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Characteristics of Lactic Acid Bacteria Isolated from Gastrointestinal Tract of Cemani Chicken and Their Potential Use as Probiotics

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ABSTRACT

The aims of this study were to screen and characterize lactic acid bacteria (LAB) isolated from gastrointestinal (GI) tract of Cemani chicken, one of Indonesian local chicken and to investigate their potential use as probiotics. LAB were isolated from GI tract using MRSA and GYPA media and incubated anaerobically. Selected LAB were determined their probiotic properties with several assays. Identification of selected LAB was based on 16S rDNA sequences, morphological and biochemical characteristics. Ninety five bacteria were isolated and characterized as lactic acid bacteria (Gram positive, catalase negative, non sporeforming and acid producing). Twenty four isolates of LAB demonstrated antimicrobial activity against *Escherichia coli* JCM 1649 and *Salmonella enteritidis* B2586, and three selected isolates, i.e. CCM011, CSP004, and CVM002 showed the highest inhibition activity. The isolates had characters of high cell surface hydrophobicity and inter-isolate coaggregation ability of LAB, high survival at low pH, high phytase and protease activity (but no amylase and lipase activity), weak coaggregation with pathogen and no resistance to the examined antibiotics. The isolates were identified based on sequence analysis of 16S rRNA gene as *Lactobacillus salivarius*, however, each isolate had different profiles of sugar fermentation. Therefore the three LAB isolates had potential application as probiotics for chicken.

Key words: Cemani chicken, gastrointestinal tract, lactic acid bacteria, probiotic

ABSTRAK

Tujuan penelitian ini adalah untuk menyeleksi dan mengarakterisasi bakteri asam laktat (BAL) yang diisolasi dari saluran pencernaan ayam Cemani, salah satu ayam asli Indonesia dan untuk mengetahui potensi penggunaannya sebagai probiotik. BAL diisolasi dari saluran pencernaan dengan menggunakan medium MRSA dan GYPA dan diinkubasi secara anaerobik. BAL terpilih kemudian ditentukan karakter probiotiknya melalui serangkaian percobaan. Identifikasi isolat BAL terpilih berdasarkan pada sekuen 16S rDNA, karakter morfologi dan biokimianya. Sembilan puluh lima isolat diisolasi dan dikarakterisasi sebagai bakteri asam laktat (Gram positif, katalase negatif, tidak membentuk endospora dan menghasilkan asam). Dua puluh empat isolat BAL memperlihatkan aktivitas antimikrob terhadap *E. coli* JCM 1649 dan *S. enteritidis* B2586, dan tiga isolat terpilih, yaitu CCM011, CSP004, dan CVM002 menunjukkan aktivitas penghambatan yang tertinggi. Isolat-isolat tersebut mempunyai karakter pelekatan terhadap permukaan sel yang tinggi, mempunyai kemampuan koagregasi antar BAL, daya tahan yang tinggi pada pH rendah, menunjukkan aktivitas enzim fitase dan protease yang tinggi (tetapi tidak mempunyai aktivitas amilase dan lipase), koagregasi yang lemah terhadap bakteri patogen dan tidak resisten terhadap antibiotik uji. Isolat-isolat tersebut diidentifikasi berdasarkan analisis sekuen gen 16S rRNA sebagai *Lactobacillus salivarius*, tetapi tiap isolat mempunyai profil fermentasi terhadap gula yang berbeda. Ketiga isolat BAL tersebut berpotensi sebagai probiotik pada ayam.

Kata kunci: ayam cemani, saluran pencernaan, bakteri asam laktat, probiotik

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INTRODUCTION

Increasing consumption of chicken and their products contributed to an increasing use of antibiotics in a poultry farm. Antibiotics that are used to improve chicken growth performance and to protect chicken from pathogenic microorganisms are known as antibiotic growth promoters (AGPs) (Gaggia *et al.,* 2010). However, application of AGPs in poultry can cause development of bacterial resistance to antibiotics and it can affect human health, due to the residues in chicken products. In European countries, application of AGPs in poultry feed is prohibited.

