PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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E-mail: editorpjn@gmail.com

OPEN ACCESS Pakistan Journal of Nutrition

ISSN 1680-5194
DOI: 10.3923/pjn.2018.131.139

Research Article
Effects of Selected *Lactobacillus plantarum* as Probiotic on *In vitro* Ruminal Fermentation and Microbial Population

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Abstract

Background and Objective: Probiotics are widely used in ruminant production, but information about the potential of *Lactobacillus plantarum* (*L. plantarum*) as a probiotic for ruminants is still limited. The aim of this research was to select *L. plantarum* strains as a probiotic for ruminants and to determine their effect on the rumen fermentation system. **Materials and Methods:** The first experiment was conducted using a randomized block design to select 14 strains of *L. plantarum* isolated from rumen cattle. The second experiment was arranged in a completely randomized design using two selected *L. plantarum* strains to determine their effects as a probiotic on rumen fermentation. The substrates used for *in vitro* fermentation were napier grass (*Pennisetum purpureum*) and concentrate in a 70:30 ratio. **Results:** From experiment 1, *L. plantarum* U32 was selected, because it produced low methane/total gas (27.39%) and strain U40 was selected because it had the highest dry matter and organic matter rumen disappearance (56.45 and 56.44%). In experiment 2, the addition of *L. plantarum* U32 and U40 as probiotics increased propionic acid and decreased acetic production ($p<0.05$), which led to a lower A:P ratio ($p<0.05$). The total volatile fatty acid and *in vitro* digestibility were not affected by the addition of *L. plantarum*. Probiotic addition increased lactic acid bacteria and the protozoa population ($p<0.05$) from the rumen fluid compared to the control. The total rumen bacteria were not significantly changed by the treatments. **Conclusion:** The addition of *L. plantarum* strains U32 and U40 as probiotics had beneficial effects for rumen fermentation due to increased propionic acid and decreased methane production.

Key words: *Lactobacillus plantarum*, probiotic, lactic acid bacteria, methane, rumen fermentation

Received: October 17, 2017 Accepted: February 08, 2018 Published: February 15, 2018

Citation: Wulansih D. Astuti, Komang G. Wiryawan, Elizabeth Wina, Yantyati Widyastuti, Sri Suharti and Roni Ridwan, 2018. Effects of selected *Lactobacillus plantarum* as probiotic on *in vitro* ruminal fermentation and microbial population. Pak. J. Nutr., 17: 131-139.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ruminant productivity can be improved by increasing feed utilization through the manipulation of the microbial ecosystem of the rumen. The use of living microbial supplements as probiotics provides a suitable alternative to antibiotics, because it does not leave residues or cause toxicity in livestock products. Probiotics are living microbial feed supplements that may beneficially affect the host animal upon ingestion by improving its intestinal microbial balance[1]. Probiotics are also defined as living microbes that when administered in adequate amounts, confer a health benefit to the host[2].

Major probiotic strains are *Lactobacillus*, *Saccharomyces*, *Bacillus*, *Streptococcus* and *Aspergillus*[3-5]. Bacterial probiotic strains may be classified as lactic acid producing bacteria, lactic acid utilizing bacteria, or other microorganisms[6,7]. Lactic acid bacteria (LAB) already used as probiotics for ruminants are species of *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus*, *Bacillus* and *Propionibacterium*[8]. LAB is naturally found in many different habitats, such as fermented foods, fruits, grains, animal digestive tract or silage and it ferments sugars or carbohydrates to produce lactic acid. In the digestive tract of cattle, LABs were found in the rumen[9,10]. They could interact with rumen microorganisms and enhanced rumen microbial activity, improved rumen degradability[11] and has shown potential to reduce methane emissions[12].

One LAB that has the potential to serve as a probiotic is *Lactobacillus plantarum*, which produces lactic acid from their metabolism. The addition of *L. plantarum* cultures to *in vitro* rumen fermentation can increase propionic production and decrease acetic acid production and cumulative methane production[13]. In contrast, another *in vitro* study has reported no reductions in methane production[14]. There is also evidence that *L. plantarum* significantly increased digested organic matter and increased total volatile fatty acid (VFA) production. The effect of *L. plantarum* on *in vitro* rumen fermentation was influenced by dosage and the bacterial strain used in the experiment[15].

