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The Use of Lactic Acid Bacteria as Ruminant Probiotic Candidates Based on *In Vitro* Rumen Fermentation Characteristics

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ABSTRACT

This research was conducted to select, to identify LAB isolates and to investigate the effects of the LAB as probiotics candidate in the rumen fermentation. Nine isolates exhibited the potency as candidate probiotics for cattle. The experiment was arranged in randomized block design with ten treatments and three different times of in vitro as a block. The substrate consisted of 70% forage and 30% concentrate proportion. The substrate was incubated at 39°C using serum bottle of 100 ml capacity for fermentation. Approximately 0.75 g of substrates was put inside the serum bottle glass and filled with 73 ml of buffered rumen fluid and 2 ml of LAB inoculant. Gas production was measured every 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of the incubation period. Gas production kinetic was estimated by the Ørskov's equation. The LAB with the highest gas production, as probiotics candidate, were identified using partial 16S rDNA sequence. The results of this research indicated that nine LAB produced high gas production in the range of 193-198 ml compare to that of control (173 ml). The addition of LAB in rumen fermentation resulted in digestibility 65-75%, organic matter digestibility 51-73%, and 6.67-6.68 pH. Based on the molecular identification, 8 isolates are *Lactobacillus plantarum* and 1 of uncultured bacteria. The LAB strain 32 *L. plantarum* showed the best for a ruminant probiotic candidate based on the in vitro rumen fermentation characteristic.

Keywords : Fermentation, Lactic acid bacteria, Rumen, Probiotics, 16S rDNA sequence

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Introduction

Ruminant productivity can be improved with higher feed digestibility by stimulating microbial activity in the rumen. Bacteria, ciliate and flagellate protozoa, and anaerobic fungi are the community of gut microbes which have an important role in nutritional, physiological, immunological, and protective functions of the host. The rumen is one of the most extensively studied gut ecosystems, because of the importance of ruminant health and productivity. Rumen microorganisms play different roles in feed digestion and mode action synergistically to ferment plant structural and nonstructural of nutrient (Durand and Ossa, 2014).

Recently, the antibiotic and growth hormone application had already banned for animal production. Addition of probiotics is alternative for antibiotic replacement in ruminant production. Probiotics are defined as live microorganisms which have been administered in adequate amounts give positive effect to microbial balance and health benefit on the animal host (Anadón *et al.*, 2006; Fuller, 1989). Seo *et al.* (2010) state that

the role of probiotics can be beneficial in the efficient use of feed in improving the productivity of livestock, to prevent gut infections and support the ecosystem regulation of microbes in the digestive tract. Lactic acid bacteria (LAB) are widely used as probiotics in cattle to contribute feed digestibility microbial balance, and health to animals host (Uyeno *et al.*, 2015). Many kinds of LAB strains, the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, are considered beneficial to the animal host and have been used as probiotics (FAO, 2016). Numerous factors such as dietary and management constraints are demonstrated strong affect about structure and activities of gut microbial communities that lead to increasing performance and health of cattle (Durand and Durand, 2010).

Probiotics from LAB has been administered with the aim of improving rumen fermentation for feed efficiency by stimulating microbial fermentation. Moreover, probiotic can increase feed digestibility by producing gas as one of a parameter of microbial activity in the rumen. The lab used in this experiment was originally isolated from the rumen of Ongole Crossbred

cattle. This research was conducted to select, identify the best LAB isolate as a ruminant probiotic candidate and to observe LAB effect as probiotics in the rumen fermentation characteristics.

Materials and Methods

Culture conditions and pre-screening of isolates

Nine LAB isolates (LABRumen26, 27, 32, 37, 38, 40, 42, 43, and 80) were isolated from rumen Ongole Crossbred cattle belong to the Research Center for Biotechnology. One loopful of the stock culture of selected LAB under anaerobic condition were streaked onto De Man Rogosa Sharpe (MRS, Fluka) agar plate and incubated at 39°C for 24 h. Inoculum of the selected LAB was prepared in a 20 ml glass tube with 10 ml MRS broth medium incubated at 39°C for 24 h with anaerobe condition.

