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16s rRNA Identification of *Pediococcus* spp. from Broiler and Studies of Adherence Ability on Immobilized Mucus

Ema Damayanti[1], Lies Mira Yusiati[2], and Achmad Dinoto[3]

[1] Technical Implementation Unit for Development of Chemical Engineering Processes, Indonesian Institute of Sciences, Yogyakarta, Indonesia
[2] Laboratory of Biochemistry and Nutrition , Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3] Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia

Abstract

The objectives of this research were to study taxonomical status of lactic acid bacteria (LAB) isolated from broiler and adherence ability on mucus in vitro. Molecular analysis was performed by analyzing 16S rRNA gene using universal primer. The adherence assay on mucus was carried out using microplate method with total plate count (TPC), absorbance (A_{550}) and confirmed by scanning electron microscopy (SEM). The results of this studies revealed that three of LAB isolates have closed relation to *Pediococcus acidilactici* (99.9%) species. Three isolates of *P. acidilactici* have adherence ability on broiler mucus higher than that on porcine mucin with an adherence percentage of 55.5% versus 50.8% and absorbance A_{550} of 0.061 versus 0.051, respectively. The highest adherence ability showed by *P. acidilactici* R02 with adherence percentage was 59.3% and absorbance A_{550} = 0.068. Adherence on mucus were affected by the addition of 3 g/1 of gastric juice and 0.3% (b/v) of bile salt. Adherence analysis using SEM also showed that the adherence on broiler mucus was higher than the adherence on porcine mucin. Altogether this adherence studies, suggest that three isolates of *P. acidilactici* LAB were capable of colonizing host intestinal mucus in vitro as important property to be promising probiotic bacteria for broiler.

Key words : adherence, broiler, *Pediococcus*, mucus, 16S rRNA

Introduction

Bacterial resistance that caused by antibiotic application in both medical and agricultural fields has become a serious worldwide problem (Tellez *et al*., 2012). The ban of antibiotics as growth promotors is a challenge for animal nutritionis to find alternative methods to control and prevent pathogenic bacterial colonization such as probiotics (Gaggia *et al*., 2010). Lactic acid bacteria (LAB) are commonly used as probiotic, which aid to maintain a balanced intestinal microbiota, excluding pathogens and helping to keep the gut homeostatis by influencing the mucosal immune system (Muñoz-Provencio *et al*., 2009). The binding of probiotic bacteria to intestinal cell is expected to have lasting beneficial effects for health. Binding is thus generally considered to be important property, and, along with survival, is often the main feature investigated in relation with probiotic characteristics of bacteria (Turpin *et al*., 2012).

The mucosa colonizing bacteria can be found both in the mucus layer and the epithelial cells. The epithelial cells of intestine are covered by a protective layer of mucus, which is a complex mixture of glycoproteins and glycolipids with the large glycoprotein

***Corresponding author:**
Ema Damayanti
Technical Implementation Unit for Development of Chemical Engineering Processes, Indonesian Institute of Sciences (UPT. BPPTK – LIPI), Yogyakarta, Indonesia, e-mail : ema_dyanti@yahoo.com

mucin being the main component (Roos and Jonsson, 2002). To asses the binding capacity of LAB, several models have been developed (Munoz-Provencio, 2009). Immobilizing commercially mucin to microtitter well plates is the most simple method to measure the adhesion of bacterial cell strains to mucus (Tassell and Miller, 2011) and it could be modified by certain conditions such as stress condition on gastrointestinal tract (GIT) (Ramiah *et al*., 2009; Sanchez *et al*., 2010). The adhesion properties showed a high intra-species variability (Muñoz-Provencio *et al*., 2009). The other factors which affecting bacterial adherence were acid pH, gastric juice and bile salt. A concentration of 0.15 - 0.3% of bile salt (Boke *et al*., 2010) and gastric juice 0.5 – 2.0 mg/ml at pH 2 – 3.5 (Zhu *et al*., 2006) were a suitable concentration for selecting probiotic bacteria. Adhesion genes expression also influenced by mucin concentration (0.01 – 0.05%) and bile salt (0.3 – 1%) (Ramiah *et al*., 2009). The selected LABs of *P. acidilactici* DB9 was potential as poultry probiotic and it would be important for further studies of adherence on mucus and affected stress factors.