The cause of improved animal performance by the addition of LAB is not completely clear and the response in ruminants is inconsistent. Strain differences affect the probiotic ability to improve rumen fermentation. Information on the effect of *L. plantarum* addition on rumen fermentation is lacking in the literature. Therefore, it is necessary to select *L. plantarum* strains that have a beneficial effect on rumen fermentation. The objective of this study was to select *L. plantarum* strains as probiotics for ruminants and to evaluate the effect of the *L. plantarum* addition on *in vitro* rumen fermentation and the microbial population.

MATERIALS AND METHODS

***Lactobacillus plantarum* strains:** Fourteen strains of *L. plantarum* were obtained from the collection of the Laboratory of Applied Microbiology, Research Center for Biotechnology, Indonesian Institutes of Sciences, Indonesia. All strains were grown in MRS (de Man Rogosa Sharpe) broth medium and incubated at 39°C for 20 h, under anaerobic conditions, using Hungate tubes flushed with CO_2.

***In vitro* rumen fermentation:** Rumen fluids were obtained from two rumen-fistulated Ongole crossbred cattle before the morning feeding (mixed in 1:1 ratio). The rumen-fistulated Ongole crossbred cattle were managed according to the protocols approved by the Ethic Clearance Committee of Indonesian Institute of Sciences (Number 9879/WK/HK/XI/2015). Rumen fluid was filtered through a double layer of cheese cloth for *in vitro* studies, pooled in pre-warmed bottles, sealed and immediately transported to the laboratory. The substrate used for *in vitro* rumen fermentation was a mixture of concentrate feed and dried milled elephant grasses (*Pennisetum purpureum*), with 70:30 ratio. The concentrate consisted of rice bran, corn, corn gluten feed, coconut meal, palm kernel meal, pollard, soybean meal, mineral mix, DCP (dicalcium phosphate) and NaCl and it contained 18% crude protein, 9.8% crude fiber and 70% TDN (Total Digestible Nutrients). Approximately 0.75 g of substrates (consisting of 70% *P. purpureum* and 30% concentrate proportion) was put inside the serum bottle glass and filled with 75 mL mixture rumen fluid and Mc'Dougall buffer. The bottle was closed with a rubber cap and an aluminum crimp after it was flushed with CO_2 gas for 30 sec to obtain anaerobic conditions. Then, the bottle was incubated in a water bath incubator at a temperature of 39°C[16].

Experiment 1: *In vitro* rumen fermentation was conducted to find the best candidates from 14 strains of *L. plantarum* as ruminant probiotics. The experimental design was arranged in a randomized block design with 3 replications and 15 treatments. The control treatment was rumen fermentation without the addition of *L. plantarum* and the other 14 treatments were rumen fermentation with *L. plantarum* addition. One mL of each *L. plantarum* strain (10^9 CFU mL^{-1}) was added to each experimental tube. Total gas and methane production were measured at 3, 6, 9, 12 and 24 h of fermentation. Dry matter and organic matter rumen disappearance (DMRD and OMRD) were measured after 24 h of fermentation.

Experiment 2: Two strains of *L. plantarum* from experiment 1 were selected as probiotic candidates and used for further analysis in experiment 2. The experiment was arranged in a completely randomized design with 5 replications and 3 treatments, consisting of a control (without addition *L. plantarum*), the addition of *L. plantarum* strain U32 and the addition of *L. plantarum* strain U40. One mL of each *L. plantarum* strain (10^9 CFU mL^{-1}) was added to each experimental tube. A rumen fluid sample was collected for NH_3, VFA, protozoal number, rumen bacterial and LAB population after 4 h of incubation. *In vitro* dry matter digestibility (DMD) and organic matter digestibility (OMD) was measured after 2×48 h of fermentation, with the addition of pepsin-HCl after 48 h of fermentation.

