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Survival of *Lactobacillus plantarum*U40 on the *in vitro* rumen fermentation quantified with real-time PCR

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ABSTRAK

Tujuan dari penelitian ini adalah untuk menganalisa daya tahan *L. plantarum*U40 menggunakan real-time PCR dalam fermentasi rumen *in vitro*. Desain penelitian yang digunakan adalah Rancangan Acak Kelompok dengan 3 perlakuan dan 4 ulangan. Perlakuan yang diberikan adalah kontrol, inokulasi dengan *L. plantarum*U40 dan*L. plantarum*U40 + glukosa. Perlakuan dengan inokulasi menghasilkan populasi *L. plantarum*U40 yang lebih tinggi. Setelah 8 jam inkubasi, pemberian glukosa cenderung menurunkan populasi *L. plantarum* U40. Perlakuan kontrol menghasilkan populasi *L. plantarum* U40 yang paling rendah selama fermentasi *in vitro.*Inokulasi *L. plantarum* U40meningkatkan populasi Bakteri Asam Laktat secara signifikan (P<0.05) sampai dengan 12 jam inkubasi dibandingkan dengan kontrol. Perlakuan kontrol mempunyai pH yang paling tinggi selama masa inkubasi. Penambahan glukosa secara signifikan (P<0.05) menurunkan pH rumen pada akhir inkubasi (24 jam) (6.30), dibandinkgkan dengan kontrol (6.85).Inokulasi dengan *L. plantarum*U40 + glukosa secara signifikan (P<0.05) meningkatkan asam propionat, menurunkan asam asetat serta nisbah A/P dibanding perlakuan lainnya.*Lactobacillus plantarum* U40 tanpa penambahan glukosa tidak memberikan efek terhadap produksi propionat secara signifikan. Dapat disimpulkan bahwa *Lactobacillus plantarum*U40 dapat bertahan hidup di dalam rumen serta mengubah fermentasi rumen ketika glukosa ditambahkan sebagai sumber karbon.

Kata kunci: Lactobacillus plantarumU40, daya tahan, glukosa,fermentasi rumen, quantitative real-time PCR

ABSTRACT

The objective of this study was to evaluate the survival of *L. plantarum*U40 quantified with real-time PCR during *in vitro* rumen fermentation. The experiment was arranged in a randomized block design with 3 treatments and 4 replications. Treatments were control, rumen fermentation inoculated with *L. plantarum*U40and *L. plantarum*U40 + glucose solution. Population of *L. plantarum* U40 was higher at inoculation treatment. After 8 hours incubation, glucose addition tended to decrease *L. plantarum* U40 population. Control treatment showed lowest population of *L. plantarum* U40 along *in*

vitro fermentation compared with other treatment. Inoculation of *L. plantarum*U40 significantly ($p<0.05$) increased population of LAB until 12 hours incubation compared with control. Control treatment had highest pH at all incubation time. Glucose addition significantly ($P<0.05$) decreased final rumen pH (24 hours) (6.30), compared with control treatment (6.85). Inoculation of *L. plantarum* U40 with glucose addition significantly ($P<0.05$)increased propionic acid, decreased acetic acid and A/P ratio compared with other treatments. *Lactobacillus plantarum* U40 without glucose addition did not affect propionic acid production significantly. As conclusion, *Lactobacillus plantarum* U40 can survive in rumen fluid and changes rumen fermentation when glucose is added as carbon source.

Keywords: Lactobacillus plantarumU40, survival, glucose, rumen fermentation, quantitative real-time PCR

INTRODUCTION

Demand of probiotic in animal production is increasing due the total ban of antibiotic application as feed additives started from 2006 onwards in European Union countries (Cheng *et al*., 2014; Yirga, 2015) and 2018 in Indonesia according to The Regulation of Minister of Agriculture number 14/Permentan/PK. 350/5/2017.According to FAO/WHO (2001) probiotics defined as living microbes which when administered in adequate amounts confer a health benefit to the host. The working of probiotics in the rumen would yield benefits such as modified fermentation patterns and enhanced fiber digestion, both of which are directly linked to improve animal performance (McAllister *et al*. 2011).