There are several potential alternative ways instead of using AGPs, one of them is using probiotics. Probiotics are live microorganisms which, when administered in adequate amount, confer a health benefit to the host (FAO/WHO, 2002). The aims of using probiotics in chickens are to prevent and combat gastrointestinal disorders based on competitive exclusion of potentially pathogenic bacteria, such as *Salmonella enteritidis* and *Escherichia coli,* to stimulate host immune response, and to secrete antimicrobial substances (Corcionivoschi *et al.,* 2010).

Many studies reported that LAB could be used as probiotics for chicken (Torshizi *et al.,* 2008; Sofyan *et al*., 2012). LAB probiotics showed beneficial effect by inhibiting growth of pathogen bacteria, such as *Escherichia coli* (Istiqomah *et al.,* 2013) and *Salmonella sp* (Nouri *et al.,* 2010). LAB have been used for fermentation of certain foods, so they are non-pathogenic bacteria and recognize as GRAS (Generally Regarded as Safe) status. These bacteria are also found normally in the gastrointestinal tract of a chicken.

Probiotics using indigenous LAB isolated from gastrointestinal tract of Cemani chicken are important as potential probiotics for Indonesian local chicken. Indonesia has thirty one local chickens with various genotypic and phenotypic characteristics (Nataamijaya, 2010). Cemani chicken is one of the Indonesian local chicken that has unique characteristics with black color on the whole body including nails, tongue, comb, beak, feet, eye-balls, legs, feathers, skin, muscles, bones, and internal organs. This chicken is recognized to have high disease resistance, and high adaptability to environmental conditions (Sulandari *et al.,* 2009; Nataamijaya, 2010). The chicken was sometimes utilized as traditional medicine (Sartika *et al*., 2011). Therefore, this study was conducted to screen and characterize LAB isolates from gastrointestinal (GI) tract of Cemani chicken for potential application as probiotics in chicken.

MATERIALS AND METHODS

Bacterial Cultures and Growth Condition

Two strains of intestinal pathogenic bacteria as indicators of bacterial strains (*Escherichia coli* JCM 1649 and *Salmonella enteritidis* B2586) were used in this experiment. These indicator bacterial cultures were grown in brain heart infusion broth (Becton Dickinson) at 37 °C for 24 h. All strains were subcultured twice prior to experiments.

Isolation of LAB

Three healthy Cemani chickens (9 to 12 months old; 1.0 to 1.5 kg body weight) were obtained from Mranggen district, Central Java, Indonesia. Those chickens received no antibiotic-feed containing rice bran and leftover rice before experiments. The chickens lumen contents including crop, ventriculus, ileum, and cecum were collected aseptically. Lumen samples were serially diluted in 0.85% (w/v) sterile NaCl solution and plated onto MRS (HiMedia Laboratories, India) agar and glucose yeast extract peptone (GYP) agar supplemented with 0.5% (w/v) $CaCO_3$ to distinguish acid producing bacteria. The plates were incubated in anaerobic jars (Merck, Germany) for 48 h at 37 °C with Anaerocult A (Merck, Germany). After incubation, the bacterial colonies were enumerated and purified on MRS agar up to three times to obtain pure LAB isolates. All isolates were examined for colony morphology and cell shape and catalase assay. For long-term storage, the bacterial isolates were kept at -80 °C in 20% (v/v) glycerol until further use (Guerin-Danan *et al.,* 1999).

Antimicrobial Activity Assay

For detection of antimicrobial activity, the well diffusion assay described by Taheri *et al.* (2009) was performed. The cultures were grown anaerobically overnight in MRS broth at 37 °C to achieve cell concentration of 10^8 CFU/mL. Bacterial culture, cell-free supernatant, and neutralized cell-free supernatant (pH 7.0, added with 2M NaOH) of different LAB isolates were determined for antimicrobial activity against indicator bacterial strains. Inhibitory zones around the wells were screened for each strain after overnight incubation at 37 °C. The experiment was carried out three times and data were displayed as the mean of radius of inhibitory zone.

pH and Bile Salts Tolerance Assay

Overnight selected LAB cultures were centrifuged at 7,500 × *g* for 5 min and washed twice with sterile phosphate buffer (PBS, pH 7.0). The washed cell density were adjusted to OD_{600}= 0.5-0.7 using spectrophotometer. For pH tolerance assay, 1 mL of cell suspensions were resuspended with 5 mL PBS at pH 2.0, 4.0, and 6.5, and incubated at 37 °C for 90 min. Meanwhile, for bile salts tolerance assay, 1 mL of the washed cell suspension was resuspended in the sterile PBS containing 0.05%, 0.08%, 0.1%, and 0.3% bile salt (Sigma) and incubated at 37 °C for 5 h. The bacterial survival under different pH conditions and concentrations of bile salts were determined by plated 0.1 mL suspension onto MRS agar and incubated in anaerobic jars for 48 h at 37 °C (Taheri *et al*., 2009).