Parameters measured: The total gas and methane production in experiment 1 was measured using a glass syringe according to the methods of Fievez *et al*.[17]. Rumen pH was measured with a pH meter. The concentration of NH_3 was measured by the microdiffusion Conway method[18]. The total VFA concentration and molar proportions of VFA were analyzed using gas chromatography (GC 8A, Shimadzu Corp., Kyoto, Japan, with capillary column type containing 10% of SP-1200, 1% of H_3PO_4 on 80/100 Cromosorb WAW and nitrogen as the gas carrier). The total rumen bacteria were quantified using 9-85 medium with rolled-tube[19]. The LAB population was quantified with total plate count (TPC) methods using an MRS agar plate in the form of Colony-Forming Units (CFU) incubated in 39°C for 24 h in an anaerobic condition using an anaerobic jar with anaeropack to reduce the oxygen. The number of protozoa in the buffered rumen fluid was counted after 4 h of incubation under a microscope. The contents of the fermentation tubes were mixed and a 1 mL aliquot was taken and mixed with 1 mL of methyl green formaldehyde saline solution, containing 35% formaldehyde, distilled water, methyl green and NaCl. The stained sample was kept at room temperature. Protozoa populations were counted with Fuchs Rosenthal Counting Chamber (4×4×0.2 mm) under a microscope (10×10)[19]. Dry matter and organic matter disappearance (DMRD and OMRD) were measured after 24 h of fermentation, while dry matter and organic matter digestibility (DMD and OMD) were measured after 2×48 h of fermentation, with the addition of pepsin-HCl after 48 h of fermentation, using the Tilley and Terry[20] method.

Statistical analysis: Data were analyzed by a one-way analysis of variance using SPSS 16 (SPSS, Inc., IBM, Chicago). Significant effects of treatments were determined by Duncan's multiple range test. Significant differences were accepted if $p<0.05$.

RESULTS

Experiment 1: The total gas production from 24 h *in vitro* rumen fermentation of each *L. plantarum* strain (14 strains) was significantly higher than the control treatment ($p<0.05$) (Table 1). The highest total gas production was observed in the treatment with the addition of *L. plantarum* strain U32 (146.67 mL), while the control treatment produced the lowest gas production (123.67 mL). A similar result also obtained from methane production and the control treatment produced the lowest methane (34.67 mL). The addition of *L. plantarum* significantly increased ($p<0.05$) methane production, but there were no significant differences among *L. plantarum* strains used in this experiment. The lowest % methane/total gas production resulted from addition of *L. plantarum* strain U90 (26.77%), which was significantly lower compared to the highest % methane/total gas produced by strain U40 (28.91%). None of the treatments were affected by the final pH of the rumen fluid after 24 h *in vitro* incubation, which ranged from 6.93-7.00.

Table 1: Total gas, methane production, methane production/total gas and pH of *in vitro* rumen fermentation with the addition of different strains of *L. plantarum*

Treatments	Total gas (mL)	Methane (mL)	Methane production/total gas (%)	pH
Control	123.67^{a}	34.67^{a}	28.06^{ab}	6.97
U26	143.33^{bc}	41.00^{b}	28.58^{ab}	7.00
U27	142.00^{bc}	40.17^{b}	28.27^{ab}	7.00
U32	146.67^{c}	40.17^{b}	27.39^{ab}	6.97
U37	139.33^{bc}	39.33^{b}	28.27^{ab}	6.97
U38	142.67^{bc}	40.17^{b}	28.16^{ab}	6.97
U40	138.33^{b}	40.00^{b}	28.91^{b}	6.97
U42	143.00^{bc}	40.50^{b}	28.35^{ab}	6.93
U43	138.33^{b}	38.83^{b}	28.10^{ab}	6.97
U72	145.67^{bc}	40.33^{b}	27.63^{ab}	6.97
U46	138.67^{bc}	38.83^{b}	28.09^{ab}	6.97
U74	141.00^{bc}	40.50^{b}	28.75^{ab}	6.93
U89	139.00^{bc}	38.83^{b}	27.98^{ab}	7.00
U90	142.33^{bc}	38.17^{b}	26.77^{a}	6.97
U115	141.67^{bc}	39.00^{b}	27.48^{ab}	6.93

$^{a-c}$ Means with different superscripts within columns significantly differed ($p<0.05$)

Table 2: Dry matter and organic matter rumen disappearance, total gas/digested organic matter and methane/digested organic matter of *in vitro* rumen fermentation with the supplementation of *L. plantarum*