Previous experiments on LAB screening from GIT of broiler chicken have obtained 3 probiotic candidates i.e: DB9 from duodenum, R01 and R01 from proventiculus (Damayanti *et al*., 2012; Damayanti *et al*., 2010). Those isolates were Gram positive, coccus or diplococcus, negative catalase, non gas producer and non motil. All of them could grow in aerobic, anaerobic and agitation condition, and could grow at temperature 30, 37 and 45 ^{0}C. Based on identification key in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 2000), all isolates were characterized as *Pediococcus* genus. Biochemical identification using API 50 CHL kit resulted in miss-identified bacteria and it was recomended to confirm with further molecular identification using 16S rRNA gene and phylogenetic analysis. The sensitivity of 16S rRNA methodology has been enhanced by the polymerase chain reaction, and new kits and equipments make genetic methodologies for bacteria classification easier to use than phenotypic methodologies (i.e: Gram stain, cell shape, motility, nutritional requirements and fermentation products) (Cobos *et al*., 2011). The objectives of this research were to study the taxonomical status of LAB isolates on species level and the adherence ability on mucus.

Materials And Methods

16S rRNA sequence analysis

Genomic DNA were isolated from fresh culture on MRS Agar according to Genomic DNA Purification Kit (K0512) (Fermentas) protocol. DNA concentrations were measured using spectrophotometer (BioSpec-DNA/protein/Enzyme analyzer-Shimadzu) at $\lambda_{260\ nm}$ and $\lambda_{280\ nm}$ that was making a reaction formula for polimerase chain reaction (PCR) (Table 1).

Primer used for PCR reaction was a universal primer 27f (5´-AGAGTTT GATCCTGGCTCAG-3´) and 1492r (5´-GGTTACCTTGTTACGACTT-3´) (Al-Jassim *et al*., 2005; Gong *et al*., 2007). Reaction volume of PCR was 50 µl which consist of 20 ng/µl of DNA concentration, 25 µl of GoTaq Green Master Mix, 1 µl of 1492 r primer (10 pm) and 1 µl of 27f primer (10 pm). PCR reaction was performed on 35 cycles for 3 h at 25 min

Table 1. Reaction composition of (PCR) to amplified 16S rRNA gene of LAB isolates

Isolate	A1 = λ_{260}	[DNA] = A1 x 50 ng/µl	Vol. DNA (µl = (20 ng/µl)/[DNA]	GoTaq (µl)+ primer 1492R (µl) +primer 27 F (µl)	Nuclease free water (µl)	Reaction volume vol. reaction (µl)
DB9	0.129	6.45	3.1	25 + 1 + 1	19.9	50
R01	0.101	5.05	4.0	25 + 1 + 1	19.0	50
R02	0.138	6.90	2.9	25 + 1 + 1	20.1	50

Table 2. Primer sequence for 16S rRNA gene sequencing

Primer	TM	Sequence
27f	56.3 °C	5´-AGAGTTTGATCCTGGCTCAG-3´
357f	54.0 °C	5´-CTAGGGGAGGCAGCAG-3´
920 f	58.2 °C	5´-AAACTCAAATGAATTGACGG-3´
520 r	58.0 °C	5´-ACCGCGGCGTGCTGGC-3´
1080r	50.0 °C	5´-CCCAACATCTCACGAC-3´
1492r	51.7 °C	5´-GGTTACCTTGTTACGACTT-3´

TM : melting point

using PCR machine (Takara-Thermal Cycler). PCR condition was set as follows : 4 °C with 94 °C for 2 min of pre-denaturation, 94 °C for 2 min of denaturation, 48,5 °C for 1 min of annealing, 72 °C for 1 min of elongation and 72 °C for 1 min of final extention (Al-Jassim *et al*., 2005). PCR product was analyzed using gel electrophoresis agarose 1% (Harisha, 2007) using electrophoresis apparatus (BioCRAFT BE 520) and 1 kb DNA ladder (Gene Rule-Fermentas) as ladder. Sequencing of 16S rRNA sequence was conducted by First BASE Laboratories (Singapore) using 6 primers as mention in Table 2.

Phylogenetic tree

Sequence data was showed in *abi format and was edited by FinchTV program and was contiq analyzed by BioEdit program. Contiq sequence was then analyzed using basic local alignment search tool (BLAST) program in Gene Bank of National Center for Biotechnology Information (NCBI) (website: www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed by Neighbor-Joining tree method using ClustalX2 and MEGA5 programs. Grouping stability was calculated using 1000 bootstrap value (Cobos *et al*., 2011).

Adhesion assay

The adhesion assay on mucus was conducted by using complete randomyzed factorial. First factor was LAB isolates, sec factor was source of mucus and third factor was stress conditions (pepsin and bile salt). Each treatments covered tree replicates. Total plate count (TPC) of adhering LAB on mucus and absorbance were observed as parameters. Microscopic visualization of LAB adhesion on mucin or mucus was observed using SEM. Experimental design was showed in Table 3.

