

Screening of Lactic Acid Bacteria from Rumen Liquor and King Grass Silage as well as Their Antibacterial Activities

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ABSTRACT

Probiotic is a live microbial culture which has positive effect on animal by improving the natural balance of microflora in the digestive tract. This experiment aimed to screen and identify indigenous lactic acid bacteria (LAB) from rumen liquor and king grass (*Pennisetum hybrid*) silage as a probiotic candidate and to evaluate their resistance in low pH, and inhibitory activities against pathogenic bacteria. The LAB isolate was characterized by a clear zone formed on MRSA medium + CaCO₃ 0.2% (w/v) and further identified by morphological and biochemical assays. The selected isolates were evaluated for their viability in low pH, pathogenic bacterial inhibition, and lactic acid production. The experimental arrangement was a factorial block design (4 x 2) consisted of four isolates and two levels of pH value (pH 2 and 3), each treatment in 3 equal replicates. The result showed that four isolates (two isolates from the rumen liquor of fistulated cattle and two isolates from silage) were identified as lactic acid bacteria. The four isolates showed inhibition activity against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and performed viability at low pH during 2 h treatment. The highest lactic acid production was obtained from isolates Sil.3 (21.42%) and followed by CR2 (19.88%), CR1 (15.40%) and Sil.9 (15.08%). Biochemical identification by standard of analytical profile index (API) 50 CHL kit showed that the selected isolates CR1 was *Lactobacillus paracasei* ssp. *paracasei* 3 (91.5%), *L. paracasei* ssp. *paracasei* 3 (76.5%), Sil.3 was *Lactobacillus brevis* (95.1%), and Sil.9 was *Lactobacillus collinoides* (92.5%). In conclusion, probiotic candidates isolated from rumen liquor are confirmed as *L. paracasei* ssp. *paracasei* (CR1 and CR2), while two other isolates from king grass silage are identified as *L. brevis* (Sil.3) and *L. collinoides* (Sil.9). *L. brevis* (Sil.3) and *L. paracasei* ssp. *paracasei* (CR1) has higher inhibition against pathogenic bacteria (*E. coli*, *S. aureus*, and *B. subtilis*) than *L. paracasei* ssp. *paracasei* (CR2) and *L. collinoides* (Sil.9).

Key words: isolation, probiotic, pathogenic bacteria, rumen liquor, silage

ABSTRAK

Probiotik merupakan kultur mikroba hidup yang dapat memberikan efek positif terhadap performa ternak dengan cara memperbaiki keseimbangan mikroorganisme alami di dalam saluran pencernaan. Penelitian ini bertujuan untuk mengisolasi, menyeleksi dan mengidentifikasi bakteri asam laktat (BAL) indigenos dari cairan rumen sapi dan silase rumput raja (*Pennisetum hybrid*) sebagai kandidat probiotik dengan mengetahui ketahanan beberapa isolat pada pH rendah dan aktivitas penghambatannya terhadap bakteri patogen. Isolat BAL dikarakterisasi daya hambatnya dengan metode cawan sebar pada media MRSA yang mengandung CaCO₃ 0,2%, dan identifikasi dilakukan berdasarkan morfologi dan uji biokimia. Seleksi isolat dilakukan dengan mengukur viabilitas pada pH rendah, kemampuan menghambat bakteri patogen dan produksi asam laktat yang disusun dalam rancangan acak kelompok pola faktorial (4 x 2) yang terdiri atas empat jenis isolat dan 2 kondisi pH (pH 2 dan 3), setiap perlakuan terdiri atas 3 ulangan. Hasil penelitian mendapatkan empat isolat BAL (dua isolat berasal cairan rumen sapi dan dua isolat dari silase). Keempat isolat menunjukkan aktivitas penghambatan terhadap pertumbuhan bakteri patogen *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* dan memiliki ketahanan hidup pada pH rendah (pH 2 dan 3) selama 2 jam. Produksi asam laktat tertinggi diperoleh dari isolat Sil.9 (21,42%) dan CR2 (19,88%) diikuti oleh CR1 (15,40%) dan Sil.9 (15,08%). Hasil identifikasi

