycin). There was a significant interaction ($P<0.05$) between antibacterial substance against bacteria. It was indicated that either bacteriocin or antibiotic had specific inhibition on the specific bacteria. Inhibition of bacteriocin on *E. coli* was higher than on *P. aerogenosa* and *S. pullorum*. Lactic acid bacteria from GIT have different characteristic from other sources such as fermented food. Grosu-Tudor *et al.* (2014) reported that 274 LAB isolates from fermented food had no inhibitory activity against *Salmonella enterica* ATCC 14028 and *E. coli* ATCC 25922. Cotter *et al*. (2013) in his review also reported that bacteriocin produced by probiotic bacteria had narrow and broad spectrum activities against pathogenic bacteria.

Antibacterial activity in LAB isolates was produced by extracellular metabolite during being grown on fermentation medium. One of extracellular metabolite was well known as bacteriocins or antimicrobial peptide. Based on the in vivo assay, Yang *et al*. (2014) reported that the antimicrobial substances (e.g. bacteriocins) produced by probiotic bacteria can have a direct impact on reduction *Salmonella* burden in the gastrointestinal tract.

Tabel 1. Antibacterial activity assay of cell free supernatant of LAB isolated from broiler proventriculus

Antibacterial substances	Clear zone diameter (mm)		
	P. aerogenosa	*S. pullorum*	*E. coli*
LAB's bacteriocin			
R01	10.03 ± 1.3^{g}	12.50 ± 1.80^{f}	23.10 ± 0.50^{b}
R02	8.73 ± 0.4^{g}	10.20 ± 2.40^{g}	22.80 ± 2.00^{b}
R03	10.23 ± 0.4^{g}	9.90 ± 0.50^{g}	21.47 ± 1.00^{bc}
R12	8.67 ± 0.6^{g}	10.10 ± 1.00^{g}	23.00 ± 0.80^{b}
Antibiotic			
Penicillin G 10 µg	6.00 ± 0.1^{h}	18.00 ± 1.20^{d}	6.70 ± 0.20^{h}
Streptomycin 15 µg	6.60 ± 0.2^{h}	20.50 ± 0.40^{c}	16.00 ± 0.30^{e}
Erythromycin 10 µg	6.90 ± 0.2^{h}	29.40 ± 1.60^{a}	10.10 ± 1.10^{g}

Note: means with different superscript at the same row and column differ significantly (P<0.05).

de Lima & Filho (2005) also explained that a wide range of bactericidal activities of bacteriocins are enzyme activity modulation, inhibition of out-growth of spores, anion carrier activity to the formation of selective or non-selective pores. These peptides may have a broad or narrow target spectrum. In the latter case, their activity is often assumed to be associated with a receptor-like molecule at the surface of the target cell. One of inhibition mechanism of bacteriocin towards bacteria was bacteriocin adsorption to specific and non-specific receptor on the cell membrane of targeted bacteria.

Two selected LAB isolates were identified based on biochemical procedures by using API 50 CH kit (bioMerieux) as shown on Table 2. In biochemical identification, R01 isolate was identified as *Leuconostoc lactis* and R02 as *Pediococcus pentosaceus*. Broth of LAB isolates had different fermentation abilities on several types of carbohydrate but they had the same positive result in D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine and D-maltose.

In the previous study, *P. pentosaceous* TMU 457 was isolated from 45-50 days old of broiler chicken and it had positive result on API 50 CH test in L-arabinose, D-xylose, L-rhamnose, N-acetyl glucosamine, Amygdalin, Arbutin, D-trehalose, Gentibiose, D-tagatose, and Potassium 2-Keto gluconate (Thorsizi *et al*., 2008). In the other study, LAB isolates from digestive tract of chicken that were screened as the best candidate was identified as *Enterococcus faecalis*, *E. durans*, *E. faecium* and *Pediococcus pentosaceus* (Musikasang *et al*., 2009). This result was different from molecular identification by using sequence 16S rRNA analysis. Damayanti *et al.* (2012a) reported that R01 and R02 were identified as *P. acidilactici* that had 99% of similarity with *P. acidilactici* DSM 20284 (T) (AJ305320) based on nucleotide BLAST on NCBI website (2011).