In vitro rumen fermentation

The rumen fluid was obtained from three fistulated Ongole Crossbred cattles before the morning feeding. The use of the cattle in this experiment was approved by the Ethic Clearance Committee of the Indonesian Institute of Sciences No. 9879/WK/HK/XI/2015. Approximately 0.75 g of substrates (consisted of 70% *Pennisetum purpureum* and 30% concentrate proportion in dry matter basis) was put inside the serum bottle glass of 100 ml capacity and filled with 73 ml mixture of 24.33 ml rumen fluid and 48.67ml of Mc'Dougall buffer (NaHCO₃ 0.98 g, Na₂HPO₄·7H₂O 0.7 g, KCl 0.057 g, NaCl 0.0472 g, MgSO₄·7H₂O 0.012 g, CaCl 0.004 g, and distilled H₂O up to 100 ml) and 2 ml (2.67%) of LAB inoculum. The bottle was closed with rubber cap and aluminum crimp after supplied (infused) with CO₂ gas to achieve anaerobic conditions (± 3 minute supply). Incubation was done at 39°C in a water bath incubator. Gas production was measured at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of incubation period with a disposable syringe. Gas production kinetics were estimated by the Ørskov's equation $p = a + b(1 - e^{-ct})$, where p is gas production, a is gas production (ml) from quickly soluble fraction, b is gas production (ml) from insoluble fraction, c is gas production rate (ml/h), and t is incubation period (Ørskov and McDonald, 1979; Jayanegara *et al.*, 2009). After *in vitro* fermentation, the samples were separated for the liquid and solid fractions by filtration using filter papers (Whatman™ 41 Cat No 1441-125). Liquid fraction or rumen buffer was used for analysis of pH and *L. plantarum* population using qPCR (Klocke *et al.*, 2006). Solid fraction for measurement of dry matter digestibility (DMD) and organic matter digestibility (OMD) (Theodorou *et al.*, 1994).

DNA extraction and identification of LAB

Nine LAB selected isolates were identified with partial 16S rDNA sequences. The DNAs of LAB grown in MRS broth medium as described above and rumen buffer from each treatment were

extracted by using Genomic DNA Mini Kit based on Buffy Coat Protocol (Geneaid). Extraction of DNAs was modified such as the addition of Proteinase K (final concentration of 2 mg/mL), Lysozyme (final concentration of 25 mg/mL) and RNase A (final concentration of 10 mg/mL), and then incubated at 60°C for 30 min. DNAs product of LAB and rumen buffer were used for identification and quantification of *L. plantarum* by qPCR method, respectively. LAB isolates were identified by a molecular approach using partial 16S rDNA sequence. The 16S rDNA amplification was performed as described previously by Ridwan *et al.* (2015). DNA was amplified by using primer 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGTTACCTTGTACGACTT3'). Amplification of PCR reaction was used in a total volume of 50 μ L consisted of 25 μ L KAPPA Ready mix (Firstbase), 5 μ L of dissolved DNA (<1 μ g), 10 pmol of each primer and up to 50 μ L of pure distilled water. The 16S rDNA were amplified by using a Techne TC-512 Thermal cycler with the following program for bacteria: 95°C for 3 min, followed by 30 cycles consisted of 95°C for 30 s, 50°C for 30 s and 72°C for 1.5 min, with a final extension at 72°C for 10 min. Amplified DNAs were verified by electrophoresis of aliquots PCR product (3 μ L) in 1.5% agarose in 1x TAE buffer. The PCR products were purified and sequenced using the DNA sequencing services (Firstbase, Malaysia). The 16S rDNA sequences were checked using BioEdit base on the sequence of primers and compared similarity using BLAST search at NCBI homepage (Zhang *et al.*, 2000). Phylogenetic relationship data were collected from RDP II genebank data base (Cole *et al.*, 2009) by using the CLUSTAL X 2.1 program and a phylogenetic tree was inferred using neighbor-joining tree algorithms.