Mucus isolation

Mucus from broiler chicken was prepared from small intestine of freshly slaughtered animals. The intestine was rinsed with cold PBST (PBS supplemented with 0.05% Tween 20) after which the mucus was released by gently scraping the mucosa and washing with PBST. Particles were pelleted by centrifugation (13,000 xg, 15 min) twice and the mucus was stored at -20 ^{0}C (Roos dan Jonsson, 2002).

Microplate adhesion assay

Adhesion assay were performed in 96-well polystyrene microplates (Corning) using mucin or mucus as the matrix (Sanchez *et al*., 2010). Mucus was diluted in PBS (Oxoid) at OD_{280} = 0.1 of concentration whereas porcine mucin (Sigma) at 0.1 % (1 mg/ml PBS) of concentration (Roos and Jonsson, 2002). One hundred microliters of a mucus/mucin solution was immobilized on the plate wells for one h at 37 ^{0}C, followed by overnight incubation at 4 ^{0}C. Well was washed twice with 200 µl of PBS and incubated with 20 g/l bovine serum albumin (BSA) fraction V (Merck), for 2 h at 4 ^{0}C. Well was washed twice with 200 µl of PBS in order to eliminate non-bound BSA, and 100 µl of treated or non treated bacterial cell suspensions, adjusted to cell counts of approximately 10^9 CFU/ml, was added to the wells and incubated at

37 ^{0}C for 1 h, anaerobically. Treated bacteria was cultured in MRS Broth (Oxoid) for 18 h at 37 ^{0}C anaerobic, than centrifuged at 5,500 rpm, 5 min and was diluted in sterile PBS pH 7.2 (Oxoid) with cell total amount 10^9 cfu/ml (OD_{550} = 2). Pepsin (Sigma) 3 g/l in PBS pH 2 (adjusted using 10 mM HCl) was used for gastric juice simulation for 2 h at 37 ^{0}C. After incubation, pepsin was removed from cell using centrifugation and cell was diluted in PBS. Bile salt (Sigma) 3% (b/v) in PBS was used for bile salt simulation whereas control was used PBS without stress condition. The amount initial of bacteria added was determined in all-cases by plating out. After incubation, wells were washed five times with 200 µl of steril citrate buffer to remove unbound bacteria. Two hundred µl of 0.5% (v/v) Triton X-100 was added to eliminate attached bacteria. The content of each well was thoroughly mixed with micropipette, and 100 µl of the resulting suspensions was sampled and plated to obtained the CFU/ well. Experiments were performed in tree replicates.

Microtitre plate binding assays

Microtitre plate binding assay used microplate 96 well (Corning) based on Munoz-Provencio *et al*. (2009) and Vesterlund *et al*. (2005) methods. Mucin porcine were used at 500 µg/ml at 50 mM carbonate/ bicarbonate buffer pH 9.6 (200 µl) whereas broiler mucus was disolved in PBS pH 7.2 (Oxoid). One hundred microliters of a mucus/mucin solution was immobilized on the plate wells for one h at 37 ^{0}C, followed by overnight incubation at 4 ^{0}C. After immobilization, well was washed tree times with PBS and were blocked for 1 h with PBS plus 1% Tween 20 (Merck). One hundred µl of each strain were added to each well in PBS, PBS plus pepsin (Sigma) 3 g/l (b/v) pH 2, dan PBS plus bile salt (Sigma) 3%, and plates were incubated overnight at 4 ^{0}C and 1 h at 37 ^{0}C. Negative control used PBS without LAB addition. Non-adhered cells were removed by washing three times with 200 µl of PBS plus 0.05% Tween 20 (Merck) then plate were dried at 55 ^{0}C for 1 - 2 h. Adhered cells were stained with crsytal violet 1 mg/ml (100 µl/well) for 45 min. After six washed with PBS, the colorant was liberated with 50 mM citrate buffer pH 5 (100 µl/ well) for 30 min and the absorbance at 550 nm was determined in microplate reader (680 XR, Bio-Rad Laboratories, Inc). Stained mucus without added bacteria was used as negative control and the absorbance value of this negative control was subtracted from the absorbance value of the samples (Vesterlund *et al*., 2005). Each treatments were performed in 3 replicates.

