Effect of lactic acid bacteria on the ensiling characteristics and *in vitro* ruminal fermentation parameters of alfalfa silage

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**ABSTRACT**

The objective of this study was to evaluate the effects of lactic acid bacteria (LAB) inoculants on fermentation quality and subsequent *in vitro* gas production, ruminal fermentation parameters, cellulolytic bacteria and their activities of alfalfa silage. Primary growth of alfalfa (*Medicago sativa* L.) was harvested at 50% flowering stage, inoculated without (control) or with *Lactobacillus plantarum*, *Enterococcus mundtii* and *Enterococcus faecalis* at $1.0 \times 10^6$ cfu/g of fresh weight (FW) in quadruplicate laboratory silos for 45 d. The silage inoculated with LAB were well preserved, indicated by the lower ($p < .05$) pH and ammonia-N content and the higher ($p < .05$) dry matter (DM), organic matter (OM), crude protein and lactic acid contents than the control silage. *In vitro* asymptotic gas and total volatile fatty acids production were higher in all LAB-treated silages ($p < .05$). All inoculants increased carboxymethyl-cellulase and $\beta$-glycosidase activities, and obtained higher DM and neutral detergent fibre degradability ($p < .05$) except *E. mundtii*. Similarly, *L. plantarum* and *E. faecalis* inoculants had higher ($p < .05$) *Ruminococcus albus* and *Fibrobacter succinogenes* relative proportions than the control. However, *L. plantarum* inoculants had lower ($p < .05$) percentage of methane (CH$_4$) in 72 h gas production than the control and *E. faecalis* inoculants. These results suggested that *L. plantarum* were more effective in enhancing alfalfa silage utilisation by promoting forage digestibility and reducing ruminal CH$_4$ emission than *E. mundtii* and *E. faecalis*.

**HIGHLIGHTS**

- Lactic acid bacteria (LAB) inoculants improved alfalfa silage quality.
- Silage treated with *Lactobacillus plantarum* or *Enterococcus mundtii* increased gas production but reduced the percentage of methane *in vitro*.
- *L. plantarum* and *Enterococcus faecalis* promoted neutral detergent fibre digestibility by increased rumen cellulolytic bacteria proportion and cellulase activity.

**INTRODUCTION**

Alfalfa (*Medicago sativa* L.) is one of the most important forages, which is widely cultivated and used as a major source of protein for ruminants in the form of hay or silage in the world (Zhang et al. 2017). Harvesting alfalfa as hay has a high risk, because its harvest season is usually rainy in northern China. So ensiling is currently increasingly popular in China. Alfalfa has high buffering capacity and low water-soluble carbohydrates (WSC), and especially a few epiphytic lactic acid bacteria (LAB), consequently, silage pH decline is often not as rapid in alfalfa as other grasses (Cai et al. 1999). Application of additives in alfalfa silage has become the consensus of researchers and producers. Homofermentative and facultative heterofermentative LAB are the important silage additives, which not only promote silage fermentation and reduce the nutrition loss of forage (Yuan et al. 2015; Zhang et al. 2016; Zheng et al. 2017), but also improve livestock performance (Kung et al. 1993; Chen et al. 1994).

Some researchers considered that the improvement in ruminant performance is due to more nutrients preserved in silage by inoculated with homofermentative or facultative heterofermentative LAB (Keady et al. 1994; Keady and Steen 1995; Keles and Demirci 2011). However, based on a meta-analysis, these inoculants did not change the fermentation quality and nutritional value of silage but still improved ruminant
performance (Oliveria et al. 2017). Weinberg et al. (2004) considered the improvement of animal performance is related to the interactions between LAB inoculants and rumen microorganism, but the mechanisms are still unclear.

For silage, improving the DM digestibility, especially the digestibility of fibre, is important for enhancing animal productivity. In rumen, the fibre degradation is positively correlated with predominant cellulolytic bacteria, such as Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens and Butyrivibrio fibrisolvens. These cellulose bacteria activities are usually evaluated by cellulase activities, including carboxymethylcellulase, β-glycosidase and xylanase (Hobson and Stewart 1997; Agarwal et al. 2002). Therefore, the objective of this study was to evaluate the effects of the three selected LAB inoculants (Lactobacillus plantarum, Enterococcus mundtii and Enterococcus faecalis) on fermentation quality, in vitro gas production, ruminal fermentation parameters, especially the ruminal cellulolytic bacteria and cellulase activities of alfalfa silage.