Cell Surface Hydrophobicity Test

Assay for microbial surface hydrophobicity was performed based on the adherence to the non polar solvent (Taheri *et al.,* 2009). Cell suspensions were prepared as above and then 3 mL of washed cells suspensions were added with 1 mL of toluene and mixed by stirring on a vortex for 2 min. The optical density of mixture was measured at 600 nm using spectrophotometer. Hydrophobicity was calculated as follows:

Hydrophobicity (%)= [(OD_{600} before mixing − OD_{600} after mixing)/ OD_{600} before mixing] × 100

Co-Aggregation Test

Equal volume (2 mL) of suspension of each indicator bacteria and the LAB isolates were placed together in a test tube and mixed by vortexing. The OD_{600} of the bacterial mixture was measured after incubation at 37 °C for 5 h (Taheri *et al.,* 2009). The percentage of co-aggregation was calculated by using the equation as follows:

$$\text{Co-aggregation (\%)} = \{[(A_x + A_y)/2 - A(_x+_y)]/[(A_x + A_y)/2]\} \times 100$$

which representing absorbance value (A) at each of the two strains examined in the control tube (x and y), and at their mixture (x + y).

Enzymatic Activities Assay

The selected LAB isolates were assayed for the presence of dietary enzymes, i.e. amylase, protease, lipase, and phytase (Taheri *et al.,* 2009). To detect the amylase, lipase, and phytase activities, the isolates were subcultured in MRS broth and then spot-inoculated onto relevant agar-based media (starch agar, skim milk agar, phytic acid enriched agar and lipid hydrolysis agar). After anaerobic incubation for 48 h at 37 °C, a clear zone surrounding each colony was measured. Lugol's solution was added over the plate surface for clear zone detection of amylase activity.

Antibiotic Sensitivity Test

Antibiotic susceptibility of selected LAB isolates was determined by using Kirby-Bauer disc method (Taheri *et al.,* 2009). As much as 0.1 mL of LAB cell suspensions were spread over the entire surface of the plates containing MRS agar. Subsequently, paper discs containing antibiotics of amoxicillin 10 μg, ampicillin 10 μg, chloramphenicol 30 μg, cefadroxil 30 μg, doxycycline 30 μg, erythromycin 15 μg, lincomycin 15 μg, rifampin 5 μg, spiramycin 30 μg, and tetracycline 30 μg were placed on the plates and incubated anaerobically at 37 °C for 24 h. Diameter of a clear zone was measured to determine antibiotic sensitivity of the isolates.

Identification of LAB Isolate

Genomic DNAs from the selected LAB isolates were extracted by using Xprep Stool DNA Mini Kit (PhileKorea Technology, INC, Korea) according to the manufacturer's instructions. DNA pellet was then resuspended in 50 μL TE buffer and stored at -20 °C. A PCR mixture was prepared from each sample using a TaKaRa PCR Thermal Cycler with forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GTTACGACTTCACCCTCCT-3') (Zhang, *et al*., 2007). PCR reaction mixture consisted of 25 μL GoTaq GreenMaster Mix (Promega, USA), 2 μL of each primer (10 pmol), and destilated water in a final volume of 50 μL and 100 ng DNA template in the final concentration. PCR condition was set up with an initial denaturation at 94 °C for 1 min 30 s, followed by 25 cycles of denaturation temperature at 95 °C for 30 s, annealing temperature of 50 °C for 30 s, and extension temperature at 72 °C for 1 min 30 s and then final elongation at a temperature of 72 °C for 5 min. PCR products were confirmed by electrophoresis by using 1% agarose gel in 1x TAE buffer and visualized with ethidium bromide staining. Purification of PCR products and sequencing were conducted by a company providing sequences services. The DNA sequences were compared with available sequences in GenBank using the BLASTN tools through the National Center for Biotechnology Information (NCBI). Sequence homology of more than 97% was regarded as belonging to the same species (Tannock, 1999). The sequence was aligned with the clustal X program, then the alignment was manually edited to the construction of phylogenetic tree. The phylogenetic tree was constructed by neigbour-joining method in MEGA program version 4. The values of branches of phylogenetic tree were determined using booststrap analysis based on 1000 resamplings (Felsenstein, 1985).