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biokimia menggunakan standar *analytical profile index* (API) 50 CHL Kit menunjukkan bahwa isolat CR1 adalah *Lactobacillus paracasei* ssp. *paracasei* (91,5%), CR2 adalah *L. paracasei* ssp. *paracasei* (76,5%), Sil.3. adalah *Lactobacillus brevis* (95,1%) dan Sil.9 adalah *Lactobacillus collinoides* (92,5%). Kesimpulan dari penelitian ini, kandidat probiotik yang diisolasi dari cairan rumen adalah *L. paracasei* ssp. *paracasei* (CR1 dan CR2), sedangkan dua isolat lainnya dari silase rumput raja adalah *L. brevis* (Sil.3) dan *L. collinoides* (Sil.9). *L. brevis* (Sil.3) dan *L. paracasei* ssp. *paracasei* (CR1) memiliki penghambatan terhadap bakteri patogen (*E. coli*, *S. aureus*, and *B. subtilis*) yang lebih tinggi dibandingkan *L. paracasei* ssp. *paracasei* (CR2) dan *L. collinoides* (Sil.9).

Kata kunci: isolasi, bakteri patogen, cairan rumen, probiotik, silase

INTRODUCTION

Since antibiotic had been banned for promoting animal growth in 2006, it implied an alternative substance to replace antibiotic by using organic additive based on natural compound, microbial culture or their combination. Due to consumer concerns to improve health and safety of animal products including those resulting from organic farming (European Commission, 2012). In fact, supply of animal product (e.g. milk production) is only covering 30% national demands and also the adequacy of meat supply needs to be increased by improvement of beef cattle productivity. Indonesian Ministry of Agriculture reported that milk production in 2010 was 0.90 million tones which only increased 9.94% from 2009 (Ditjen PKH, 2012) and also meat was imported to fulfill the national demand.

Beside the nutritional problem, ruminant disease caused by pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* implied on low production and immune-suppression of animals. *S. aureus* is known as a Gram positive bacteria, opportunistic pathogen and a major concern for both animal and human health (Charlier *et al.*, 2009), and it has been found associated with certain gastrointestinal disease that is responsible for mastitis in dairy herds (Bouchard *et al.*, 2013). *E. coli* infection caused disruptions of intercellular tight junctions, leading to clinical sequelae including acute diarrhea, hemorrhagic colitis, and the hemolytic-uremic syndrome (Johnson-Henry *et al.*, 2008). *B. subtilis* has also been reported associated with bovine mastitis although a limited number of cases (USEPA, 2012).

Administration of direct feed additive based microbes is potential to improve feed digestibility. Probiotic contains life microbial and favorable for improving the digestive tract function especially in digesting forage. Furthermore, probiotic is expected to modulate the immune system and inhibit pathogenic bacteria. Probiotics or direct feed microbes (DFM) are dietary supplements that inhibit gastrointestinal infection and provide optimally regulated microbial environments in the digestive tract (Seo *et al.*, 2010). Administration of probiotics in livestock in the period of growth appears to be a real impact. Lactic acid bacteria as probiotic, are able to produce lactic acid, reuterin, diacetyl, bacteriocins and other metabolic functions as an antimicrobial (Rattanachaiakunsopon & Phumkhachorn, 2010), enhance fiber and starch digestion (Fraga *et al.*, 2013) and improve animal health (Bayatkouhsar *et al.*, 2013).

Lactic acid bacteria (LAB) ferments sugars or carbohydrates to produce large amounts of lactic acid. Lactic acid bacteria are naturally found in many different habitats such as fermented foods, fruits, grains, animal digestive tract or silage. In the digestive tract of cattle, lactic acid bacteria were found in the rumen (Bureenok *et al.*, 2011; Cobos *et al.*, 2011). Lactic acid bacteria in forage feed live as epiphytic bacteria in limited colony.

In order to optimize probiotic functions specific for host animal, administration of lactic acid isolated from indigenous sources will have promise more adaptable probiotic in the ruminant digestive tract. However, scientific publication related to exploration of indigenous microorganisms used as probiotics candidate from the host animal (rumen liquor) or animal feed (forages) is still limited. Therefore it has required to isolate and characterize lactic acid bacteria from cattle rumen and king grass (*Pennisetum hybrid*) silage and determine their antibacterial activity against pathogenic bacteria.