Materials and methods

Silage preparation

Alfalfa was harvested at the 50% flowering stage with hand clippers in the experimental field of Shanxi Agriculture University (37°25’08” N, 112°35’25”E, elevation 783 m, Shanxi province, China), and immediately chopped into approximately 2 cm length with a fodder chopper. After mixing, alfalfa was divided into 16 equal parts and any 4 parts were randomly added with one of the 4 additions: L. plantarum (KC479667), E. mundtii (KC479665), E. faecalis (KC479663) or equal volume of distilled water (Control). All inoculants were applied at a rate of 1.0 × 10⁶ cfu/g fresh weight (FW).

Approximately 680 g alfalfa was ensiled in a 1 L plastic laboratory silo (Guo et al. 2015), sealed immediately and stored at ambient temperature for 45 d for subsequent tests. Chemical composition and microbial population of fresh material are shown in Table 1.

In vitro ruminal fermentation

Incubation fluid consisted of rumen fluid and artificial buffer solution at a proportion of 1:2 (v/v). Rumen fluid was obtained from three rumen-cannulated Jinnan cattle before the morning feeding. The experimental protocol was approved by the Animal Care and Use Committee of Shanxi Agriculture University. The cattle were fed with TMR that consisted of corn silage (300 g/kg DM), alfalfa hay (50 g/kg DM), alfalfa silage (100 g/kg DM), corn grain (320 g/kg DM), soybean meal (100 g/kg DM), whole cottonseed (60 g/kg DM), wet brewer grain (50 g/kg DM) and a supplement with vitamin and minerals (20 g/kg DM). The mixture of the rumen fluid was filtered through four layers of gauze, mixed with buffer solution and kept at 39°C in a water bath while continually flushed with CO₂. The artificial buffer solution was prepared by the method of Menke and Steingass (1988) and kept in water bath at 39°C.

After 45 d ensiling, a portion of the fresh silage samples was wet-ground in a mixer (FJ2005, Hangzhou Qiwei Instrument Co., Ltd., China) to a particle size of approximately 1–4 mm. Approximately 0.5 g fresh ground silage samples were weighed in triplicate into calibrated 100 mL glass syringes (Häberle Labortechnik, Lonsee, Germany). Syringes were pre-warmed to 39°C, and then 30 mL of the incubation fluid was inhaled. A total of 51 syringes (Four treatments × four individual samples × three glass syringes per sample, with three syringes as blanks without substrate) were prepared. The gas production was recorded at 0, 4, 8, 12, 24, 48 and 72 h incubation, and at each time point gases were collected with gas sampling bags (E-Switch, volume 500 mL, Shanghai ShenYuan Scientific Instrument Co., Ltd., China) for determining methane production. Cumulative gas production data were fitted to the exponential equation (Ørskov and McDonald 1979):

\[ Y = b \left(1 - e^{-ct}\right) \]

where \( Y \) is the gas production at time \( t \), \( b \) is the asymptotic gas volume (mL), \( c \) is the gas production rate constant, and \( t \) is the incubation time (h). After incubation, each incubation fluid was immediately measured for pH, then collected into a falcon tube (10 mL) and stored at −80°C for the following tests with ammonia nitrogen (NH₃-N), volatile fatty acid (VFA), enzyme activity and DNA extraction. The residue was collected into a nylon bag, washed with

| Table 1. Chemical composition and microbial population of fresh alfalfa. |
|-----------------|-----------------|
| Item            | Values          |
| Dry matter, g/kg FW | 325 ± 5.12      |
| Organic matter, g/kg FW | 316 ± 5.33      |
| Crude protein, g/kg DM | 173 ± 0.87      |
| Water soluble carbohydrates, g/kg DM, | 61.0 ± 1.98      |
| Neutral detergent fibre, g/kg DM | 423 ± 9.23      |
| Acid detergent fibre, g/kg DM | 237 ± 6.71      |
| Acid detergent lignin, g/kg DM | 59.7 ± 2.35      |
| Ether extract, g/kg DM | 9.88 ± 0.73     |
| Ash, g/kg DM | 9.18 ± 0.58     |
| Lactic acid bacteria, lg cfu/g FW | 3.99 ± 0.06     |
| Aerobic bacteria, lg cfu/g FW | 7.67 ± 0.30      |
| Mould, lg cfu/g FW | 4.17 ± 0.07     |
| Yeast, lg cfu/g FW | 4.83 ± 0.13     |

DM: dry matter; FW: fresh weight

Values are mean ± standard deviation of three samples.
Chemical and microbial quantitative analysis