Scanning electron microscopy (SEM)

For the qualitative examination of adhesion by scanning electron microscopy (SEM), 5 x 5 mm coverslips were placed in the bottom of tissue culture plate 24 wells (SPL life sciences) before seeding with mucin or mucus. Three hundred microliters of mucin or mucus solutions in PBS pH 7.2 (Oxoid) were immobilized at 4 °C, overnight. Non adhere mucus was removed by pippeting aid. LAB used in this method was treated as mention previously in adhesion assay. Three hundred microliters of LAB solution in PBS (OD_{550} = 2) was placed into coverslip surface until completely submerged that incubated for 1 h at 37 °C. After incubation, coverslip was added by gutaraldehyde 2% at 4 ^{0}C overnight to stop reaction. Sample in coverslip was prepared according specimen preparation for SEM analysis (Moussavi and Adams, 2010). SEM (JSM-5000) analysis was conducted in Zoology Division, Research Center for Biology, Indonesian Institute of Sciences, Cibinong Indonesia.

Statistical analysis

TPC and absorbance data were statistical analyzed using ANOVA and Duncan post hoc test in CoStat program (free license). TPC data in cfu/well was converted to logaritmic value (log 10 cfu/well) and transformed to percentage of adherence (%) which was

obtained from LAB total after incubation divided by initial LAB and multiplied with 100% (Moussavi and Adams, 2010).

Results and Discussion

Gene of 16S rRNA was amplified a using universal primer set (27f and 1492r), with PCR product of ~1,500 bp in size (Figure 1).

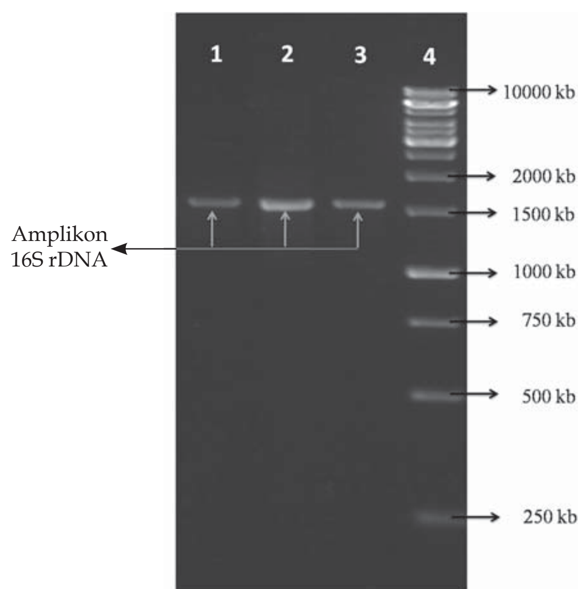

Figure 1. PCR product of 16S rDNA gene from LAB isolates : DB9 (1), R01 (2), R02 (3) and DNA ladder (1 kb) (4)

Using a 1,500 bp fragment from the LAB isolates, the 16S rRNA gene sequencing revealed 99,9% similarity with the species *Pediococcus acidilactici* (Table 3). Based on BLAST analysis on NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, was accesed on 02/10/2011) revealed nucleotide homology as strain identity (Table 3).

Identification result on biochemical analysis using API 50 CHL kit showed 85.1 – 99.9% of similarity. The difference between biochemical and molecular analysis was previously obtained by Olaoye *et al*. (2008) and Balcázar *et al*. (2007). Biochemical identification using API 50 CHL kit showed that R isolate as *P. pentosaceous* whereas using molecular identification revealed that R was *P. acidilactici* (Olaoye *et al*., 2008). Similar result was obtained for two isolates that showed the difference between biochemical analysis (*L. fermentum* (82.6%) and *L. fermentum* (80.2%)) and 16S rRNA analysis (*L. sakei* (99.8%) and *L. plantarum* (99.8%)) (Balcázar *et al*., 2007).

The phylogenetic tree of some *P. acidilactici* species clearly showed the position of LAB isolates in the *P. acidilactici* cluster (see Figure 2). Previous phylogenetic tree revealed that *Pediococcus* genus and *Lactobacillus* genus included in *Lactobacillaceae* family (Zhang *et al*., 2011; Makarova and Koonin, 2007). Some *P. acidilactici* species reported in the Gen Bank has been isolated from baby feces (EF059987), human (AJ305320), paddy rice silage (AF515229) and traditional fermented milk (FJ844982) (http://www.ncbi.nlm.nih.gov/). Based on figure 2, *P. acidilactici*, *P. pentosaceus* and *L. salivarius* were grouped in one group and separated with *Leuc. lactis* and *Leuc. mesenteroides*. This analysis showed that R01 was not *Leuconostoc lactis* (99.5%)