Biochemical Characterization of LAB

Biochemical characterization of the selected LAB was determined by using API® 50 CHL for performance of carbohydrate metabolism tests (bioMérieux, Inc, Durham) according to the manufacturer's instructions. Fermentation profiles of the isolates were determined by using the API web software (Pelinescu *et al.,* 2009).

Statistical Analysis

All quantitative data were subjected to ANOVA analysis by using IBM SPSS statistics 21.0. A test of least significant differences was used to separate means; differences between means were considered statistically significant $P<0.05$.

RESULTS AND DISCUSSION

Population of Culturable LAB in Chicken Gastrointestinal Tracts

By culturing method, the content of gastrointestinal tract of chicken had LAB number 7.12±0.63 up to 9.07±0.17 log CFU g^{-1} and the highest number was found in crop region (Table 1), followed by cecum, ileum and ventriculus, respectively. The pH of all regions were below 7. The number of LAB was not influenced by the pH value. There were many other factors affecting such

Table 1. Total culturable lactic acid bacteria incubated in MRS agar for 48 h and pH from gastrointestinal tract of Indonesian Cemani chicken

Sample	log cfu/g ±SD (n=3)	pH ±SD (n=3)
Crop	9.07±0.17	5.05±1.48
Ventriculus	7.12±0.63	4.50±0.71
Ileum	7.25±0.22	6.20±0.14
Caecum	8.57±0.64	6.55±0.64

as diet, age, presence of barrier in the intestine and different physiological functions within the organ system (Dumonceaux *et al*., 2006).

Ninety-five LAB colonies were randomly picked based on clear zone and colony morphology on MRSA or GYPA medium supplemented with $CaCO_3$. All isolates had specific characteristics of lactic acid bacteria, such as positive Gram stain, catalase negative reaction and no endospore forming, and mostly bacterial cell had rod-shaped (88 isolates, 92.6%) and 7 isolates (7.4%) had round shaped.

Assessment of Probiotic Characteristics

For antimicrobial activity assays, *Escherichia coli* and *Salmonella enteritidis* were used, because they were common potentially intestinal pathogenic bacteria causing problem of gastro intestinal tract in chicken. Antimicrobial activity of 24 LAB isolates against *E. coli* and *S. enteritidis* was shown in Table 2. All bacterial cultures had antimicrobial activity against *E. coli* and *S. enteritidis*, however there were only 10 LAB isolates (41.7%) and 7 LAB isolates (29.2%) that their cell-free neutralized supernatant exhibited antibacterial activity against *E. coli* and *S. enteritidis*, respectively.

Three LAB isolates, i.e. CSP004, CCM011 and CVM002 that had the highest inhibition zone of their cell-free neutralized supernatant were selected for further assays. The neutralized supernatant of these isolates were able to inhibit the growth of the Gram negative pathogen tested in this study. The antimicrobial activity of neutralized cell-free supernatants of CSP004 isolate to *S. enteritidis* was higher than that of non neutralized supernatant. Antimicrobial activity of neutralized supernatant of CCM011 isolate was not significantly different from that of non neutralized supernatant (Table 3). According to Nouri *et al.* (2010) and Heravi *et al.* (2011) study, *L. salivarius* had the highest inhibitory activity against *S. enteritidis* and *E.coli*.

Table 2. Number of lactic acid bacteria having antimicrobial activity against *E. coli* and *S. enteritidis* using diffusion agar test

Bacterial indicator	Number of lactic acid bacteria having antimicrobial activity (n (%)) from:		
	Bacterial cell	Supernatant	Neutralized supernatant
E. coli	24(100)	20(83.3)	10(41.7)
S. enteritidis	24(100)	21(87.5)	7(29.2)

LAB isolates showed ability to inhibit pathogen growth possibly through cell competitiveness, decreasing pH environment and producing organic acids and bacteriocin. Organic acids produced by LAB such as acetic and lactic acid inhibited the growth of many bacteria, especially pathogenic gram-negative types, like *E. coli* and *S. enterica*, due to its ability to undissociate to penetrate the cytoplasmic membrane, resulting in reduction of intracellular pH and disruption of the transmembrane proton motive force (Alakomi *et al.*, 2000).