The fresh material and silage were dried in an oven at 65 °C for 48 h, and ground to pass a 1-mm screen with a mill (FZ102, Shanghai Hong Ji instrument Co., Ltd., Shanghai, China) for chemical analysis. Analytical DM, ether extracts (EE), ash and total nitrogen (TN) contents of each sample were determined according to the AOAC (2012). Organic matter (OM) content was calculated as the difference between DM and ash contents. The WSC content was determined according to method of Van Soest et al. (1991). Thirty grams of each silage sample was blended with 60 mL of distilled water for 24 h and filtered through two layers of cheesecloth (Guo et al. 2019). The filtrate was used for determining pH, lactic acid, NH₃-N and VFA contents. Lactic acid and NH₃-N contents were determined colorimetry (Kleinschmit et al. 2005), and VFA determined according to method of Van Soest et al. (1991). Thirty grams of each silage sample was blended with 60 mL of distilled water for 24 h and filtered through two layers of cheesecloth (Guo et al. 2019). The filtrate was used for determining pH, lactic acid, NH₃-N and VFA contents. Lactic acid and NH₃-N contents were determined colorimetry (Kleinschmit et al. 2005), and VFA contents were determined by gas chromatography (Thermo T1300; Guo et al. 2015). The methane (CH₄) content in gas samples was determined by gas chromatography with flame ionisation detector (Thermo T1300, USA; Kougiás et al. 2014). Enumeration of aerobic bacteria, LAB, moulds and yeasts was performed by the method of Guo et al. (2015), and these data were log10 transformed. The incubation fluid samples were used to analyse the cellulase activities (carboxymethyl-cellulase, β-glycosidase, xylanase and pectate) as described by Agarwal et al. (2002).

Ruminal microbial DNA was extracted from 1 mL of incubation fluid using TIANamp Stool DNA Kit (DP302-02, Tiangen, Beijing, China). The relative abundance of R. albus, R. flavefaciens, Ruminobacter amylophilus, Prevotella ruminicola, F. succinogenes and B. fibrisolvens was determined by qPCR, and calculated by a proportion of total bacterial 16SrDNA according to the formula: Relative quantification = 2^{-ΔΔCt}, where Ct was threshold cycle (Pei et al. 2013), and the heat map was made by GraphPad Prism 7 software. The primers used for qPCR were forward primer 5’-CCC TAA AAGCAG TCT TAG TGG TTG-3’ and reverse primer 5’-CCT CTTGC GGT TAG AAC A-3’ for R. albus (Koike and Kobayashi 2001), forward primer 5’-TAA CAT GAG AGT TTG ATC CTG GCT C-3’ and reverse primer 5’-CGT TAC TCA CCC GTC CGC-3’ for B. fibrisolvens (Ma et al. 2016), forward primer 5’-CTG GGG AGC TGC CTG AAT G -3’ and reverse primer 5’-GCA TCT GAA TGC GAC TGG TTG-3’ for R. amylophilus, forward primer 5’-GAA AGT CGG ATT AAT GCT CTA TGT TG -3’ and reverse primer 5’-CAT CCT ATA GCG GTA AAC CTT TGG-3’ for P. ruminicola (Stevenson and Weimer 2007), forward primer 5’-CGA ACG GAG ATA ATG TTA TTG CC-3’ and reverse primer 5’-CGG CTT CTG TAT GGT ATG AGG TAT TAC C-3’ for R. flavefaciens, forward primer 5’-GGT CGG AAT TAC TGG GCG TAA A-3’ and reverse primer 5’-CGC CGT CCC CTG AAC TAT C-3’ for F. succinogenes, forward primer 5’-CGG CAA CGA GCG CAA CCC-3’ and reverse primer 5’-CCA TTG TAG CAC GTG TGT AGC C-3’ for total bacteria (Denman and McSweeney 2006). qPCR was carried out on an Applied Biosystems stepone plus Fast Real-Time PCR System (Applied Biosystems Co., Foster City, CA). The reaction mixture and PCR programs referred to the literature of Denman and McSweeney (2006).

Statistical analyses

Analyses were performed using the GLM procedure and correlation procedure of SAS version 9.1 (SAS Institute, Cary, NC). Data on the silage fermentation quality and in vitro ruminal fermentation characters were analysed by one-way analysis of variance (ANOVA) with treatments as main effect. Each replicate served as an experimental unit. The means were compared by Duncan’s multiple test, and differences were considered significant when p < .05. The relationship of rumen cellulolytic bacteria population and cellulase activity was evaluated by Pearson correlation coefficient.

Result

Silage fermentation characteristics

Fermentation characteristics of alfalfa silage are presented in Table 2. LAB treated silage had higher (p < .05) DM, OM, CP, lactic acid and acetic acid contents, lower (p < .05) LAB counts and similar (p > .05) ADL, EE and WSC contents compared with those of control silage. In addition, the butyric acid content in control silage was 1.26 g/kg DM, but it was not detected in all LAB-treated silages. Alfalfa silage inoculated with L. plantarum had lower (p < .05) NDF and ADF contents compared with the control silage.