Table 3. Identification of lactic acid bacteria strains

Strains[a]	Biochemical identification	Similarity (%)[b]	16S rRNA Identification[c]
	Species		Species
DB9	*Pediococcus pentosaceus*	85,1	*Pediococcus acidilactici* (99,9)
R01	*Leuconostoc lactis*	99,5	*Pediococcus acidilactici* (99,9)
R02	*Pediococcus pentosaceus*	99,9	*Pediococcus acidilactici* (99,9)

Note :

a. Lactic acid bacteria isolated from duodenum (DB9), proventriculus (R01 and R02) of broiler chicken
b. Similarity percentage based on biochemical profiles using API 50 CHL kit which showed in API website (apiweb™)
c. Analysis result of 16S rRNA sequence with similarity percentage based on nucleotide BLAST on NCBI website (http://www.ncbi.nlm.nih.gov/)

as previously was revealed by biochemical identification. Curently there are 400 LAB which were devided into 4 families and 7 genera i.e: *Lactobacillaceae* family (genera *Lactobacillus* and *Pediococcus*), *Leuconostocaceae* family (genera *Oenococcus* and *Leuconostoc*), *Enterococcaceae* family (genus *Enterococcus*) and *Streptococcaceae* family (genera *Lactococcus* and *Streptococcus*) (Zhang *et al*., 2011). In evolution of LAB, *Bacillus subtilis* was an ancestor for *Lactobacillus*. The common ancestor of *Lactobacillales* has at least ~2,100 to 2,200 genes, having lost 600 to 1,200 genes (~25 to 30%) and gained ~100 genes after the divergence from the *Bacilli* ancestor, for which the genome size of ~2,700 to 3,700 genes was estimated. Many of the changes mapped to this stage of evolution seem to be related to the transition made by the LAB to existence in nutritionally rich medium. *Bifidobacterium* belongs to a different major bacterial branch, the actinobacteria (Makarova and Koonin, 2007). *P. acidilactici* species have *Generally Regarded as Safe* (GRAS) status from Food and Drug Administration United States (FDA-USA) and *Qualified Presumption of Safety* (QPS) status from European Food Safety Authority (EFSA) (Gaggia *et al*., 2010).

The adherence of *P. acidilactici* isolates (R01, R02 and DB9) on porcine mucin or broiler mucus with TPC parameter was showed in Table 4. Adhesion assay showed that all isolated had adhesion properties on both of porcine mucin and broiler mucus with adhesion percentage ranging from 46.6 – 59.3%. This result similar with Roos and Jonsson (2002) study showed that *L. reuteri* strain 1063 isolated from small intestine of pig have adhered efficiently to both pig and hen mucus indicates that the bacterium had