Survival assay under acid condition showed that all isolates survived at pH 4 and pH 2, however, there were decreasing viability at pH 2 comparing with control (pH 6.5). The CVM002 isolate had the highest survival ability at low pH (Table 4). Most LAB grow more slowly at low pH, probably caused by acid that can damage and loss of cell viability. However, LAB had ability to regulate their cytoplasmic or intracellular pH at near neutral during growth or storage at low extracellular pH (Konings *et al*., 1997).

Survival assay under bile salts condition showed that all isolates did not survive under 0.3% bile salts. However the isolates could survive under 0.1% bile salts. The survival of the isolates under 0.1% bile salts was decreased after 5 hours incubation at 0.1% bile salts suspension (Table 4). Iniguez-Palomares *et al.* (2007) reported that LAB isolates had no resistance to CPBS (conjugated porcine bile salts) at concentration more than 0.1% bile salt. Bile released in the small intestine, could damage bacteria because its destroying effect to cell membrane. Some bacteria, like lactic acid bacteria had bile salt hydrolase enzyme (BSH), so that had ability hydrolyzing bile salt and reducing their solubility. To enhance survival of bacterial passage through upper gastrointestinal tract could be conducted by encapsulating bacteria with alginate and skim.

One benefit of probiotics is production of extracellular enzymes supporting the host to digest their nutrients.

Table 3. Inhibitory activity of cell-free supernatants of selected lactic acid bacteria (LAB) isolates against *E. coli* and *S. enteritidis*

LAB isolate	Source of isolate	Diameter of inhibitory zone (mm) against			
		E. coli		*S. enteritidis*	
		Untreated	Neutralized	Untreated	Neutralized
CCM011	Crop	7.3±0.6^{a}	8.3±0.6^{a}	7.3±1.2^{a}	6.3±0.6^{a}
CSP004	Cecum	9.3±0.6^{a}	5.3±1.2^{b}	8.3±0.6^{a}	16.0±2.0^{b}
CVM002	Gizzard	8.3±0.6^{a}	3.3±1.2^{b}	18.7±0.6^{a}	8.3±0.6^{b}

Note: Means in the same row with different superscripts differ significantly (P<0.05)

Table 4. Survival of selected LAB Isolates on inhibitory substances conditions

Inhibitory condition of	Viable LAB isolate (Log10 CFU/mL)		
	CCM011	CSP004	CVM002
pH			
pH 2.0	5.71±0.01[a]	4.68±0.03[a]	5.85±0.01[a]
pH 4.0	6.11±0.10[b]	5.79±0.01[b]	6.16±0.02[b]
pH 6.5	6.73±0.02[c]	6.25±0.07[c]	6.2±0.040[b]
Bile salts			
0%	6.73±0.11[a]	6.60±0.31[a]	6.87±0.03[a]
0.05%	3.68±0.06[b]	4.04±0.62[b]	4.04±0.37[b]
0.08%	1.45±0.04[c]	2.39±0.30[c]	1.57±0.04[c]
0.10%	0.74±0.06[d]	0.59±0.16[d]	0.45±0.21[d]
0.30%	ND	ND	ND

Note: Means in the same column with different superscripts differ significantly (P<0.05); ND = Not detected.

Chicken feed especially grains contains many substrates like starch, protein and fats, and other components such as mannans, cellulose, lignin and phytic acid, which are difficult to be digested by monogastric animals like chicken. API 50 CHL assay confirmed that all isolates did not grow on starch agar (Table 5). The isolates also did not have lipase enzyme. However, the isolates could produce protease and phytase (Table 6). Musikasang *et al.* (2012) found that LAB isolates showed the proteinase activity, but neither starch nor lipid digestions were detected.