Treatments	Dry matter rumen disappearance (%)	Organic matter rumen disappearance (%)	Total gas/digested organic matter (mL/100 mg)	Methane/digested organic matter (mL/100 mg)
Control	50.84^{ab}	51.41^{ab}	37.49^{a}	10.54^{a}
U26	47.33^{ab}	48.75^{ab}	46.10^{c}	13.20^{b}
U27	51.21^{ab}	53.96^{ab}	41.06^{abc}	12.14^{ab}
U32	52.24^{ab}	53.33^{ab}	43.02^{abc}	11.79^{ab}
U37	51.08^{ab}	52.08^{ab}	41.84^{abc}	11.82^{ab}
U38	49.75^{ab}	50.76^{ab}	44.36^{abc}	12.52^{ab}
U40	56.45^{b}	56.44^{b}	38.59^{ab}	11.20^{ab}
U42	51.09^{ab}	51.49^{ab}	44.01^{abc}	12.54^{ab}
U43	49.12^{ab}	50.67^{ab}	42.69^{abc}	11.96^{ab}
U72	48.22^{ab}	49.82^{ab}	45.67^{bc}	12.65^{ab}
U46	50.90^{ab}	51.45^{ab}	42.12^{abc}	11.85^{ab}
U74	50.58^{ab}	51.08^{ab}	43.22^{abc}	12.40^{ab}
U89	50.47^{ab}	51.11^{ab}	42.53^{abc}	11.87^{ab}
U90	46.73^{a}	48.52^{a}	45.96^{c}	12.34^{ab}
U115	47.67^{ab}	48.52^{a}	45.59^{bc}	12.53^{ab}

$^{a-c}$Means with different superscripts within columns significantly differed ($p<0.05$)

Table 3: Volatile fatty acid (VFA) production of *in vitro* rumen fermentation with the addition of *L. plantarum*

Treatments	Total VFA (mol L^{-1})	Propionic acid (%)	Iso-butyric acid (%)	Butyric acid (%)	Iso-valeric acid (%)	Valeric acid (%)	Acetic acid (%)	A/P	Methane (mol L^{-1})
Control	52.67	21.18^{a}	3.07^{a}	9.04^{a}	2.04^{a}	1.07^{a}	63.60^{c}	3.01^{b}	13.93
L. plantarum U32	49.15	25.28^{b}	3.58^{a}	10.51^{ab}	2.52^{a}	1.37^{ab}	56.74^{b}	2.25^{a}	11.37
L. plantarum U40	51.06	25.42^{b}	4.79^{b}	12.64^{b}	3.79^{b}	1.64^{b}	51.71^{a}	2.04^{a}	10.92

$^{a-c}$Means with different superscripts within columns significantly differed ($p<0.05$), A/P: Acetic acid/propionic acid

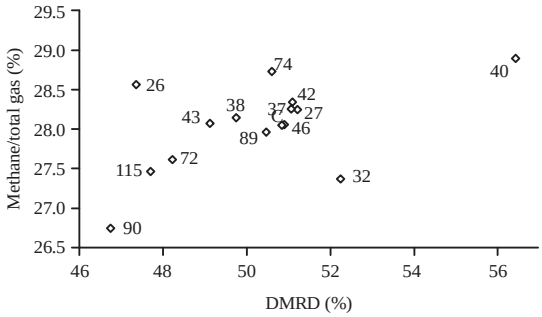

Fig. 1: Correlation of DMRD (%) and methane/total gas production (%) from *in vitro* rumen fermentation with the addition of *L. plantarum*

The addition of *L. plantarum* as a probiotic did not significantly affect *in vitro* dry matter and organic matter rumen disappearance (DMRD and OMRD) (Table 2) compared to the control. *L. plantarum* strain U40 resulted in the highest DMRD and OMRD (56.45 and 56.44%, respectively), which were significantly higher than the lowest DMRD and OMRD (46.73 and 53%, respectively) that resulted from strain U90. The production of methane from each 100 g DMRD of feed was calculated to determine which *L. plantarum* strain produced the lowest methane. Among all strains used in this experiment, *L. plantarum* strain U40 produced the lowest methane/100 mg digested organic matter, although it was not significantly lower than the highest methane production from *L. plantarum* strain U26. However, from all treatments, the control without the addition of *L. plantarum* produced lower methane/100 mg digested organic matter, although it was not significantly different from the other treatments, except from *L. plantarum* strain U26.

The correlations between *in vitro* DMRD and % methane/total gas production of each *L. plantarum* strain used in this experiment are shown in Fig. 1. *L. plantarum* strain U40 showed the highest DMRD among treatments, but high % methane/total gas production, while strain U32 showed the lower % methane/total gas production but also lower DMRD. *L. plantarum* strain U26 showed the lowest % methane/total gas production but also had the lowest DMRD among treatment.