The probiotic effect of lactic acid bacteria (LAB) has been documented in human and animals. Species of *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus*, *Bacillus* and *Propionibacterium* are LAB that already used as probiotics for ruminants (Seo *et al*., 2010). Lactobacillus species stimulate indigenous LAB and the production of short-chain fatty acids, and may modulate the intestinal immune response (Ohashi and Ushida, 2009). Several LAB species including *Lactobacillus plantarum, Lactobacillus acidophilus* and *Enterococcus faecium* were evaluated as potential probiotics, as they stimulate the activity of lactic acid utilizing rumen microbes and thereby ameliorate and prevent acidosis (McAllister *et al*. 2011).

In order to affect rumen microbes as beneficial effects of probiotic, the survival of LAB in the rumen needs to be addressed. Weinberg *et al*. (2003) examined the changes in populations of silage LAB (*L. plantarum, E. faecium* and *Pediococcus pentosaceus*) during *in vitro* rumen incubation and concluded that acceptable numbers of LAB could survive, particularly when sugar substrates were fortified. Similarly, Rodriguez-Palacios *et al.* (2009) isolated *L. plantarum* from caecum content and both *P. pentosaceus* and *P. acidilactici* from bovine faecal matter, suggesting that LAB species used as inoculants may survive and confer probiosis in both the rumen and intestine.

One hypothesis about the mechanism of *L. plantarum* as probiotic is its interaction with rumen microbes to enhance rumen functionality (Weinberg *et al*., 2004). Only limited studies have been reported about the survival of *L. plantarum* in rumen fluid, mostly because its' role as probiotic for ruminant is not clear yet. For that reason, the survival of the strain during rumen fermentation as one of a desirable probiotic property for ruminant (FAO/WHO, 2002), need to be examined. Real-time PCR has the ability to enumerate species specific targeted bacteria with high sensitivity and has been used to analyze rumen microbes samples (Wanapat and Cherdthong, 2009; Singh *et al*., 2014; Grilli *et al*., 2016). Therefore, the objective of this study was to evaluate the survival of *L. plantarum*U40 quantified with real-time PCR during *in vitro* rumen fermentation.

MATERIALS AND METHODS

The study was conducted at the Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, West Java, Indonesia.

*Lactobacillus plantarum S*train and Culture Conditions

Strain *L. plantarum*U40 used in this study was collection of Laboratory of Applied Microbiology, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). It was isolated from rumen of Ongole Breed cattle (Ridwan *et al*., 2018). It was cultured

in deMan Rogosa Sharpe (MRS) broth medium (Merck, Darmstadt, Germany) (Weinberg *et al*., 2003; Jiao *et al*., 2017) at 39°C under anaerobic conditions.

***In vitro* Rumen Fermentation and Experimental Design**

Rumen fluid was obtained from two rumen-fistulated Ongole crossbred cattle before morning feeding, mixed in equal portion. 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 cheesecloth for *in vitro* studies, pooled in pre-warmed bottles, sealed and immediately transported to the laboratory.

The experiment design was arranged in a randomized block design with 3 treatments and 4 replications. The experiment was repeated 4 different times which treated as block. Rumen fermentation without any addition served as a control (C). Two other treatments were inoculation of *L. plantarum* U40 (RP) and inoculation of *L. plantarum* U40+ glucose solution (RPG). Rumen fluid for RPG treatments was added with sterile 50% (w/v) glucose solution to a final concentration of 5 g/L according to Weinberg *et al*. (2003). One ml of *L. plantarum*U40 strain (10^9 cfu/mL) was inoculated to each experimental bottle with RP and RPG treatments.

In vitro rumen fermentation was conducted using serum bottle glass, filled with 75 ml mixture rumen fluid and Mc'Dougall buffer (1:2 ratio). The bottle was closed with rubber cap and aluminum crimp after flushed with CO_2 gas for 30 s to get anaerobic conditions and incubated in water bath incubator at temperature of 39°C (Theodorou, 1994). At 0, 4, 8, 12 and 24 h incubation, bottles from each treatment were sampled for pH, and LAB population measurement.VFA concentration and NH3 were measured from rumen fluid at 8 h incubation. Some rumen fluid from each treatment at 0, 4, 8, 12 and 24 h incubations were kept in -20°C for further analysis.