The difference result between biochemical and molecular analysis in the previous research. Balcázar *et al.* (2007) showed that two isolates have different result between biochemical analysis using API 50 CHL (*L. fermentum* (82.6%) and *L. fermentum* (80.2%)) and molecular analysis using 16S rRNA gene (*L. sakei* (99,8%) and *L. plantarum* (99.8%)). Zhang *et al*. (2011) and Makarova & Koonin (2007), also reported that based on phylogenetic tree analysis using 16S rRNA sequence and 232

Table 2. Identification of LAB Isolates using API 50 CHL kit

No	Type of test	R01	R02	No	Type of test	R01	R02
0	Temoin	-	-	25	Esculine	-	+
1	Glycerol	-	*	26	Salicin	-	+
2	Erythritol	-	-	27	D-Cellibiose	-	+
3	D-arabinose	-	-	28	D-Maltose	+	+
4	L-arabinose	-	+	29	D-Lactose	+	-
5	D-ribose	-	+	30	D-Melibiose	+	-
6	D-xylose	-	+	31	D-Sacharose	+	-
7	L-xylose	-	-	32	D-Trehalose	-	+
8	D-adonitel	-	-	33	Inulin	-	-
9	Methyl-βD-xylopyranoside	-	-	34	D-Melezitose	-	-
10	D-galactose	+	+	35	D-Raffinose	+	-
11	D-glucose	+	+	36	Amidon	-	-
12	D-fructose	+	+	37	Glycogen	-	-
13	D-mannose	+	+	38	Xylitol	-	-
14	L-sorbose	-	-	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	-	-

Note: (+): positive reaction (yellow), no. 25 (black); (-): negative reaction (violet); (*): weak reaction. R01: *Leuconostoc lactis* (99.5%); R02: *Pediococcus pentosaceus 1* (99.9%)

orthologous gene of LAB showed that *Pediococcus* genus and *Lactobacillus* genus was grouped into *Lactobacillaceae* family.

The result of low pH tolerant assay (pH 1, 2, and 3), both of isolates had high viability percentages (79.07%-121.17%) that were shown on Figure 1. Based on the cell viability percentages, the viability of *P. acidilactici* R02 was higher than that of *P. acidilactici* R01 at pH 2 for 1 h incubation. Viability of LAB in low pH condition is affected by pH and strain variations. This study has the same result with that reported by Sofyan *et al*. (2013). The viability percentages of four LAB strains in various pH conditions have different viabilities. Viability percentage in pH 2 for 1 h incubation time revealed that *L. paracasei* subsp. *Paracasei* (CR1 and CR2) was higher than *L. brevis* Sil.3 and *L. collinoides* Sil.9. The two of LAB isolates in this research have the high viability on acid pH simulation because they have been adapted on low pH condition as proventriculus of broiler chicken.

On gastric juice (pH 2) tolerant assay, both of LAB isolates showed cell viability percentage between 40.84%-76.76% during 1-2 h incubation. *P. acidilactici* R02 viability decreased whereas the viability of *P. acidilactici* R01 increased after 2 h incubation. In the previous research, *P. pentosaceus* Db9 isolated from duodenum of broiler chicken and *L. salivarius* I72 isolated from ileum of native chicken had the highest viability (75.66%-81.76%) (Damayanti *et al*., 2012b) but *P. acidilactici* R01 and *P. acidilactici* R02 in this research had higher viability than *P. pentosaceus* TMU457, *L. rhamnosus* TMU094 and *L. fermentum* TMU121 which had 55.89%-67.76% of cell viability (Thorshizi *et al*., 2008).

Based on Figure 1, it was concluded that both of LAB isolates had equal viability on 0.3% of bile salt for 1 and 2 hours incubation (100.35%-102.71% and 100.02%-102.65%, respectively for R01 and R02). This result similar with *L. fermentum* TMU121 (Torshizi *et al*., 2008) and *L. salivarius* I72 (Damayanti *et al*., 2012b) which have the viability percentage higher than 100%. The ability to survive on bile salt would be possible for LAB to deconjugate bile salt and would be effective to reduce serum cholesterol in broiler chicken. The high activity of bile salt hydrolyzed in lumen of intestine could reduce bile salt conjugation ability to break down lipid (Torshizi *et al*., 2008). Using different methods for bile salt tolerance assay, *Lactobacillus* isolates from chicken (L3, LB1, LB2 and LB4) had the same ability to grow with OD_{600} value was greater than 1.0 at concentrations of bile salt from 0.3% to 1.5% (Yang *et al*., 2014). The same viability of *P. acidilactici* R01 and R02 in bile salt condition might be caused by natural habitat in proventriculus. The result of this study was similar with that of Takanashi *et al.* (2014). They said that *Lactococcus lactis* strains from the various sources had different viabilities. This fact indicated that each strain had adapted to its particular environment.

Based on the ability to resist on bile salt, gastric juice and low pH, both of LAB isolates had the same adaptation mechanism toward gastrointestinal stress condition. Pfeiler & Klaenhammer (2007) explained that BAL probiotic species differed from the common LAB in the food fermentation process like cheese and milk. Turpin *et al*. (2011) in their molecular study showed that in *P. acidilactici* strains were detected several genes which linked with acid pH and bile salt like *groEL* gene (heat shock protein), *dltD* gene ($_D$-Alanine transfer protein), *clpL* gene (ATPase), *bsh* gene (conjugated bile salt acid hydrolase) and others.