Experiment 2: The results from experiment 2 found that *L. plantarum* strains U32 and U40 were candidates for probiotics. The total and individual VFA productions from *in vitro* rumen fermentation are shown in Table 3. The total VFA production was not significantly affected by the addition of both *L. plantarum* strains U32 and U40. The addition of *L. plantarum* as a probiotic changed the composition of VFA. Acetic acid significantly decreased ($p<0.05$), while propionic acid significantly increased ($p<0.05$) compared to the control treatment. *L. plantarum* strain U40 produced the lowest

Table 4: Rumen fermentation characteristic from *in vitro* rumen fermentation with the addition of *L. plantarum*

Treatments	DMD (%)	OMD (%)	NH_3 (%)	pH
Control	77.68	78.43	8.48[a]	6.98
L. plantarum U32	76.51	75.86	17.17[b]	6.95
L. plantarum U40	74.62	74.20	16.37[b]	6.95

DMD: Dry matter digestibility, OMD: Organic matter digestibility, [a-c]Means with different superscripts within columns significantly differed (p<0.05)

Table 5: Rumen microbes population of *in vitro* rumen fermentation with the supplementation of *L. plantarum*

Treatments	Total bacteria	Lactic acid bacteria	Protozoa
	(log 10 CFU mL^{-1})		
Control	10.57	6.64[a]	3.72[a]
L. plantarum U32	10.32	8.92[c]	4.10[b]
L. plantarum U40	9.90	8.10[b]	4.12[b]

[a-c]Means with different superscripts within columns significantly differed (p<0.05)

proportion of acetic acid (51.71%), followed by *L. plantarum* strain U32 (56.74%), which was significantly higher than U40. The control treatment without the addition of *L. plantarum* produced the highest proportion of acetic acid (63.60%). The highest propionic acid was produced by the addition of *L. plantarum* strain U40 (25.42%), which was not significantly different from *L. plantarum* strain U32 (25.28%). The lowest propionic acid was produced by the control, which was significantly lower than the other treatments (21.18%).

Butyric and valeric acid significantly increased (p<0.05) with the addition of *L. plantarum* strain U40 compared to the control treatment. Although the addition of *L. plantarum* strain U32 also increased butyric and valeric acid, it was not significantly compared to the control treatment. Higher propionic acid and lower acetic acid production by *L. plantarum* addition resulted in a significantly (p<0.05) lower acetic:propionic acid (A:P) ratio compared to the control. The predicted methane production was calculated from acetic, propionic and butyric acid production using the Moss equation[21]. The addition of *L. plantarum* strains U32 and U40 decreased methane production from *in vitro* rumen fermentation between 18.43-21.62%.

The *in vitro* dry matter digestibility (DMD) and organic matter digestibility (OMD) were not significantly affected by the addition of *L. plantarum* as a probiotic and the values were comparable to the control (Table 4). The production of NH_3 significantly increased (p<0.05) with the addition of *L. plantarum* compared to the control, but both strains showed comparable production of NH_3. The NH_3 production of the control was 8.48% and it increased to 17.17 and 16.36% with the addition of strains U32 and U40, respectively. Treatments were not significantly effect pH of rumen fluid, which varied between 6.95 (*L. plantarum* U32 and U40) to 6.98 (control).

The populations of total rumen bacteria, LAB and protozoa are shown in Table 5. Rumen bacteria were not significantly affected by the addition of *L. plantarum* U32 (10.32 CFU mL^{-1}) and U40 (9.90 CFU mL^{-1}), although it was lower compared to the control (10.57 CFU mL^{-1}). The addition of *L. plantarum* significantly increased (p<0.05) the LAB population in the rumen. The highest LAB population (log 10) resulted from the addition of *L. plantarum* strain U32 (8.92 CFU mL^{-1}), which was significantly higher than strain U40 (8.10 CFU mL^{-1}). The control treatment without the addition of *L. plantarum* resulted in the lowest LAB population (6.64 CFU mL^{-1}), which was significantly lower than the other treatments. Protozoal number (log 10) also significantly increased (p<0.05) with the addition of LAB as probiotics, from 3.72 CFU mL^{-1} (control) to 4.099 and 4.121 CFU mL^{-1} with the addition of *L. plantarum* strains U32 and U40, respectively.