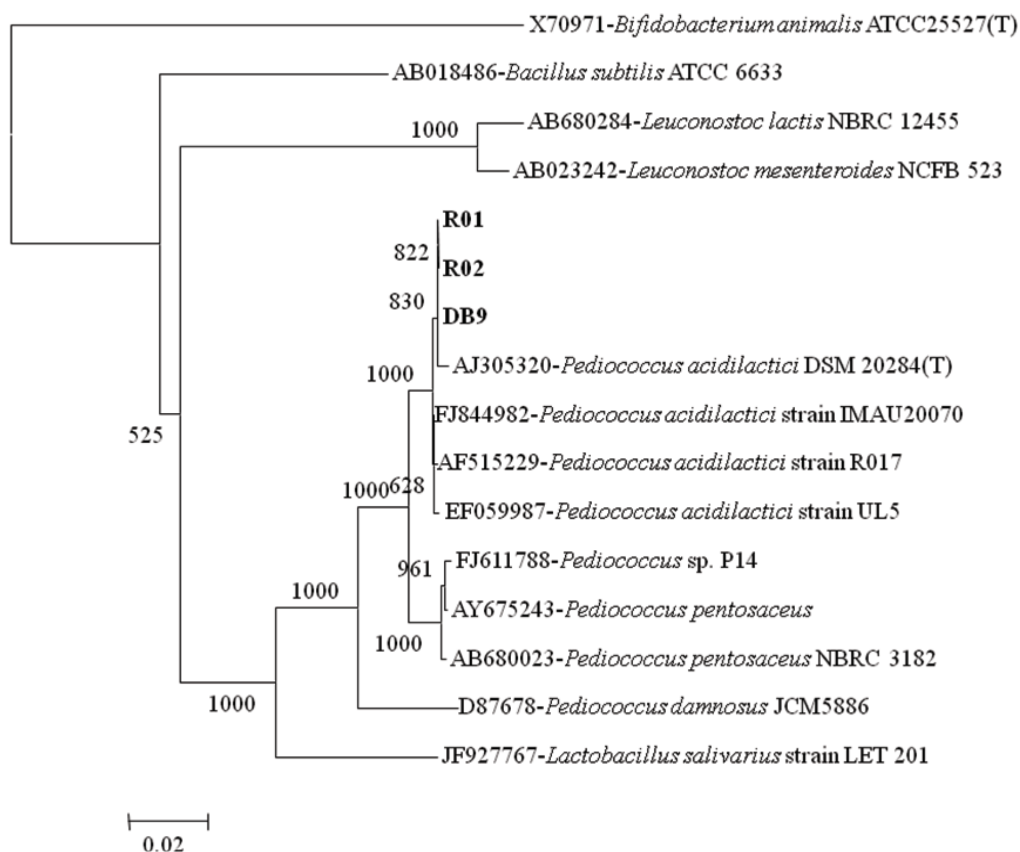

Figure 2. Phylogenetic tree of LAB isolated from duodenum (DB9) and proventriculus (R01 and R02) of broiler chicken using Neighbor-Joining tree method with grouping stability was 1000 of bootstrap value.

Table 4. Adhesion percentage of *Pediococcus acidilactici* on porcine mucin and broiler mucus

Strain (A)	Mucus (B)	Percentage of adherence (C)			average (A)
		PBS	Stress		
			Pepsin 3 g/l	Bile salt 0.3% (b/v)	
R01	mucin porcine	46.6 ±4.0	56.1 ± 8.8	44.3 ±3.1	53.5^{b} ± 8.5
	Mucus broiler	65.0 ±5.0	68.9 ± 2.7	49.8 ±1.4	
R02	Mucin porcine	47.5 ±1.2	72.8 ±11.9	48.3 ±2.8	59.3^{a} ±11.5
	Mucus broiler	68.9 ±2.7	51.4 ± 6.5	67.1 ±3.7	
DB9	Mucin porcine	52.8 ±3.4	42.7 ±17.5	46.4 ±1.3	46.6^{c} ± 5.5
	Mucus broiler	49.8 ±1.4	38.0 ±16.9	50.2 ±3.2	
Average (C)		55.1^{a} ±9.5	51.4^{a} ±12.2	52.9^{a} ±9.2	
Average (B)	Mucin porcine				50.8^{b} ± 9.2
	Mucus broiler				55.5^{a} ±10.5

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

little or no host specifity regarding adhesion to mucus.

The statistical analysis showed that there was significantly differences among all of *P. acidilactici* strain (P<0.05). *P. acidilactici* R02 and R01 from proventriculus had higher percentage of adherence than DB9 strain from duodenum. This difference assosiated with the difference condition in proventriculus and duodenum. Rynsburger and Classen (2007) studied that pH of chicken gastrointestinal tract from crop, proventriculus, gizzard, duodenum, jejunum and ileum have changed during growth stages. The proventriculus decreased if chicken get older (5.2 – 3.37), and pH duodenum was stable relatively (6.57 – 6.40). In the proventriculus and gizzard, initial protein digestion involves hydrochloric acid denaturation of protein and conversion of pepsinogen to its active form. Jacob *et al*. (2011) suggest that the proventriculus (also known as the 'true stomach') is the glandular stomach. Hydrochloric acid and digestive enzymes (e.g., pepsin) are added to the feed and digestion begins.

The adherence ability of *P. acidilactici* on broiler mucus (55.5%) was higher than on mucin porcine (50.8%) and it was significantly different (P<0.05) (Table 4). This result showed a host specificity between LAB and its host. In stress condition treatment, there was no difference of adherence between with or without stress conditions (P<0.05) (Table 4). This mechanism corelated with adaptation ability of intestinal LAB. Pfeiler and Klaenhammer (2007) cited that intestinal *Lactobacilli* adapted to survive in extrime digestive system such as acid pH and bile salt. Begley *et al*. (2006) described that intestinal LAB have bile salt hydrolase (BSH) enzyme to break peptide bond from bile salt than liberated amino acid from core steroid. The final product was non conjugated bile salt which precipitated in acid pH. Merrit and Donaldson (2009) explained that, bile salt resistance was assosiated with combination mechanism between defence and repair mechanism. One of these mechanisms was efflux pumps to remove bile salt from the cell and to protect membrane injuries. Another mechanism corelated with exopolisaccharide (EPS) production which function as barrier from bile salt exposure (0.15 – 0.3%) and acid pH (2.0 - 3.0) (Boke *et al*., 2010).