Quantitative Real-Time PCR (qPCR)

The analysis of qPCR was performed as described previously (Klocke *et al.*, 2006) by using the Rotorgene Q Qiagen in accordance with the manufacturer's instructions and the dsDNA-binding dye SYBR GreenI with pair specific primers of *L. plantarum* L.pla f- 5'TTACATTTGAGTGAGTGCCGAAC-3' and L.pla r- 5-AGGTGTTATCCCCGCTTCT-3'. The total qPCR reaction was determined in a 20 μ L final volume consisted of 10 μ L of Quantifast® SYBR® Green PCR Kit, 1 μ L of each specific primer (F & R), 6 μ L of H₂O, and 2 μ L of extracted DNA sample from each treatment. The copy number of *L. plantarum* in samples was determined by using *L. plantarum* cells as a standard. Data were analyzed by using the Rotor-Gene Q Series Software 1.7. Ink version. The qPCR analysis was used for quantification the real specific population of the LAB in the rumen fermentation.

Statistical analysis

The experiment design was a randomized block design (RBD) with ten treatments and three different times of *in vitro* as a block. The treatments

consisted of the different LAB isolate as inoculant and control without LAB. The fermentation parameters measured were pH, total gas, potential gas production (a+b), the rate of gas production (c), DMD, OMD, DNA copy numbers (qPCR). Data were analyzed by using ANOVA with SPSS 23 for windows. Significant effects of each treatment were further analyzed by using the least significant difference by Duncan multiple range test ($P < 0.05$), except microbial diversity data, were analyzed descriptively. Kinetic parameters of Ørskov's equation were obtained by non-linear regression procedure in SPSS 23.

Results and Discussion

Nine isolates were selected as candidates for ruminant probiotic, based on the rumen fermentation. Effect of LAB addition on *in vitro* rumen fermentation is shown in Table 1. The addition of LAB in rumen fermentation did not significantly change pH ($P > 0.05$). All treatments showed normal pH range (6.67-6.70) during rumen fermentation. The DMD and OMD were significantly affected ($P < 0.05$) by addition of LAB to rumen fermentation.

The highest values of DMD and OMD resulted by addition of LAB isolate 32. It is significantly higher compare to the lowest DMD resulted by addition of LAB isolate 37, and to the lowest OMD resulted by addition of LAB isolate 43. From nine isolates added to *in vitro* fermentation system, seven isolates gave higher DMD than control. A different result was observed for OMD where only isolate 32 can increase OMD from 68.47% for control to 74.03%. Isolate 32 also gave the highest DMD, even compared to control. These results were indicated that the LAB affected to stimulated rumen fermentation to increase feed degradation (Lettat *et al.*, 2012).

The results of this research indicated that nine LAB were significantly ($P < 0.05$) produced higher total gas production than control. Addition of LAB isolate 42 produced the highest value of gas production compared to the other treatments, while isolate of 80 produced the lowest. The gas production was fitted to the Ørskov's equation

(Ørskov and McDonald, 1979) to find gas kinetics as presented in Figure 1. The maximum gas production (b) and rate of gas production (c) from the equation were shown in Table 1. From the equation can be found that addition of LAB isolate 40 produced the highest gas production rate.

Nine isolates of the LAB with the high gas production were identified with partial 16S rDNA sequences database of bacteria (Table 2). Eight isolates were identified having high similarity to *Lactobacillus plantarum*, and one isolate was similar to an uncultured clone of bacteria.

Analysis of qPCR was done to find out the population of *L. plantarum* in the rumen buffer because most of the isolates identified in this experiment were *L. plantarum*. Addition of LAB significantly increased *L. plantarum* population (Table 2) in rumen buffer after 72 h incubation. LAB isolate 40 resulted the highest *L. plantarum* population significantly compared to other isolates except with isolate 43. All isolates used were significantly increased *L. plantarum* population compared to control.