DISCUSSION

The selection of *L. plantarum* strains as candidates for ruminant probiotics was based on their gas production and rumen disappearance during *in vitro* rumen fermentation. The total gas production results showed that *L. plantarum* strain U32 produced the highest gas. The addition of a probiotic must have a beneficial effect for rumen fermentation, such as increased feed digestibility or lowered methane production. A different result was obtained when % methane/total gas production was calculated, which showed strain U90 as the lowest methane producer. Experiment 1 did not measure VFA production, therefore, methane gas production becomes an important parameter for selecting the best candidates for ruminant probiotics.

The increased total gas production was usually associated with the increase of dry matter and organic matter rumen disappearance (DMRD and OMRD). The effects of *L. plantarum* addition on gas and methane production may appear during the initial stages of *in vitro* fermentation but are largely absent at the end of the incubation[15]. From the DMRD parameter, methane production/100 mg digested organic matter can be calculated. The results showed that the lowest amount of methane was produced by *L. plantarum* strain U40 in this experiment. Lower methane production indicates more efficient rumen fermentation, but low % methane production from strain U90 resulted from poor feed digestibility in the rumen. The digestibility from strain U90 was the lowest among all treatments. Therefore, although *L. plantarum* strain U90 produced the lowest % methane production, it would not be selected as a probiotic candidate.

The correlation of DMRD and % methane/total gas production is shown in Fig. 1. The selection of the best candidates of *L. plantarum* strains for probiotics for ruminants was based on the highest DMRD and % methane/total gas production. It can be seen that *L. plantarum* strain U40 showed the highest DMRD, while strain U32 showed the lowest % methane/total gas production. As previously mentioned, although strain U90 resulted in the lowest methane production, it resulted from poor digestibility. U90 was not selected, because it showed the lowest DMRD. Therefore, *L. plantarum* strains U40 and U32 can be selected as candidates for ruminant probiotics.

A further experiment on *Lactobacillus plantarum* strains U40 and U32 was conducted to reveal more details about their effects on *in vitro* rumen fermentation. In this experiment, the addition of *L. plantarum* strains U40 and U32 significantly changed the composition of VFA produced from rumen fermentation to higher propionic acid and lower acetic acid production. Total VFA production was affected by the addition of *L. plantarum* strains U40 and U32, although they were slightly reduced. Increased propionic acid, with the addition of LAB, was reported within the study conducted by Soriano *et al*.[22], but in that experiment, there was no decrease in acetic acid production. The addition of LAB may stimulate the growth of lactic acid utilizing bacteria[23], leading to increased production of propionic acid as a result of their metabolism. When higher propionic acid was produced, the supply of H_2 for methane production in the rumen was reduced.

The changes in rumen fermentation products to higher propionic acid and lower acetic acid, followed with a lower A:P ratio, indicated the potential of *L. plantarum* added as a probiotic for ruminants toward more efficient rumen fermentation by lower methane production. More efficient rumen fermentation by increased propionic acid could improve growth efficiency[24]. Methane produced by ruminants represents an energy loss for the host animal of 2-12% of dietary energy[21]. Therefore, decreased methane production will increase the energy supply of the animal, followed by increased productivity. O'Brien *et al*.[13] reported high methane reduction with the addition of *L. plantarum*, with more than a 60% decrease in cumulative methane production from *in vitro* rumen fermentation, but total VFA production (mM) was significantly reduced. The reduction of methane production was due to the general suppression of the fermentation process, when *L. plantarum* was directly added into the *in vitro* bottle. In this experiment, the reduction of methane was approximately 18-20%, without a significant reduction of total VFA production. This result indicated that there was no negative impact on rumen fermentation from the addition of *L. plantarum* strains U32 and U40.

VFA production in the rumen highly depends on the degree and rate of fermentation[25,26]. *In vitro* dry matter digestibility (DMD) and organic matter digestibility (OMD) were not significantly affected by the addition of *L. plantarum* as a probiotic and the values were comparable with the control (Table 4). In this experiment, the digestibility results were in line with total VFA production. There were no significant changes in either parameters compared with control, which indicated that rumen microbes were still working normally with the addition of *L. plantarum* as a probiotic measured from rumen fermentation products. An experiment by Contreras-Govea *et al*.[14] reported significantly increased digestibility with the addition of *L. plantarum*. Increased digestibility showed that the addition of LAB can stimulate fibrolytic bacteria in the rumen. These results showed that the effect of the addition of *L. plantarum* as a probiotic on rumen fermentation depends on the type of strains, dose and substrate incubated [27].