Selected LAB isolates can produce phytase that hydrolyzes phytic acid to myo-inositol and phosphoric acid. This enzyme is needed for chicken, because chicken do not capable to metabolize phytate phosphorus due to the lack of digestive enzymes hydrolyzing the substrate. In addition, phytic acid that is the storage form of phosphorus in cereal, oil and legume, act as an antinutritional agents forming complexes with proteins and various metal ions, thereby decreasing the dietary bioavailability of these nutrients (Raghavedra & Halami, 2009). *Bifidobacterium dentium*, *L. reuteri* L-M15 and *L. salivarius* L-ID15 isolated from gastrointestinal tract of chickens showed the highest phytate degrading activity (Palacios *et al.*, 2007).

Microbial adhesion to hydrocarbon of the LAB isolates indicated the hydrophobicity of cell surface properties. The LAB isolates exhibited high hydrophobicity that was determined by microbial adhesion to toluene in the range of 77%-82%. The strong hydrophobicity ability was shown by CVM002 isolate. As much as 82% cells of the isolate could adhere to hydrocarbon toluen. It was suggested that CVM002 isolate showed high adhesion ability to mucus and epithelial cells in the intestine. *L.*

Table 5. Sugar fermentation pattern of selected lactic acid bacteria (LAB) isolates using API 50 CHL Kit

No	Substrate	Reaction of isolates			No	Substrate	Reaction of isolates		
		CCM011	CSP004	CVM002			CCM011	CSP004	CVM002
0	Control	-	-	-	25	Esculin ferric citrat	-	-	-
1	Glycerol	-	-	-	26	Salicin	-	-	-
2	Erythritol	-	-	-	27	D-cellobiose	-	-	-
3	D-arabinose	-	-	-	28	D-maltose	+	+	+
4	L-arabinose	-	-	-	29	D-lactose (bovine origin)	-	+	+
5	D-ribose	-	-	-	30	D-melibiose	-	+	+
6	D-xylose	-	-	-	31	Saccharose (sucrose)	+	+	+
7	L-xylose				32	D-trehalose	+	+	+
8	D-adonitol	-	-	-	33	Inulin	-	-	-
9	Methyl -β-d xylopyranoside	-	-	-	34	D-melezitose	-	-	-
10	D-galaktose	-	+	+	35	D-raffinose	-	+	+
11	D-glukose	+	+	+	36	Amidon (starch)	-	-	-
12	D-fructose	+	+	+	37	Glycogen	-	-	-
13	D-manose	+	+	+	38	Xylitol	-	-	+
14	L-sorbose	-	-	-	39	Gentiobiose	-	-	-
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 -α-d mannopyranoside	-	-	-	45	D-arabitol	-	-	+
21	Methyl -α-d glucopyranoside	-	-	-	46	L-arabitol	-	-	-
22	N-acetyl glucosamine	+	+	+	47	Potasium gluconat	-	-	-
23	Amygdalyn	-	-	-	48	Potasium 2-keto- gluconat	-	-	-
24	Arbutin	-	-	-	49	Potasium 5-keto-gluconat	-	-	-

Note : + : positive reaction, - : negative reaction

Table 6. Enzymatic activities of selected lactic acid bacteria (LAB) isolates

Enzyme	LAB isolate		
	CCM011	CSP004	CVM002
Amylase	-	-	-
Protease	++	++	+++
Phytase	+++	+++	++
Lipase	-	-	-

Note: +++ = clear zone ≥ 3 mm; ++ = clear zone ≤ 3 mm; - = not detected.

salivarius has capability to produce bacteriosin and exopolysaccaharide (EPS) that helps to adhere to intestinal mucus (Raftis *et al*., 2011). The ability of bacteria to produce biofilm and adhesion are able to increase the gut residence time of commensal bacteria and promote pathogen exclusion.

Inter-isolate coaggregation ability of LAB was needed to evaluate the effectiveness of probiotics adhesion in the intestine. Meanwhile, coaggregation between LAB isolates and bacterial pathogens to avoid adhesion of pathogen on host intestinal cells. Inter-isolate coaggregation of LAB was from 26.64% to 81.56%, and the highest coaggregation was found on CSP004 and CVM002 isolates (Figure 1). Coaggregation ability between pathogenic bacteria, *E. coli* and CCM011 isolates showed weak coaggregation, however there was no coaggregation of *E. coli* with CSP004 and CVM002 isolates (Figure 2). In addition, coaggregation of LAB isolates with *S. enteritidis* was 4%-20% (Figure 2).