MATERIALS AND METHODS

Sample Preparation

Rumen liquor and king grass (*P. hybrid*) silage were prepared as inoculant sources. Rumen liquor was obtained from the fistulated cattle (Ongole Crossbred) which daily consumed diet consisted of forage and concentrate (70:30 dry matter basis). Silage was made from the chopped king grass which packed in a plastic bag and incubated during 21 d at room temperature (25-30 °C) (Sofyan *et al.*, 2011).

Isolation and Screening of Lactic Acid Bacteria

Collected fresh rumen liquor (100 mL) was strained through cheesecloth to obtain supernatant consisting microbial cells (rumen supernatant). Collected fresh silage (100 g) was added by 500 mL of distilled water and then mashed in a blender, then strained through cheesecloth to obtain supernatant. An amount of 50 ml of supernatant was added by 1 g of d-glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$) (w/v) to stimulate the bacterial growth. Supernatant from the rumen and silage were grown on the selective medium of MRSA (*de Man Rogosa Sharpe* Agar) supplemented with $CaCO_3$ 0.2% (w/v) using a spread plate method and anaerobically for 24 h at 37 °C in an anaerocult chamber with an atmosphere of CO_2 .

Primary screening of LAB was performed by evaluating the clear zones on selective medium. LAB colony with the largest clear zone diameter was chosen as the selected isolates.

Characterization of Lactic Acid Bacteria

Selected isolates from rumen liquor and king grass silage were characterized by catalase test, Gram staining, morphology, motility test, and gas production test. Motility test isolates was observed on SYP medium (sucrose-yeast extract-peptone), incubated for 24-48 h at 37 °C. Gas production test performed by growing cultures in 5 mL of liquid SYP for 2-3 d at 37 °C with a Durham tube which placed in reverse to capture the gas production (Damayanti *et al.*, 2012).

Viability in Low pH

The viability of lactic acid bacteria was evaluated by cultivating the isolate on acidity medium (pH 2 and pH 3). Evaluation of viability was arranged a factorial block design (4 × 2) consisted of the screened isolates (four isolates) and pH condition (pH 2 and 3), each treatment in 3 equal replications. Isolates were grown in MRS broth at 37 °C for 18 h then centrifuged using a refrigerated centrifuge 'Centrifuger® BL-II series' at 4137 × g for 5 min at 4 °C to obtain the biomass (pelleted). Pelleted were washed twice with sterile phosphate buffered saline (PBS, 0.1 M phosphate buffer with 0.8% NaCl, pH 7.0) referred to Bakari *et al.* (2011).

The strain was diluted 1/100 in sterile PBS adjusted to pH 2 and 3 using 0.1 N HCl. Incubation times were 0 and 1 h, then continued 2 h incubation in pH 3 conditions. Serial decimal dilutions in sterile PBS were prepared, and aliquots (0.1 mL) from the dilutions was used for point inoculation on the surface of MRS agar plates to determine the number of surviving cells (Rehaieem *et al.*, 2014).

The number of colonies were converted into log cfu/ml for the calculation of viability percentage according to the following equation; $[(\text{Log cfu Nt}) / (\text{Log cfu N0})] \times 100\%$, where Nt represented total colonies at 1, 2 h and N0 represented total colonies at 0 h (Bao *et al.*, 2010).

The Growth Curve of Lactic Acid Bacteria and Lactic Acid Production

Prior to cultivation LAB isolate into the medium, each of the four isolate was pre-cultured in MRSB, then incubated at 37 °C for 24 h (culture stock). An amount of 1% (v/v) culture stock was inoculated into MRSB using a Hungate tube then incubated at 37 °C for 48 h for analyzing growth kinetics. The control tube contained MRSB without the inoculation of LAB cultures. Bacterial growth was evaluated at 0, 3, 6, 9, 12, 18, 24, 30, 36, and 48 h after incubation to obtain the optical density (OD) using a spectrophotometer (Dynamica® Halo RB10) at $\lambda_{625 \text{ nm}}$. Viable cell was confirmed by spread plate count method to determine the number of colony at 0, 24, and 48 h after incubation. Total lactic acid and pH were measured at last incubation time (48 h). Measurement

of lactic acid production was conducted by method referred to AOAC (2005) using the equation as followed; Where:

Vts : Volume of titrant sample (mL)

Vto : Volume of titrant blank (mL)

N : Normality of titrant (0.1 N NaOH)

Mr : Molecular weight of lactic acids (90.0 g/mol)

Df : Dilution factor (10x)

Vs : Volume of sample (mL)

Antibacterial Activity Assay against Pathogenic Bacteria

In vitro assay of LAB inhibition activities against pathogenic bacteria (*E. coli* FNCC 194, *B. subtilis* FNCC 0060, *S. aureus* FNCC 6049) were performed using agar diffusion methods (Bonev *et al.*, 2008; Cizekiene *et al.*, 2013). The supernatant of 18 h from MRSB cultures was prepared using a refrigerated centrifuge 'Centrifuger® BL-II series' at 4137 × g for 5 min at 4 °C. The supernatant was collected and neutralized by adding 0.5 N NaOH up to pH 7. The antibacterial activities were observed using the paper disc diffusion method. The paper disc (5 mm) placed on the MRSA surface which was inoculated with 100 µL pathogenic bacteria cultures and then embedded with 50 µL of neutral LAB supernatant. Control was made by adding the antibiotic discs (15 mg Erythromycin, 10 mg Penicillin G, 10 mg Streptomycin) for each of the tested pathogenic bacteria. Observation of clear zones (inhibition zone) for 24 h at 37 °C.

Biochemical Identification

Biochemical tests were performed by fermentation pattern using standard analytical profile index (API 50 CHL, bioMérieux®) using the manual standard of API CHL 50 kit (Muñoz-Quezada *et al.*, 2013). Pure culture of LAB biomass from 24 h culture was inoculated into API 50 CHB/E medium. The result of the biochemical profile was used to identify the species of lactic acid bacteria using software product identification e.g. APIWEB (bioMérieux®, Marcy l, Etoile, France).

Data Analysis

Qualitative data consisted of cell morphology and biochemical characteristic were analyzed using descriptive method. Data from quantitative parameter consisted of clear zone diameter, cell viability, lactic acid and pH were analyzed using analysis of variance (ANOVA) and followed by Duncan's Multiple Range Test to determine the effect of differences between treatments mean (Gomez & Gomez, 2007). The statistical analyses of parameters were performed using the CoSTAT® Statistical Software (Cohort, 2008).

RESULTS AND DISCUSSION

Isolation and Selection of Lactic Acid Bacteria

In preliminary stage, LAB was screened by evaluating clear zone of each isolate grown on MRS

Table 1. Clear zone diameter of colony grown on MRSA+0.2% CaCO₃

Isolate number	Inoculant sources	
	Rumen liquor (CR)	Silage (Sil.)
1	2.13 ; 1.33 ^{*)}	1.02 ; 0.67
2	2.19 ; 3.42 ^{*)}	1.09 ; 1.62
3	2.45 ; 0.76	0.96 ; 2.02 ^{*)}
4	1.41 ; 2.06	0.78 ; 1.67
5	1.18 ; 1.43	1.19 ; 1.77
6	1.22 ; 0.64	1.12 ; 0.94
7	0.83 ; 0.86	0.79 ; 1.31
8	0.93 ; 1.79	0.78 ; 0.67
9	1.93 ; 1.74	1.01 ; 1.94 ^{*)}
10	1.11 ; 1.78	0.68 ; 0.57

Note: ^{*)} The selected isolate based on the largest of clear zone, the value indicated that two clear zones.

agar. Initial screening were obtained 2 isolates from rumen liquor (CR), 2 isolates silage (Sil). The clear zone diameter of the selected isolates consisted of CR1, CR2, Sil.3, and Sil.9 are presented in Table 1. Characterization of the screened isolates consisted of catalase test, Gram staining, morphology, motility test, and gas production test are shown in Table 2.

Resistance of lactic acid bacteria in low pH was performed by cultivation isolates on a medium that was adjusted at pH 2 and 3 for 0 and 1 h. Viability of four isolates were affected by pH and incubation time

significantly ($P < 0.05$). However, there was no interaction ($P > 0.05$) between isolates and medium acidity condition (pH value) on cell viability. Based on the viability in pH 2 and 3 indicated that the four isolates were able to survive in low pH condition (Table 3).