The phylogenetic tree of nine LAB isolates was constructed by alignment of the partial 16S rDNA sequences database from Ribosomal Database Project II type strains (Figure 2). All LAB isolates showed similar group with cluster of *L. plantarum* except for isolate 80 which identified as uncultured bacteria.

The breakdown of readily fermentable materials can lead to critical changes in rumen conditions, such as decreasing pH and increase lactic acid levels, which contribute to metabolic acidosis (Chiquette *et al.*, 2008). The addition of LAB in this research resulted in stable pH of rumen buffer and improved feed digestibility. Rumen fermentation showed that the rumen microorganism could function properly and improved by the addition of LAB (Seo *et al.*, 2010). This caused by lactic acid produced by LAB that might trigger other rumen microbes and also proved that LAB could be survived in rumen fluid (Weinberg *et al.*, 2007). Changed of pH caused by the addition of LAB also reported by Soriano *et al.* (2014). The pH value did not

Table 1. Effect of lactic acid bacteria addition to the *in vitro* rumen fermentation

Isolates Code	pH	Total gas (ml)	a+b (ml)	c (ml/h)	DMD (%)	OMD (%)
Control	6.69 ^a	173.33 ^a	176.79 ^a	0.0527 ^a	68.47 ^{ab}	60.36 ^{ab}
LABRumen26	6.68 ^a	197.17 ^b	192.52 ^b	0.0610 ^b	67.57 ^{ab}	56.23 ^{ab}
LABRumen27	6.70 ^a	195.67 ^b	190.66 ^b	0.0623 ^{bc}	69.26 ^{ab}	57.48 ^{ab}
LABRumen32	6.68 ^a	194.67 ^b	190.19 ^b	0.0610 ^b	74.04 ^b	73.67 ^b
LABRumen37	6.67 ^a	194.67 ^b	189.85 ^b	0.0617 ^b	65.97 ^a	57.84 ^{ab}
LABRumen38	6.68 ^a	197.67 ^b	193.22 ^b	0.0600 ^b	69.17 ^{ab}	59.98 ^{ab}
LABRumen40	6.68 ^a	196.33 ^b	190.39 ^b	0.0663 ^c	70.64 ^{ab}	55.92 ^{ab}
LABRumen42	6.67 ^a	198.17 ^b	193.20 ^b	0.0620 ^b	69.45 ^{ab}	55.61 ^{ab}
LABRumen43	6.67 ^a	196.83 ^b	192.27 ^b	0.0610 ^b	68.61 ^{ab}	51.08 ^a
LABRumen80	6.67 ^a	194.00 ^b	189.22 ^b	0.0617 ^b	69.45 ^{ab}	55.74 ^{ab}

Control; treatment without LAB addition, (a+b); potential gas production, c; gas production rate, DMD; dry matter digestibility, OMD; organic matter digestibility.

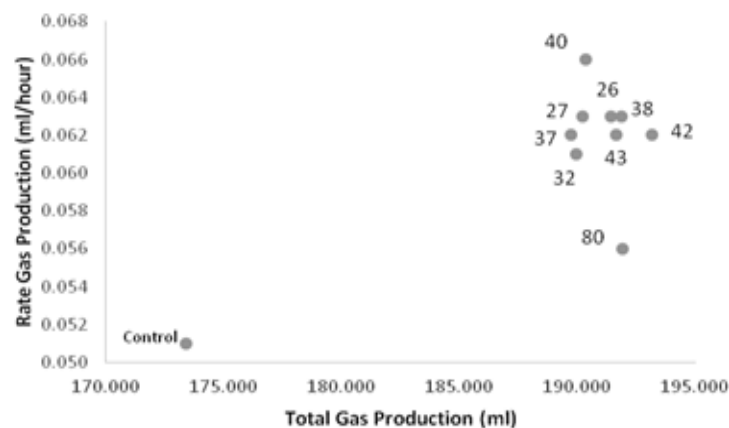


Figure 1. *In vitro* gas production kinetics of selected lactic acid bacteria by Ørskov's equation.