Rumen pH was measured with pH meter (Cyberscan 310, Eutech Instruments, Singapore). Concentration of NH_3 was measured by the microdiffusion Conway method (General Laboratory Procedures, 1966). Total VFA concentration and molar proportions of VFA were analyzed by using gas chromatography (GC 8A, Shimadzu Corp., Kyoto, Japan with capillary coloumn type containing 10% SP-1200, 1% H3PO4 on 80/100 Cromosorb WAW and nitrogen as gas carrier). LAB population was quantified with Total Plate Count (TPC) method using MRS agar plate in the form of colony-forming units (cfu) (Cappucino and Sherman, 2001), incubated at 39°C for 24 hours in anaerobic condition using anaerobic jar with anaeropack (AnaeroGen™, Thermo Scientific, Japan) to reduce the oxygen.

DNA Extraction

Genomic DNA was extracted from 0.5mL aliquots of rumen fluid from *in vitro* fermentation using *Geneaid™ DNA* isolation *kit (Geneaid Biotech Ltd., Taiwan)*, following manufacturer's recommendation. Some modifications were applied according to Ridwan *et al*. (2015). The DNA quality and quantity were checked by agarose gel electrophoresis and NanoDrop spectrophotometer (P-330, Implen NanoPhotometer, Germany) by the absorbance at 260 nm.

Real-Time PCR

All quantification of Real-time PCR amplification and detection were performed using Bio-Rad CFX 96 Touch™ Real time PCR Detection System.Species-specific PCR primers for *L. plantarum* used to amplify partial 16S rDNA regions (target DNA) were F:3'-TTACATTTGAGTGAGTGGCGAACT-5' for forward primer and R:3'AGGTGTTATCC CCCGCTTCT-5'for reversed primer (Klocke *et al*., 2006). The reaction was conducted inafinal volume of 20 µL, carried out in duplicate, containing the following: 10 µL SsoFast SYBR Green Real-Time PCR master mix (product of BioRad), 0.4 µL forward primer, 0.4 µL reverse primer, 7.2 µL Nucleus-Free Water (NFW), and 2 µL DNA template. Amplification programme included an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 30 s and extension at 95°C for 30 s. Negative control without DNA template were run with every assay to assess the overall specificity. All PCR were performed in duplicate. Absolute quantification involved the use of standard curves for *L. plantarum*U40. The construction of standard curves using 10-fold serial dilutions in NFW prior to real-time PCR.

Statistical Analysis

Data were analysed by one way analysis of variance using SPSS 16 (SPSS, Inc., IBM, Chicago). Significant effects of treatments were determined by Duncan's multiple range test method. Significant differences were accepted if $P<0.05$.

RESULTS AND DISCUSSIONS

The isolate *L. plantarum* U40 used in this experiment was selected as best candidates as probiotic for ruminants as the result of previous study (Astuti *et al*., 2018). Investigating the survival of this strain in rumen fluid is important to strengthen its potency as probiotic for ruminant. Figure 1 shows the population of *L. plantarum* U40 in rumen fluid during 24 hours *in vitro* fermentation, as quantified by the real-time PCR assays. Although it was not significant, control treatment without inoculation tended to had the lowest population of *L. plantarum*U40 along *in vitro* fermentation, indicating that *L. plantarum*U40 in the control treatment originated only from rumen fluid. In early stage of fermentation (0-8 hours), population of *L. plantarum* U40 with or without glucose addition were equal. After 8 h, *L. plantarum* U40 population tended to decrease when glucose was added as carbon source, and it continued until the end of incubation. Although it was not significant, decreased of *L. plantarum* U40 in the present of glucose was surprising, considering glucose addition was expected to serve as carbon source for *L. plantarum* U40 to ensure their survival in the rumen fluid. After 8 h, glucose in the rumen fluid was totally used by rumen microbes. Decreased of *L. plantarum* U40 population possibly was an adaptation reaction for lacked of glucose which usually consumed in this treatment. Inoculation of *L. plantarum* U40 without glucose also decreased *L. plantarum* U40 population after 8 h, but not as much as treatment with glucose.