Adherence on mucus was confirmed by microtitre plate method using absorbance of adherence as parameter. Adherence data for 1 h at 37 ^{0}C incubation produced low absorbance (data not shown) because absorbance on LAB addition was lower than control without LAB addition. Vesterlund *et al*. (2005) in they review stated that staining with crystal violet in adherence method at 37 ^{0}C for 1 h was not a sensitive-enough method to detect low levels of adherent bacteria as the signal was not different from

Table 5. Adherence absorbance of *Pediococcus acidilactici* on mucin porcine and broiler mucus

Strain (A)	Mucin (B)	Adherence absorbance (A_{550}) (C)			Average (A)
		PBS	Strees condition		
			Pepsin 3 g/l	Bile salt 0.3% (b/v)	
R01	Mucin porcine	0.024 ±0.012	0.076 ±0.014	0.037 ±0.021	0.051^{ab}±0.026
	Mucus broiler	0.091 ±0.009	0.036 ±0.034	0.043 ±0.021	
R02	Mucin porcine	0.087 ±0.014	0.094 ±0.011	0.029 ±0.009	0.068^{a} ±0.023
	Mucus broiler	0.063 ±0.006	0.060 ±0.043	0.077 ±0.026	
DB9	Mucin porcine	0.066 ±0.021	0.028 ±0.002	0.018 ±0.006	0.049^{b} ±0.022
	Mucus broiler	0.051 ±0.006	0.074 ±0.081	0.057 ±0.020	
Average (C)		0.064^{a} ±0.025	0.061^{a} ±0.025	0.043^{b}±0.021	
Average (B)	Mucin porcine				0.051^{b} ±0.030
	Mucus broiler				0.061^{a} ±0.017

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

the background. Other data was resulted at 4 ^{0}C for overnight incubation (Table 5). Three of LAB isolates had significantly different among each isolates (OD_{550} = 0.049 – 0.068 in range) (P<0.05). Munoz-Provencio *et al*. (2009) showed that some *Lactobacillus* strain showed a different absorbance on OD_{595} = 0.01 – 0.28 in range.

Adherence absorbance on broiler mucus was higher than that on porcine mucin (P<0.05). This fact showed a host specificity as resulted by Roos and Jonsson (2002) which showed that *L. reuteri* 1063 (ATCC 53608) isolated from porcine instestinal have adherence (OD_{430} = 0.169) higher than hen mucus (0.136). The difference data was