Antibiotic resistance assay of the LAB isolates showed that there was no resistant to examined antibiotics, but each isolate at least showed intermediate status to one of antibiotics. Isolates of CCM011, CSP004, and CVM002 were detected to be intermediate resistant to lincomycin, tetracyclin and erythromycin, respectively.

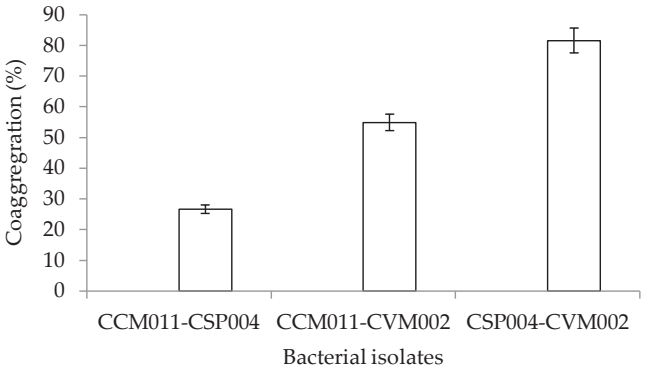

Figure 1. Inter-isolate coaggregation ability of the LAB isolates

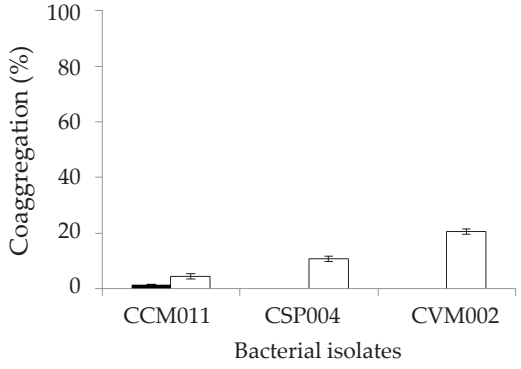

Figure 2. Coaggregation ability of LAB isolates (n=3) and indicator bacteria (*E. coli* (■) and *S. enteritidis* (□)).

Identification of Selected LAB Isolates

Genotypically, the three selected LAB isolates i.e. CCM011, CSP004 and CVM002 were identified based on sequence analysis of 16S rRNA gene and showed that DNA fragments of amplification products were about 1500 bp (Figure 3). The analysis of 16S rRNA gene of isolates has been successfully sequenced, aligned and compared. The isolates were identified as *Lactobacillus salivarius* with sequences similarity 99% (GenBank accession number, KC020621.1 and AB612967.1). Phylogenetic tree based on 16S rRNA gene sequences analysis showed that the isolates were clustered in one group (Figure 4). *L. salivarius* was known as the indigeneous strain of gastrointestinal tract and has probiotic properties (Nouri *et al*., 2010, Raftis *et al*., 2011).

Phenotypic identification based on carbohydrate fermentation assay by the API 50 CH system showed that the three selected isolates were closely related to *L. salivarius* with a 99.9% similarity. This result confirmed genotypic identification. However, there were traits differences in consuming various carbon sources among the LAB isolates. CSP004 and CVM002 isolates showed relatively similar carbon fermentation profiles, however they had different carbon fermentation profiles from CCM011 isolate. CCM011 isolate was lack ability to use d-galactose, d-lactose, d-mellibiose, and d-raffinose as carbon sources. *L. salivarius* CCM011 lacked ability to use galactose and lactose. It was possible that CCM011 did not have an intracellular transport system to take up the lactose and cannot produce the enzyme β-galactosidase/ lactase.

Meanwhile, there were traits differences between CSP004 and CVM002 isolates. CVM002 could use rhamnose, xilitol and arabitol for carbon sources, however CSP004 could use inositol for carbon source (Table 6).