Begley *et al.* (2006) explained that the peptide linkage of bile acids can be broken down by BSH enzyme of LAB, which resulted in elimination of amino acid group from its steroid core and these unconjugated bile acids precipitate at low pH. Merrit & Donaldson (2009) reviewed that the resistance in Gram positive or Gram negative bacteria models was likely an association of defense and/or repair mechanisms, not only specific to control breakage to the membrane or the DNA. Efflux pump is one mechanism owned by some enteric bacteria to dismiss bile salt from the cell, so can avoid potential damage to the membrane. Several LAB in viability test, produced exo-polysaccharides (EPSS) which had function as protection agent against bile salt (0.15%–0.3%) and low pH (2.0–3.0) (Boke *et al*., 2010).

Table 3 shows the susceptibilities of two LAB isolates to three different antibiotics. The result of antibiotic sensitivity assay showed that both of LAB had different resistance levels on each tested antibiotic. *P. acidilactici* R01 had resistant level on streptomycin whereas *P. acidilactici* R02 had resistant level on streptomycin and penicillin. As was mentioned by Stanley *et al.* (2014) chicken-indigenous LAB such as *Lactobacillus* strain possessed high antibiotic resistance. The high antibiotic resistance of microbiota from chicken GIT may be related to the

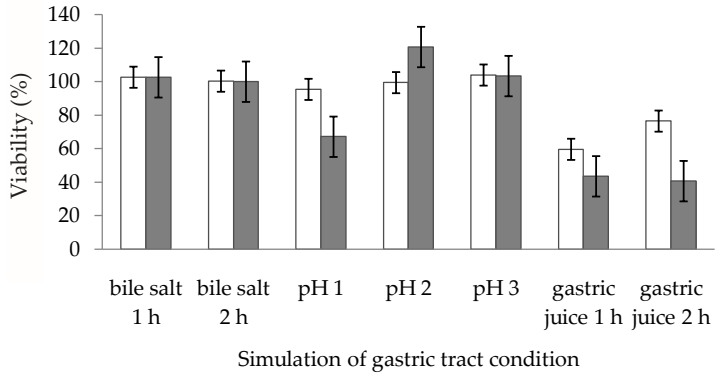

Figure 1. Viability percentage of *P. acidilactici* isolated from proventriculus of broiler chicken. □: *P. acidilactici* R01, ■: *P. acidilactici* R02.

Table 3. Antibiotic sensitivity test of LAB isolates (diameter of clearing zone/mm)

Antibiotic	*P. acidilactici* R01	*P. acidilactici* R02
Penicillin G 10 μg	23.37 ± 1.70 (I)^{C}	14.80 ± 0.60 (R)^{B}
Streptomycin 10 μg	7.30 ± 1.30 (R)^{A}	6.43 ± 0.70 (R)^{A}
Erythromycin 15 μg	18.10 ± 1.10 (S)^{C}	16.45 ± 0.40 (I)^{BC}

Note: Means in the same column with different superscript differ significantly P<0.01). I: intermediet, R: resistance, S: sensitive.

intensive usage of antibiotic in feed for growth promotor and terapeutic agent.

In this result, both of *P. acidilactici* isolates (R01 and R02) from proventriculus of broiler chicken had characteristic as probiotic candiates. LAB which had ability to produce bacteriocins had the potency to be used in the feed industry to substitute chemical preservation. Bacteria intended for probiotic should be screened for antibiotic resistance to avoid any potential carriage of undesirable antibiotic resistance into the intestinal environment (Musikasang *et al*., 2012) but the genetic exchange may be occured between native GIT strains (Stanley *et al*., 2014). For the application, the selected probiotics which have tolerance to low pH and bile salt condition and have antibiotic resistant are possible to be used in combination with appropiate antibiotics.

CONCLUSION

There were two isolates of LAB isolated from proventriculus of broiler chicken which had antimicrobial activities against *E. coli*, *S. pullorum* and *P. aerogenosa*. Molecular identification using 16S rRNA sequence analysis showed that R01 and R02 as *Pediococcus acidilactici*. *P. acidilactici* R01 and *P. acidilactici* R02 had a high viability in acid pH condition and 1-2 h on bile salt simulation, but the *P. acidilactici* R01 has higher viability than *P. acidilactici* R02 on gastric juice simulation for 1-2 h. In the antibiotic sensitivity test, *P. acidilactici* R02 showed resistant to Streptomycin and Penicillin while *P. acidilactici* R01 only resistant to Streptomycin. It was concluded that *P. acidilactici* R01 and *P. acidilactici* R02 which were isolated from proventriculus of broiler chicken were potential as probiotic candidates for chicken.

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