A number of initial colonies were similar around 5.00–6.42 log cfu/mL. Incubation time for 1 h showed that percentage of isolates colonies survived in pH 2 was 68% for CR1 and CR2, followed by Sil.3 and Sil.9. Meanwhile, the percentage of isolates colonies survived in pH 3 were 89%, 66%, 65%, and 63% respectively for CR2, Sil.9, CR1, and Sil.3. Furthermore, incubation time for 2 h in pH 3 showed that viability of fourth isolates were not significantly different. In general, the highest viability of isolates obtained from CR1, the lowest obtained from Sil.9, except for CR1 and Sil.3 were similar. It is indicated that CR2 was more able to survive in low pH than other isolates, consequently it could be used as a probiotic candidate.

An ability bacterium to survive in acid medium was closely related to cell stability which was influenced by many factors consisted of temperature, pH and nutrient availability. Moreover, adaptability bacteria in environmental condition were closely affected on growth and survivability. Romero-Pérez *et al.* (2011) revealed that environmental factor influenced ability bacteria to grow in acidity medium which was related to previous natural habitats.

In general, the pattern of LAB growth following sigmoid curve (S) was measured by turbidimetry method (Optical Density/OD) and further confirmed by total plate count (TPC) to evaluate the cell viability (Hogg, 2013; Rehaem *et al.*, 2014). The growth rate was indi-

Table 2. Characterization of the selected isolate

Isolates	Morphological test			Motility	Gas production test (fermentation type)
	Gram	Catalase	Shape		
CR1	Positive	Negative	Rod	Non-motile	Heterofermentative
CR2	Positive	Negative	Rod	Non-motile	Homofermentative
Sil.3	Positive	Negative	Rod	Non-motile	Homofermentative
Sil.9	Positive	Negative	Rod	Non-motile	Heterofermentative

Table 3. Cell viability of isolates incubated at low pH during 0, 1, and 2 h

Isolates	Initial colonies (0 h)		Viability (0 h)		Viability after 1 h		*Viability after 2 h		Average (Isolate factor)
	pH 2	pH 3	pH 2	pH 3	pH 2	pH 3	**pH 2	pH 3	
CR1	6.39±1.1	6.06±0.8	100.0± 0.0	100.0±0.0	68.3±03.5	65.0±32.1	nd	52.5± 4.7	83.3±21.6
CR2	5.26±0.2	5.00±0.8	100.0± 0.0	100.0±0.0	68.9±14.5	89.8±25.8	nd	53.2±16.8	89.7±17.6
Sil.3	5.35±0.1	6.07±0.5	100.0± 0.0	100.0±0.0	38.3±54.2	62.5±21.0	nd	31.0±43.9	75.2±35.6
Sil.9	5.02±1.4	6.42±0.2	100.0± 0.0	100.0±0.0	60.4±12.2	66.4± 5.9	nd	15.5±21.9	81.6±21.3
Incubation (block)	(0 h)		100.0± 0.0 ^a	(after 1 h)	64.9±23.8 ^b				
pH factor	(pH 2)		79.5±27.7 ^a	(pH 3)	85.4±20.9 ^a				
Interaction	Isolat x pH = non significant (P=0.877)								

Note: Mean in the same row with different superscripts differ significantly ($P < 0.05$). Initial colonies was similar population in log cfu/mL ($P > 0.05$).

* Data from the cell viability assessment after 2 h was not included in average calculation and statistical analysis. ** nd : not available data.

cated from absorbance of 48 h incubation at 37 °C. Based on the Figure 1, the high growth rate of LAB isolates was CR2, and Sil.3. Bacterial growth was influenced by pH, nutrient content, and temperature. The population of LAB after 48 h incubation showed that CR2 and Sil.3 were similar and higher than others (Figure 2). Cousin *et al.* (2012) stated that lactic acid bacteria require carbon and nitrogen sources for optimizing growth and produce lactic acid as major metabolites. Characteristic of fermentability from each isolate to ferment medium (glucose and its derivatives) was explained in Table 4.