Inoculation of *L. plantarum* U40 affected population of LAB in the rumen fluid for 12 h incubation on *in vitro* fermentation (Table 1). At 0 h incubation, LAB population from rumen with inoculation *L. plantarum* U40 treatment was higher than control, because of *L. plantarum*U40 added to those treatments was living culture and increased LAB population. After that (4 h), population of LAB in the rumen fluid with inoculation *L. plantarum* U40 treatment was decreased, showing adaptation period needed by *L. plantarum* U40 to survive in the rumen fermentation environment. During adaptation period, glucose addition provides *L. plantarum*U40 and other LABs a substrate to help them survived. As a result, decrease LAB population during adaptation period in glucose addition treatment was not as much as in treatment without glucose. The highest population of LAB at 4 h incubation was resulted from glucose addition treatment (7.85), significantly

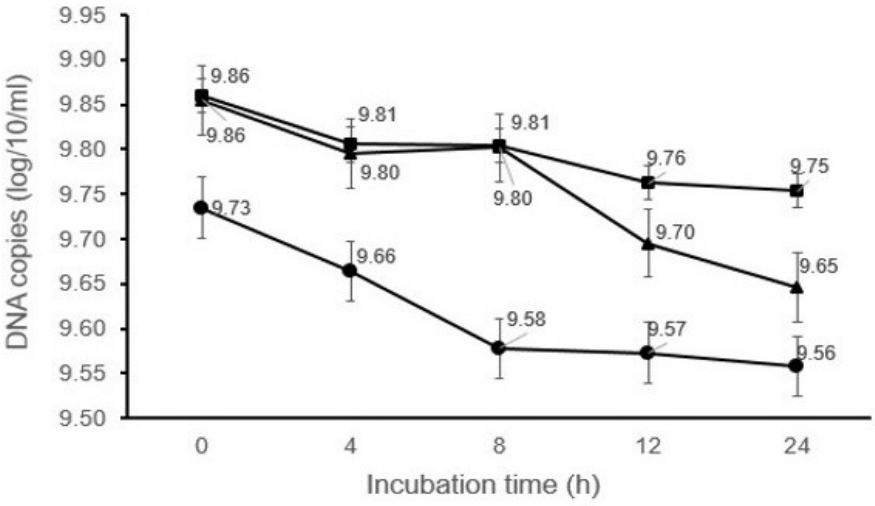

Figure 1. Population of *L. plantarum* in Rumen Fluid during 24 Hours *in vitro* Fermentation. The Symbols Represent Control (●), *L. plantarum* (■) and *L. plantarum* + glucose (▲). Data Points are Mean Values based on Four Replicates. Error Bars represent standard deviation of the Mean.

Table 1. Effect of *L. plantarum* U40 on LAB Population (log 10 cfu/mL) during *In vitro* Fermentation

Treatments	Incubation time (h)				
	0	4	8	12	24
Control	6.48[a]	6.45a	6.64[a]	6.13[a]	5.72
*L. plantarum*U40	8.36[b]	7.16b	8.04[b]	7.97[b]	6.73
*L. plantarum*U40+ glucose	8.28[b]	7.85c	7.73[b]	7.66[b]	6.07

Different superscripts within the same column indicate significantly different at P<0.05

higher than control (6.45) and *L. plantarum* U40 without glucose addition (7.16).

At 8 and 12 h incubation, there was no significant different of LAB population between inoculation of *L. plantarum* U40 with or without glucose addition. Glucose addition in this experiment was only significant during adaptation period to reduce the decreased of LAB population. After that, with or without glucose, LAB population was equal. Both treatments have significantly higher LAB population than control, showing that inoculated *L. plantarum* U40 was survive in rumen. After 24 h incubation, there was no significant different of LAB population between all treatments, although control still tended to have the lowest LAB population. It can be concluded that *Lactobacillus plantarum* U40 could affect LAB population in rumen ecosystem only for 12 h. Ellis *et al.* (2016) also reported that effects of LAB inoculants may appear at initial stages on *in vitro* fermentation, and largely absent at the end of the incubation. Anaerobic condition of rumen was not the most suitable condition for facultative-anerobic bacteria such as *Lactobacillus plantarum* U40 (Smetanková, *et al*., 2012), although it's varies between strains.