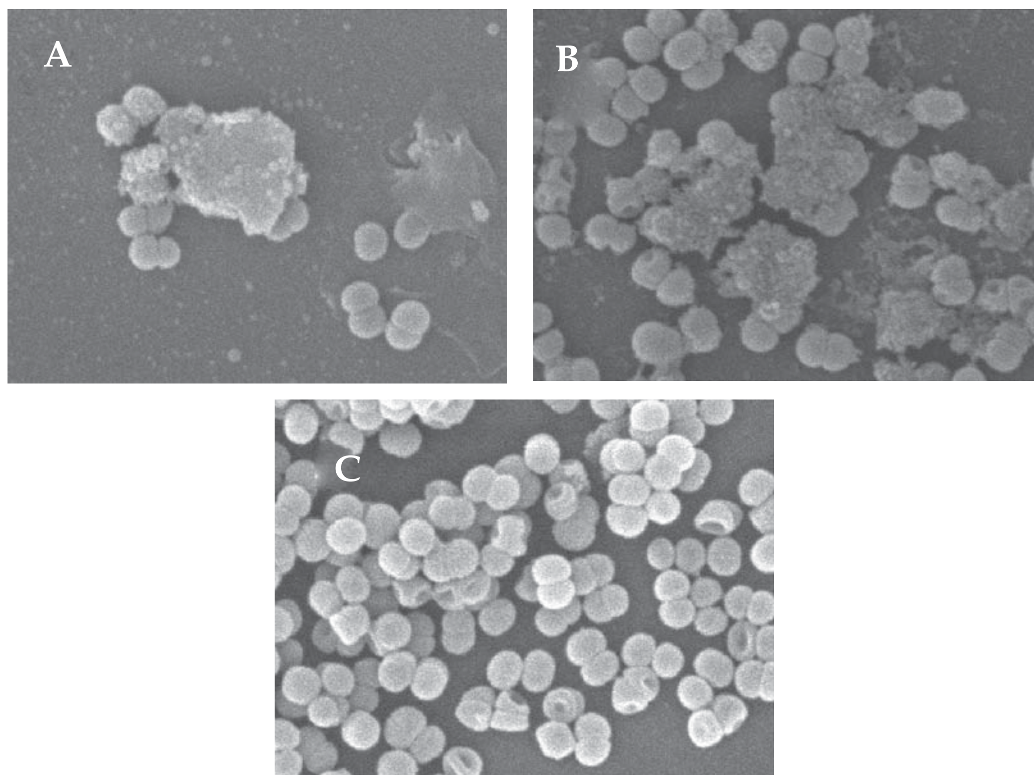

Figure 3. Scanning electron micrograph of Pediococcus acidilactici R01 (MAG X10,00, ACCV 20 kv, width 13.2μm). (A) adherence on porcine mucin, (B) adherence on broiler mucus and (C) cell cluster

revealed in stress condition. The absorbance of *P. acidilactici* in the bile salt treatment was lower than pepsin and PBS (non stress) ($P<0.05$). This result assumed that adherence absorbance was affected by cell wall stability to absorp crystal violet. As was mentioned by Pfeiler and Klaenhammer (2007), bile is a multifaceted stressor, which can disrupt cell membranes and cause damage to DNA and proteins. If bile salt bind in cell wall surface, integrity and permeabilize of cell will be disrupted. This assumed that there was no repair mechanism for cell wall recovery so there was dissability to absorp crystal violet. Ramiah *et al*. (2009) revealed that bile salt was effecting on genes expression of adhesion protein in *L. plantarum* 423 surface which showed that *mub* gene expression decreased by bile salt 3.0 g/l at pH 6.5 treatment.

Micrograph data of *P. acidilactici* adherence on mucin and mucus showed in Figure 3. *P. acidilactici* R01 cell was coccus or diplococcus (Figure 3C). *P. acidilactici* R01 adherence on broiler mucus (Figure 3B) was higher than porcine mucin (Figure 3A). This result was similar with percentage of adherence (TPC) and absorbance data.

In the upper intestinal tract, acidity in the stomach, and bile salts and pancreatic secretions in the small intestine are key antagonistic agents. Consequently, bile salts and acid resistance have become selection criteria for probiotic and vaccine delivery by LAB strains. Thus, adherence to intestinal cells is also considered a very valuable criterion for a new probiotic, and this ability is regarded as a prerequisite to exert certain beneficial effects, such as immunomodulation of the host or enteropathogenic exclusion (Vaughan *et al*., 2002). In the recent review, at least 20 genes are reported to be functionally important in the binding of *Lactobacillaceae* to the digestive tract. Turpin *et al*. (2012) performed a series of analyses of a collection of 162 LAB strains to assess their binding potential as part of the selection of new probiotic candidates. Several genes that assosiated with *P. acidilactici* adhesion were *ef-TU* gene (elongation factor TU), *eno* gene (enolase), *gap* gene (glyceraldehyde-3-phosphate dehydrogenase), *groEl* gene (heat shock protein 60) and *srtA* gene (sortase) as a housekeeping genes and *apf* gene (aggregation-promoting factors), *cnb* gene (collagen-binding protein), *fpba* gene (fibronectin-binding protein), *mub1* gene (mucin-binding protein) and *gen mub2* gene (mucin-binding protein) as a binding related gene. The adhesion assays of 30 selected LAB using mucus producing HT29-MTX cells and to non-mucus producing HT29 cells were evaluated. The *Pediococcus* genus (n = 9) showed higher binding ability than *Lactobacillus* (n = 20) with an average binding ability of 12.51% ±61.4% versus 4.8%± 61.6%, respectively. Like the HT29 model, *Pediococcus* tend to show higher binding ability to HT29-MTX cells than *Lactobacillus*, with an average binding capacity of 13.5% ±62.0% versus 10.3%±62.4%, respectively. *P. acidilactici* adhesion on mucus in this studies show adherence ability on intestinal mucosa when it was feed as probiotic.

It was concluded that three of LAB isolates from GIT of broiler have closed relation to *Pediococcus acidilactici* (99.9%) species. Three isolates of *P. acidilactici* have adherence ability on broiler mucus higher than that on porcine mucin. Adherence on mucus were affected by the addition of 3 g/l of gastric juice and 0.3% (b/v) of bile salt. Based on adherence studies, three isolates of *P. acidilactici* LAB were capable colonize of colonizing the host intestinal mucus in vitro.

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