At the end of incubation (48 h), levels of lactic acid and pH of the medium were measured to determine the type of fermentation from each isolate which obtained from the gas production test as shown in Table 2. Hogg (2013) stated that lactic acid levels are different depending on the type of fermentation. A homofermentative type of lactic acid bacteria produces high levels of lactic acid due to converting 95% glucose into lactic acid, a small amount of CO₂ and volatile acids. While the heterofermentative types of lactic acid bacteria produce lactic acid in low concentration and amount of CO₂.

Based on the test results of fermentation type, isolates CR2, and Sil.3 were homofermentative that had high levels of lactic acid. Isolates CR1 and Sil.9 had low levels of lactic acid and indicated heterofermentative type. Levels of lactic acid production were closely related to pH value. König & Fröhlich (2009) stated that fermentation type of lactic acid produced dominantly lactic acid was a homofermentative however heterofermentative of LAB produce not only lactic acid but also acetic or alcohol.

Biochemical characteristic of LAB evaluated using API CHL 50 are presented in Table 5. All of the isolates have a similar result from sugar fermentation test on API CHL 50 except for substrates consisted of D-galactose, D-lactose, D-Saccharose, inulin and D-rafinose. Based on analysis using software APIWEB (Table 6), isolates CR1 was *Lactobacillus paracasei* ssp. *paracasei* 3 (91.5%),

CR2 was *Lactobacillus paracasei* ssp. *paracasei* 3 (76.5%), Sil.3 was *Lactobacillus brevis* (95.1%), and Sil.9 was *Lactobacillus collinoides* (92.5%). Lactic acid bacteria require different carbohydrate sources for growth and to produce substances that can be used to defend cells from pathogenic bacteria invasions (bacteriocins).

This biochemical test result supported by Gulahmadov *et al.* (2009) revealed that *L. brevis*, and *L. collinoides* were isolated from Azerbaijan cheeses have activity to ferment D-ribose, D-glucose, D-fructose, N-acetylglucosamine, D-Maltose, D-trehalosa, however, D-celobiose, D-lactose, sucrose and inulin were only fermented by *L. brevis*. Muñoz-Quezada *et al.* (2013) identified *Lactobacillus paracasei* which was able to utilize inulin and sucrose. Although phenotypic identification using a biochemical test is accurate methods, molecular identification need to conduct for the confirmation of bacteria species as a probiotic candidate (Ouoba *et al.*, 2010).

Inhibitory Against Pathogenic Bacteria

The inhibition activity from tested isolates was showed by clear zone diameter (Figure 3). Antimicrobial assay results indicated that the isolates were able to inhibit the growth of pathogenic bacteria. Based on the data, all isolates inhibited *B. subtilis*. LAB isolated

Table 4. Lactic acid production and pH of media inoculated with the selected isolates

Isolate	Lactic acid (%)	pH
CR1	15.40±1.56 ^b	4.03±0.01 ^b
CR2	19.88±0.81 ^a	3.75±0.02 ^c
Sil.3	21.42±0.14 ^a	3.71±0.02 ^d
Sil.9	15.08±0.25 ^b	4.18±0.01 ^a

Note: Mean in the same column with different superscripts differ significantly (P<0.05).

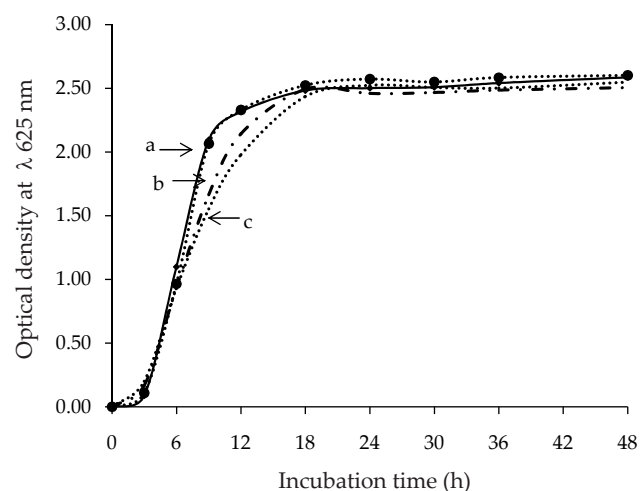


Figure 1. Growth curve of isolates (..... for CR1,•..... for CR2, -♦- for Sil. 3, - - - for Sil. 9) during 48 h of incubation. a, b, c indicated significant difference (P<0.05).