Table 2. Similarity of lactic acid bacteria isolates and quantification of *Lactobacillus plantarum* in the rumen fermentation

Isolates code	Similarity	Identity	Accession no.	DNA copy number (Log10)
Control	-	-	-	9.52 ^a
LABRumen26	<i>Lactobacillus plantarum</i>	100	KJ026610	10.89 ^{bc}
LABRumen27	<i>Lactobacillus plantarum</i>	98	KM497504	11.25 ^{bcd}
LABRumen32	<i>Lactobacillus plantarum</i>	100	KJ914901	10.73 ^{bc}
LABRumen37	<i>Lactobacillus plantarum</i>	100	KP763909	11.52 ^{cd}
LABRumen38	<i>Lactobacillus plantarum</i>	100	KP763909	11.87 ^d
LABRumen40	<i>Lactobacillus plantarum</i>	100	KM497504	12.09 ^d
LABRumen42	<i>Lactobacillus plantarum</i>	99	KP262340	10.63 ^{bc}
LABRumen43	<i>Lactobacillus plantarum</i>	100	KJ026610	12.00 ^d
LABRumen80	Uncultured clone	99	GQ079352	10.52 ^b

change significantly because rumen buffer has buffering capacity to keep pH stay in certain range.

The fermentation kinetics in the rumen and the gas production level correlated with the activities of rumen microbes. The high quality of feed, sustainability of feed resources, and rumen microbial balance may show the adverse effect to improve ruminant productivity. The most limiting factors in cattle feeding are digestibility and quality of nutrient. Feed quality can be used in the *in vitro* fermentation system for determining the nutritive values of ruminant feed (Theodorou *et al.*, 1994). This research found the relationship between LAB addition and substrates degradation which effected to DMD and OMD improvement. Weinberg *et al.* (2007) stated that some LAB increases digestibility when added directly to the rumen fluid. The feed digestibility is an important indicator for the requirement of probiotic effect from LAB addition to ruminant as host. Generally, LAB in the rumen is minority in population which contribute to rumen metabolisms. Lactobacilli are commonly found in young ruminants and particularly can be available in adult ruminants with high concentrate diets (Stewart *et al.*, 1997). The isolates of the LAB used in this research were isolated from adult cattle with high concentrate ration (70% proportion in the diets). Live microorganisms can be described as probiotics when giving positive effect for host animal. The increasing of feed digestibility

confirmed that LAB can act as probiotic by stimulating rumen bacteria activity (Table 1). To have a probiotic effect, LAB have to survive when added to fermentation. Higher LAB population from the qPCR analysis (Table 2) showing that the LAB used in this research can survive in the rumen condition. The beneficial effects come from the additional supply of protein, vitamin, and short chain organic acids from microorganism added which contributes to stimulate rumen microbial activities (Pinloche *et al.*, 2013; Soe *et al.*, 2010).

Substrate fermentability in the rumen was an indication that LAB can stimulate the activity of rumen microorganism to produce significantly higher ($P < 0.05$) total gas production. Gas production supplemented by LAB was higher ($P < 0.05$) about 13.2% than control. Gas production was measured at 72 h because incubation time of *in vitro* fermentation more than 24 hours is more accurate for measurement of rumen metabolisms (Jayanegara *et al.*, 2009). These finding suggested that the LAB used in this research are safe to use as probiotics candidate for cattle and give positive effect to the animal host.