*Lactobacillus plantarum*U40 was homogenous lactic acid bacteria. It produces lactic acid as their metabolism product. Lactic acid in rumen fermentation could decreased pH of rumen fluid. Changes of rumen pH during *in vitro* fermentation was observed at 0,4,8,12 and 24 hours incubation (Figure 2). Control treatment had the highest pH at all incubation times. At 0 h, inoculation of *L. plantarum* U40, either with or without glucose addition had significantly (P<0.05) lower pH than control. After that, glucose addition gave significant differences on rumen pH. Inoculation of *L. plantarum* U40 without glucose addition resulted similar pattern of rumen pH with control treatment. While rumen pH with glucose addition continue decreased until 24 h incubation, significantly (P<0.05) lower than other treatments. It confirmed that lower pH in treatment with glucose addition caused by lactic acid produced by *L. plantarum* U40. In this experiment, there was no other source of acid, other than lactic acid produced from LAB, that could decrease rumen pH. This result was an evident that *L. plantarum* U40 can survive in the rumen fluid as long as there was enough supply of substrates to growth. Decreased of rumen pH after inoculation of LAB also reported by Ellis *et al*. (2016). Although inoculation of *L. plantarum* U40 caused rumen pH decreased, but it is still in the normal range which is suitable for growth and activity of rumen microbes. Krause and Oetzel (2006) mention that a physiological range of ruminal pH is between 5.5 – 7.0.

Proposed mechanism for *L. plantarum* as ruminant probiotic was affects rumen microbes leading to changes on rumen fermentation products. Effects of *L. plantarum*U40 inoculation on *in vitro* rumen fermentation was shown in Table 2. Final pH of rumen fluid after 24 hours *in vitro* fermentation was significantly (P<0.05) lowered by glucose addition (6.30), compared with control treatment (6.85). Without glucose addition, inoculation of *L. plantarum* U40 did not change rumen final pH. Decreased of rumen final pH could be resulted from lactic acid produced by *L. plantarum* U40. It seems that glucose addition provides substrate for *L. plantarum* U40 so they could survive and produced more lactic acid than without glucose addition.

Total VFA was not affected by inoculation of *L. plantarum*U40, although glucose addition tended to decrease VFA production. Glucose addition resulted in different effects of *L. plantarum* U40 on composition of VFA. Glucose addition decreased acetic acid production (41.40%), significantly lower (P<0.05) than

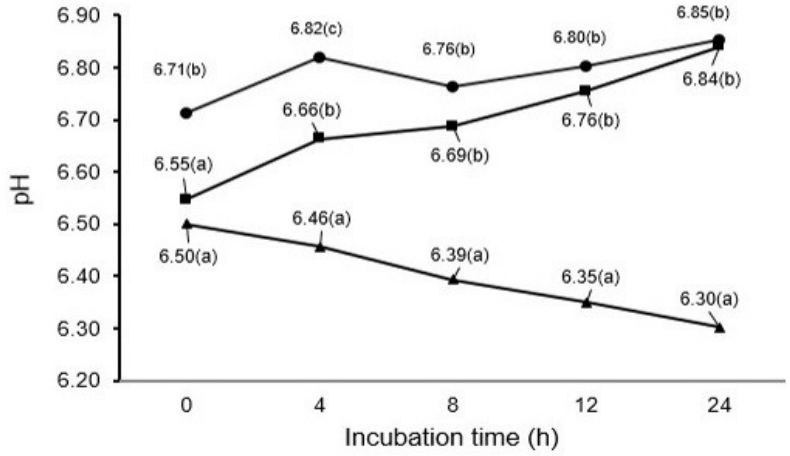

Figure 2. Effect of *L. plantarum*U40 on pH of Rumen Fluid during *In vitro* Fermentation. The Symbols Represent Control (●), *L. plantarum* (■) and *L. plantarum* + glucose (▲). Data Points are Mean Values based on Four Replicates. Error Bars Represent Standard Deviation of the Mean. Different Superscripts within the Same Incubation time (0, 4, 8, 12, 24 h) Indicate Significantly Different at P<0.05 between Treatments

Table 2. Effects of *L. plantarum*U40 on Rumen Fermentation Product from 8 Hours Incubation

Parameters	Treatments		
	Control	*L. plantarum*U40	*L. plantarum*U40 + glucose
pH	6.85^{b}	6.84^{b}	6.30^{a}
Total VFA (mM)	63.95	70.42	60.46
Acetic acid (%)	45.11^{ab}	47.71^{b}	41.40^{a}
Propionic acid (%)	24.65^{a}	23.18^{a}	27.91^{b}
Butyric acid (%)	11.91	11.99	12.77
Isobutyric acid (%)	9.89	8.66	10.04
Valeric acid (%)	2.91	2.82	2.54
Isovaleric acid (%)	5.56	5.64	5.37
A/P ratio	1.85^{b}	2.07^{c}	1.49^{a}
NH_3 (mM)	20.01^{a}	23.06^{b}	19.53^{a}