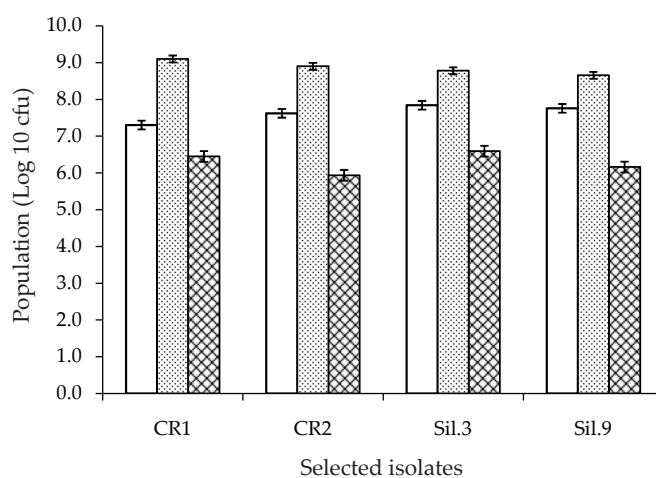


Figure 2. Population of the selected isolates after 0 (□), 24 (▨) and 48 (▩) h of incubation

Table 5. Biochemical identification of the selected isolates using API 50 CHL Kit

No	Type of substrate test	Reaction of isolates				No	Type of substrate test	Reaction of isolates			
		CR1	CR2	Sil.3	Sil.9			CR1	CR2	Sil.3	Sil.9
0	Control	-	-	-	-	25	Esculin ferric citrate	+	+	+	+
1	Glycerol	-	-	-	-	26	Salicin	+	+	+	+
2	Erythritol	-	-	-	-	27	D-cellobiose	+	+	+	+
3	D-arabinose	-	-	-	-	28	D-maltose	+	+	+	+
4	L-arabinose	-	-	-	-	29	D-lactose(bovin arginin)	+	+	-	+
5	D-ribose	+	+	+	+	30	D-melibiose	-	-	-	-
6	D-xylose	-	-	-	-	31	D-saccharose (sucrose)	+	+	-	+
7	L-xylose	-	-	-	-	32	D-trehalose	+	+	+	+
8	D-adonitol	-	-	-	-	33	Inulin	+	-	-	+
9	Methyl-bd-xylopyranoside	-	-	-	-	34	D-melezitose	-	-	-	-
10	D-galactose	-	+	+	-	35	D-raffinose	+	-	+	+
11	D-glucose	+	+	+	+	36	Amidon (starch)	-	-	-	-
12	D-fructose	+	+	+	+	37	Glycogen	-	-	-	-
13	D-mannose	+	+	+	+	38	Xylitol	-	-	-	-
14	L-sorbose	-	-	-	-	39	Gentibiose	+	+	+	+
15	L-rhamnase	-	-	-	-	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-aD-mannopyranoside	-	-	-	-	45	D-arabitol	-	-	-	-
21	Methyl-aD-glucopyranoside	-	-	-	-	46	L-arabitol	-	-	-	-
22	N-acetylglucosamine	+	+	+	+	47	Potassium gluconate	-	-	-	-
23	Amygdalin	+	+	+	+	48	Potassium-2 celogluconate	-	-	-	-
24	Arbutin	+	+	+	+	49	Potassium-5 celogluconate	+	+	+	+

Note: + : positive reaction (red changed into yellow color) , - : negative reaction (red color not changed)

Table 6. Species identification results analyzed by APIWEB software

No	Isolate code	Significant taxa value	Species name
1	CR1	91.50%	<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> 3
		5.10%	<i>Lactobacillus brevis</i> 1
2	CR2	76.50%	<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> 3
		21.50%	<i>Lactobacillus brevis</i> 1
3	Sil.3	95.10%	<i>Lactobacillus brevis</i>
		4.20%	<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> 3
4	Sil.9	92.50%	<i>Lactobacillus collinoides</i>
		4.30%	<i>Lactobacillus lactis</i> ssp. <i>lactis</i> 1

from rumen liquor (CR1 and CR2) had higher inhibition against *E. coli* than silage isolate, however isolate Sil.3 had the highest inhibition activity against *S. aureus*.