The use of Ørskov's equation for selection of the best LAB isolates based on gas production kinetics in the rumen fermentation is appropriate. It is showed that specific strain of LAB gave different effect to stimulate the activity of other

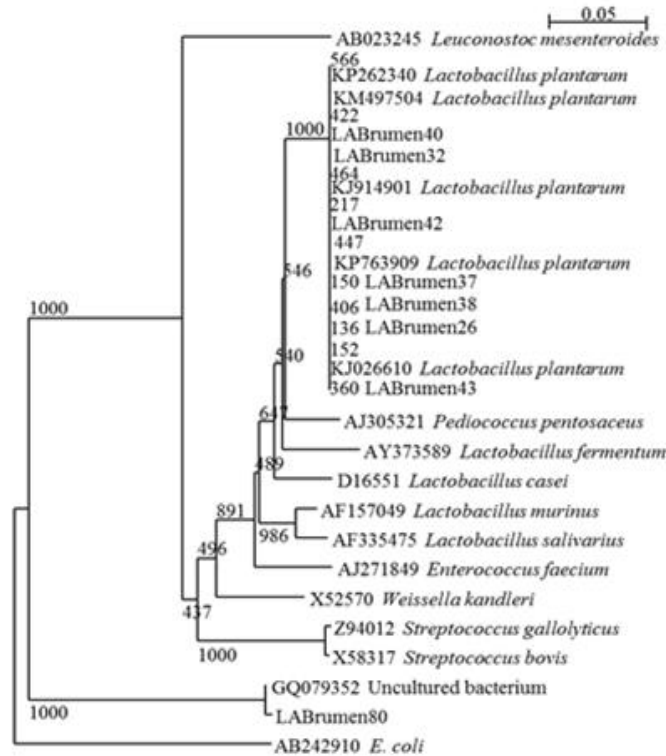


Figure 2. Phylogenetic relationships of lactic acid bacteria base on partial 16S rDNA sequences.

rumen microorganism. Other evidence that inoculum can survive in rumen ecosystem is the *L. plantarum* population measured by quantitative real-time PCR, which showed significantly higher population compared to control by addition of LAB (Klocke *et al.*, 2006).

The specific strain of LAB was chosen as probiotic as it can give benefit for the host animal. In ruminant, feed digestibility is important parameter to measured rumen fermentation. Higher feed digestibility will supply more energy for the animal, and metabolize it as fuel for body maintenance and animal production. *L. plantarum* strain 42 produced higher gas production compared to control. However, the highest DMD and OMD was obtained from *L. plantarum* strain 32. Both isolates showed good effects in feed digestibility by stimulating rumen bacteria activity. *L. plantarum* strain 32 produced total gas not significantly different from strain 42. It can be considered that *L. plantarum* strain 32 was more suitable as probiotic candidate for ruminant. All measured parameters from the addition of *L. plantarum* strain 32 showed the highest result for rumen fermentation characteristic based on feed digestibility.

Although research about the use of LAB as probiotic still gave variable results, this research showed that addition of LAB could change rumen fermentation based on gas production as an indicator. To ensure the beneficial effects of probiotic, the survival of LAB in the rumen is very important. Some LAB strains used as inoculants

may survive and show probiotics effect in the rumen and the intestine (Rodriguez-Palacios *et al.*, 2009). Soriano *et al.* (2014) said that the use of organisms isolated from the rumen itself as directly fed microorganism can be considered advantageous to produce higher gas production at 3.67 ml compared with control, as these microbes are readily adapted to the rumen environment. The LAB used in this research was isolated from rumen fluid, so it is possible these bacteria can survive in the rumen. This can be proved by the qPCR result in this research (Table 2), that showed the total number of the LAB increased significantly compared to control. The addition of LAB in the rumen can stimulate rumen fermentation and gives beneficial effects in the feed digestibility.

Conclusion

The LAB isolated from rumen cattle were associated with rumen fermentation products. *L. plantarum* isolate strain 32 gave the highest digestibility and increased total gas production compared to control. DNA identification analysis revealed that isolate 32 is closely related to *L. plantarum*. *L. plantarum* strain 32 was selected as the best candidate for ruminant probiotic based on in vitro fermentation characteristics. For the further research, the screening parameters should be evaluated mainly base on competitive exclusion, bacterial antagonism, and immune modulation.

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