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 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

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). See 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 cattle 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 48.67 ml 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, CaCl₀.004 g, and distilled H₂O up to 100 ml) and 2 ml (2.67%) of LAB in inoculum. The bottle was closed with rubber cap and aluminium 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 used for analysis of pH and organic matter digestibility (OMD) (Theodorou et al., 2006). **Liquid fraction** or rumen buffer was used for analysis of pH and **solid fraction** for dry matter digestibility (DMD) and **concentration of 25 mg /mL) and RNAse A (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 rRNA amplification was performed as described previously by Ridwan et al. (2015). DNA was amplified by using primer 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGTTACCTTGTTAGACTT3'). Amplification of PCR reaction was used in a total volume of 50 μL consisted of 25 μL KAPPA Ready mix (Firstbase), 5 μL of dissolved DNA (<1 μg), 10 pmol of each primer and up to 50 μL 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 μL) 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.

**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 rRNA amplification was performed as described previously by Ridwan et al. (2015). DNA was amplified by using primer 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGTTACCTTGTTAGACTT3'). Amplification of PCR reaction was used in a total volume of 50 μL consisted of 25 μL KAPPA Ready mix (Firstbase), 5 μL of dissolved DNA (<1 μg), 10 pmol of each primer and up to 50 μL 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 μL) 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* 

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**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
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 compared 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*| 173.33*       | 176.79*  | 0.0527*  | 68.47ab | 60.36bc |
| LABRumen26    | 6.68*| 197.17*       | 192.52*  | 0.0610*  | 67.57ab | 56.23ab |
| LABRumen27    | 6.70*| 195.67*       | 190.66*  | 0.0623*  | 69.26ab | 57.48ab |
| LABRumen32    | 6.68*| 194.67*       | 190.19*  | 0.0610*  | 74.04b  | 73.67ab |
| LABRumen37    | 6.67*| 194.67*       | 189.85*  | 0.0617*  | 65.97*  | 57.84*  |
| LABRumen38    | 6.68*| 197.67*       | 193.22*  | 0.0600*  | 69.17ab | 59.98ab |
| LABRumen40    | 6.68*| 196.33*       | 190.39*  | 0.0663*  | 70.64ab | 55.92ab |
| LABRumen42    | 6.67*| 198.17*       | 192.27*  | 0.0620*  | 69.45*  | 55.61ab |
| LABRumen43    | 6.67*| 196.83*       | 192.27*  | 0.0610*  | 68.61ab | 51.08ab |
| LABRumen80    | 6.67*| 194.00*       | 189.22*  | 0.0617*  | 69.45ab | 55.74ab |

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