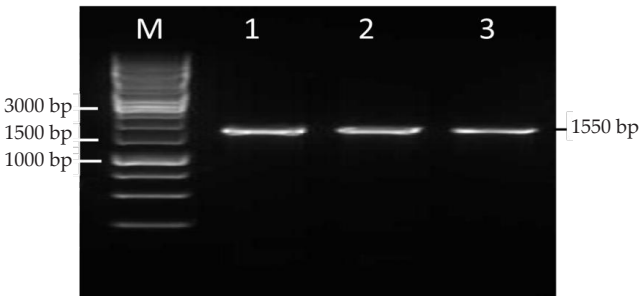

Figure 3. Agarose gel (1%) analysis of PCR amplification products of 16S rRNA gene of lactic acid bacteria isolates. M= Molecular marker (1 kb ladder), lane 1= CSP004; lane 2= CCM011 and lane 3= CVM002 isolate.

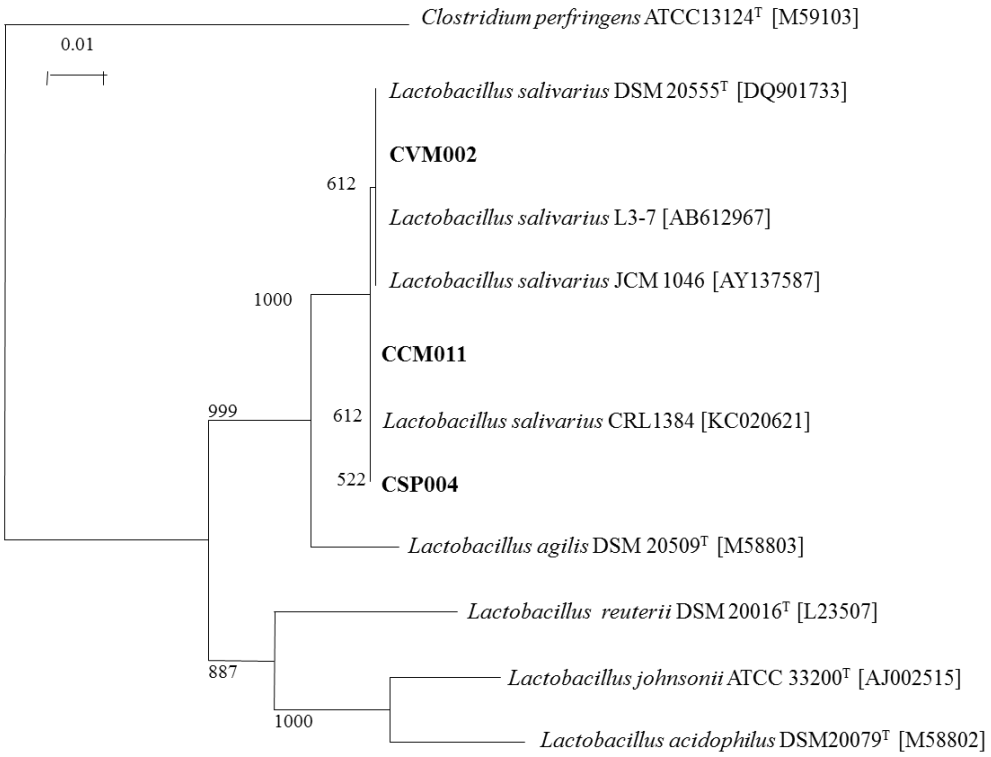

Figure 4. Phylogenetic tree derived from fulllength of 16S rDNA sequences analysis of LAB isolates showing the position of *Lactobacillus salivarius* CCM011, CSP004 and CVM002 among selected lactobacilli. The tree was generated by the neigbour-joining method and *Clostridium perfringens* was used as the out group. Bootstrap value based on 1000 replications are given at nodes. Bar 0.01 substitutions per nucleotide position. Accession numbers of sequences obtained from NCBI database.

CONCLUSION

The three selected LAB isolates (CCM011, CSP004, and CVM002 demonstrated antimicrobial activity against *E. coli* and *S. enteritidis*, resistance to low pH, strong hydrophobicity to hydrocarbon and coaggregation of inter-LAB isolates, but weak coaggregation with the bacterial pathogens and susceptible to examined antibiotics. The isolates were identified as *Lactobacillus salivarius* with 99% similarity. However, there were different characteristics of sugar fermentation profiles among the isolates. Considering the characteristics above, the isolates may be used as probiotics in poultry farm. *In vivo* assay will be required for evaluation of the isolates as a probiotic supplement in chicken.

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