The difference ability of the isolates to inhibit patho-

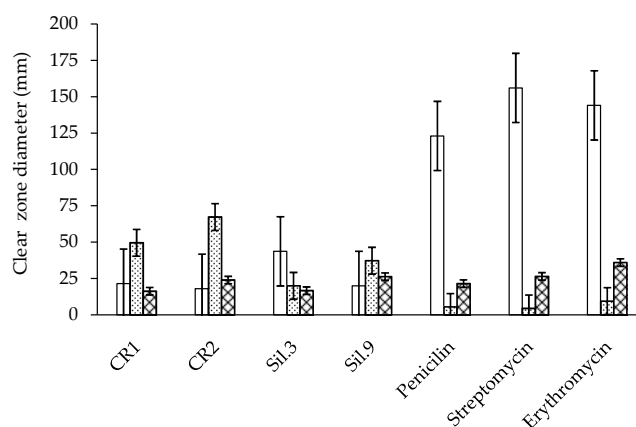


Figure 3. Antibacterial activity of LAB isolates and antibiotics (control) against pathogenic bacteria. *S. aureus* (□), *E. coli* (▨) and *B. subtilis* (▩).

genic bacteria could be influenced by many factors such as lactic acid and bacteriocine production from LAB and resistance of pathogenic bacteria to grow at low pH (acid stress). Acidity condition can damage the mem-

brane cytoplasmic Gram negative bacteria. Due to Gram positive bacteria cell encapsulated by thick layer of peptidoglycan that **strengthened cell wall** (Derouaux *et al.*, 2014). It implied ability LAB to inhibit gram positive bacteria (*S. aureus* and *B. subtilis*) seem lower than gram negative pathogenic bacteria (*E. coli*). Acuña *et al.* (2012) revealed that an antimicrobial peptide 'microcins' had better performance killing *E. coli* (Gram negative) than *Listeria innocua* (Gram positive) cells.

Charlier *et al.* (2009) reported that bacteriocin-producing strains are indeed of considerable interest for the preservation of fermented products which may be achieved by inhibiting the growth of pathogens such as *Listeria monocytogenes* and/or *S. aureus*. Vlková *et al.* (2010) reported that lactic acid bacteria have antimicrobial activity, and were bile and acid tolerant; they exhibited a decrease in viability <1 log CFU/mL after incubation for 3 h for bile tolerance and 2 h for low pH tolerance **significantly lower amounts of *E. coli***. In recent studies, application of 8.0 log cfu/g LAB produced significant reductions in *E. coli* O157:H7 (Cálix-Lara *et al.*, 2014). Moreover, Arief *et al.* (2013) studied that LAB (*L. plantarum*) was able to produce antimicrobial 'plantaricin' peptide which inhibited pathogenic bacteria (*E. coli*, *B. cereus*, *S. aureus* and *S. thymurium*).

The highest inhibition of antibiotic against *S. aureus* growth was closely related to a spectrum of antibiotic activity. Based on Figure 3, Penicilin, Streptomycin and Erythromycin seem to have **higher inhibition against *S. aureus* growth than LAB metabolite**. Compare to chemical antibiotic, pathogenic bacteria inhibition by LAB metabolite was related to the **chemical antibiotic compound in pure substance and the ability of antibiotic to penetrate pathogenic bacteria cell easily** (Bonev *et al.* 2008), however antibacterial substance from lactic acid bacteria as probiotic was **more safety than antibiotic due to its lower risk of residual and has no resistance effect on pathogenic bacteria**.

CONCLUSION

Lactic acid bacteria isolated from rumen liquor and king grass (*P. hybrid*) could be used as probiotic. Probiotic candidates isolated from rumen liquor are confirmed as *L. paracasei* ssp. *paracasei* (CR1 and CR2), while two other isolates from king grass silage are identified as *L. brevis* (Sil.3) and *L. collinoides* (Sil.9). *Lactobacillus brevis* (Sil.3) and *L. paracasei* ssp. *paracasei* (CR1) has higher inhibition against pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*) than *L. paracasei* ssp. *paracasei* (CR2) and *L. collinoides* (Sil.9).

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