The addition of *L. plantarum* strains U40 and U32 did not significantly change the total bacteria population, although a numerical decrease was observed. This result was in agreement with another experiment[28] that reported decreased total rumen bacteria at 3 h after the addition of a probiotic consisting of *L. plantarum*+*Propionibacterium*. Although other bacteria were added from outside into the rumen ecosystem as a probiotic, it is rare that it can cause significant changes to the total bacteria population. The lack of differences in the total bacteria population caused by treatments could be a reason why the total VFA and digestibility were also not affected by treatments.

Lactic acid bacteria can only have beneficial effects as a probiotic in the rumen if they could survive in the rumen. Although this experiment did not measure the population of *L. plantarum* specifically, a higher population of LAB by the addition of *L. plantarum* indicated that LAB added from outside can survive during rumen fermentation and stimulate other LABs to survive in the rumen. Although LAB species are facultative anaerobic bacteria, they survive in both the rumen and intestine[29]. Lactic acid bacteria could survive in the rumen during *in vitro* fermentation, particularly when sugar substrates were used for fortification[30]. The survival of *L. plantarum* used in this experiment might be because they were isolated from rumen cattle. It might help them to adapt more easily in an anaerobic rumen environment.

The increased protozoal number with the addition of *L. plantarum* as a probiotic can be correlated with the increased in NH_3 production. An increased protozoal number was also reported after the probiotic addition consisted of LAB and *Propionibacterium* after acidosis induction[28]. Ammonia was produced by protozoa as a result of the digestion of bacteria by protozoa. The increased protozoal number, along with decreased methane production, was unpredictable, because 9-25% of methanogenesis in the rumen was produced by methanogens associated with protozoa[31]. In addition, the, defaunation of protozoa has been known as a method to decrease methane production in the rumen. In this experiment, decreased methane production may not be caused by the decrease in methanogens, but it more likely is caused by the change in rumen microbial diversity, because *L. plantarum* addition stimulated propionate producer and fermentation shifts that divert H_2 away from methanogenesis[32,33]. Supplementation of live LAB was assumed to modify the rumen microbial population and help rumen microbes adapt to lactic acid in the rumen, in order to reduce the incidence of acute ruminal acidosis[34-37]. Lactic acid utilizer was probably stimulated by lactic acid produced by the *L. plantarum* addition and produced propionate production as metabolites products.

The implication of this experiment is that the addition of *L. plantarum* as a probiotic beneficially affected rumen fermentation, which changed to propionate production. Rumen fermentation is more efficient by producing less methane. These results showed that the application of *L. plantarum* as a probiotic is feasible and negative effects caused by *L. plantarum* addition were not detected. More experiments regarding an *in vivo* trial of *L. plantarum* addition is recommended to fulfill the effects of *L. plantarum* as a probiotic for ruminants.

CONCLUSION

Based on results from the experiments, *L. plantarum* strains U32 and U40 were selected as probiotics for ruminants based on their potential to affect rumen fermentation. The results also indicated that the addition of *L. plantarum* has beneficial effects in the rumen. The addition of *L. plantarum s*trains U32 and U40 on *in vitro* rumen fermentation as probiotics changed toward more efficient rumen fermentation by increased propionic acid and lowered acetic acid proportions, indicating lower methane production, which provides higher energy for the animal.

SIGNIFICANCE STATEMENT

This study discovers probiotics for ruminants that contain *L. plantarum* as a lactic acid producer. This study will help the researchers to uncover the effect of *L. plantarum* addition as a probiotic for rumen fermentation and rumen microbes that many researchers were not able to explore. Thus, a new theory on the role of *L. plantarum* as a probiotic for ruminants may be arrived at.

ACKNOWLEDGMENT

The authors wish to acknowledge the financial support received from "DIPA Unggulan-LIPI (2015-2017) Food Security and Drug's Discovery" of the Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI) and LPDP (Lembaga Pengelola Dana Pendidikan) scholarship.

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