Different superscripts within the same row indicate significantly different at P<0.05.
VFA=Volatile Fatty Acids; A/P ratio= Acetic acid/propionic acid ratio

inoculation of *L. plantarum* U40 without glucose addition (47.71%). Control treatment did not significantly different compared with both inoculated treatments. Glucose addition provides inoculated *L. plantarum* U40 with nutrition, to ensure they can survived and interact with rumen microbes to shifts rumen fermentation. Without glucose, *L. plantarum* U40 can survived in rumen fluid, but they could not produce lactic acid as much as with glucose addition. This hypothesis

was in line with value of rumen pH with and without glucose addition. This suggests that shifts of rumen fermentation by inoculation of *L. plantarum* U40 influenced by their survival and activity in rumen fluid.

Propionic acid production was significantly increased ($P<0.05$) by inoculation of *L. plantarum* U40 with glucose addition (27.91%), compared with control (24.66%). But inoculation of *L. plantarum* U40 without glucose addition did not affect propionic acid production significantly. Increased propionic acid could be caused by increased lactic acid produced by *L. plantarum* U40. Higher lactic acid in rumen fluid trigger lactic acid utilizer growth. Some lactic acid utilizer such as *Megasphaera elsdenii* and *Propionibacterium* will consume lactic acid, leading to increased production of propionic acid (Seo *et al*., 2010; Luo *et al*., 2017). Increased propionic acid after inoculation of *L. plantarum* also reported by Weinberg *et al*. (2003). Butyric and valeric acid were not affected by *L. plantarum* U40 inoculation. The changes in VFA composition showed one beneficial role of *L. plantarum*U40 as probiotic for ruminant. But other research also reported that inoculation of *L. plantarum* or other LAB did not affect rumen fermentation, more specific on VFA composition. This inconsistency can be concluded that the effect of *L. plantarum* inoculation as probiotic on rumen fermentation depends on the type of strains, dose and substrate utilization (Jiao et al., 2017).

Concentration of NH_3 in the rumen fluid increased significantly ($P<0.05$) by inoculation of *L. plantarum*U40 without glucose addition (23.06 mM), compared both with control (20.01) and *L. plantarum*U40 with glucose addition (19.53 mM). Rumen NH_3 is needed for microbial protein synthesis. Deficiency of NH_3 will inhibit microbial synthesis, while high concentration of NH_3 also inhibit microbial utilization of this compound (Hristov *et al*., 2011). Rumen NH_3 was products from protein degradation. This in vitro study did not add any protein source, therefore NH_3 in this case was resulted from rumen microbial cell. Lower NH_3 in treatment with glucose addition strengthen hypothesis that high NH_3 after inoculation of *L. plantarum*U40 without glucose addition was resulted from digestion of death rumen microbes caused by lack of nutrient source.

Glucose addition resulted in different A/P (acetic acid/propionic acid ratio), due to different acetic and propionic acid production from *L. plantarum*U40 inoculation. Compared with control (1.85), *L. plantarum*U40 without glucose addition significantly increased A/P ratio (2.07), while glucose addition decreased A/P ratio (1.49). Other research also reported decreased A/P ratio by inoculation of several different LAB on *in vitro* fermentation system (Jiao *et al*., 2017). Decreased A/P ratio indicated higher efficiency of rumen fermentation which correlated with improved growth efficiency (Kenney *et al*., 2015). Increased propionic acid proportion lead to decreased methane production in the rumen because of hydrogen was used for propionic acid production. Decreased of methane by LAB inoculation reported by previous studies, with highest reduction up to 60% in cumulative methane (O'Brien *et al*., 2013; Chang *et al*., 2014; Soriano *et al*., 2014; Astuti *et al*., 2018). Methane produced by ruminant represents an energy loss for the host animal of 2-12% of dietary energy (Moss *et al*., 2000), therefore decreased methane production will increase energy supply for the animal, followed by increased productivity. Other researchers also focused on lowering methane production because of its contribution to global warming (Martin *et al*., 2010).

CONCLUSION

Lactobacillus plantarum U40 can survive in rumen fluid. It changes rumen fermentation by increasing propionic acid proportion when glucose is added as carbon source. Therefore *Lactobacillus plantarum* U40 can be a potential probiotic for ruminant.

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