In vitro ruminal fermentation characteristics

The inoculants significantly (p < .05) increased the asymptotic gas production, total VFA and acetate
concentrations, but did not have effects (p > .05) on the rate of gas production, pH, NH3-N, propionate and butyrate concentrations of incubation fluid (Table 3). Alfalfa silage inoculated with L. plantarum or E. mundtii had lower (p < .05) percentage of CH4 in 72 h gas production, and L. plantarum or E. faecalis groups had higher (p < .05) dry matter (DM) degradability (DM-D) and neutral detergent fibre degradability (NDF-D) than those of the control in vitro. The rate of ratio of acetate to propionate did not show a significant difference among all silages.

The activities of carboxymethyl-cellulase and β-galactosidase, and relative proportion of F. succinogenes and R. albus were higher (p < .05), but the relative population of R. flavefaciens was lower (p < .05) for silage inoculated with L. plantarum or E. faecalis as compared to those of control silage (Figures 1 and 2). Compared with the control, inoculants slightly increased the relative population of B. fibrisolvens and R. amylophilus. Moreover, the two strains of enterococci had a slightly higher relative population of P. ruminicola than that of L. plantarum and control.

### Table 2. Fermentation characteristics of alfalfa silage inoculated with or without lactic acid bacteria.

| Item | control | Lactobacillus plantarum | Enterococcus mundtii | Enterococcus faecalis | SEM (n = 4)b |
|------|---------|-------------------------|---------------------|---------------------|-------------|
| pH   | 5.48b   | 4.58b                   | 4.66b               | 4.60b               | 0.12        |
| Dry matter, g/kg FW | 310b | 321b | 316b | 316b | 1.41 |
| Organic matter, g/kg FW | 295b | 310b | 304b | 305b | 1.65 |
| Crude protein, g/kg DM | 175b | 183b | 186b | 185b | 1.72 |
| NH3-N, g/kg TN | 128b | 73.8b | 85.4b | 76.4b | 6.82 |
| Ether extract, g/kg DM | 11.7 | 10.8 | 11.4 | 11.3 | 0.56 |
| Water soluble carbohydrates, g/kg DM | 11.6 | 12.1 | 11.0 | 12.1 | 0.39 |
| Neutral detergent fibre, g/kg DM | 441b | 428b | 436b | 434b | 3.08 |
| Acid detergent fibre, g/kg DM | 249b | 238b | 244b | 243b | 1.55 |
| Acid detergent lignin, g/kg DM | 64.5 | 61.7 | 62.8 | 62.5 | 0.96 |
| Lactic acid, g/kg DM | 42.1b | 86.9a | 97.3a | 88.2a | 6.84 |
| Acetic acid, g/kg DM | 19.8b | 44.0a | 45.0a | 37.9a | 3.40 |
| Butyric acid, g/kg DM | 1.26 | ND | ND | ND | 0.16 |
| Lactic acid/acetic acid | 2.13 | 1.98 | 2.16 | 2.33 | 0.15 |
| Lactic acid bacteria, lg cfu/g FW | 8.65a | 7.95b | 7.77b | 7.72b | 0.14 |

*CP: crude protein; DM: dry matter; FW: fresh weight; Lactic acid/acetic acid, the radio of lactic acid to acetic acid; NH3-N: ammonia-N; TN: total nitrogen; WSC: water soluble carbohydrates; ND: not detected.

| Item | Control | Lactobacillus plantarum | Enterococcus mundtii | Enterococcus faecalis | SEM (n = 4)c |
|------|---------|-------------------------|---------------------|---------------------|-------------|
| Asymptotic gas production, mL/g DM | 218b | 239a | 235a | 235a | 3.04 |
| Rate of gas production, /h | 0.075 | 0.076 | 0.077 | 0.074 | 0.003 |
| 72 h methane production, %GP | 38.2 | 35.5 | 35.3 | 38.9 | 1.40 |
| DM-D, % | 72.8b | 74.7a | 73.5ab | 74.3a | 0.62 |
| NDF-D, % | 43.1b | 44.9a | 43.6a | 45.2a | 0.32 |
| pH | 6.79 | 6.72 | 6.73 | 6.70 | 0.02 |
| NH3-N, mg/100 mL | 45.2 | 45.8 | 46.6 | 47.9 | 0.41 |
| Total VFA, mmol/L | 120b | 131a | 127a | 129a | 1.12 |
| Acetate, mmol/L | 76.8b | 84.2a | 81.9a | 82.3a | 1.04 |
| Propionate, mmol/L | 21.6 | 22.6 | 22.9 | 22.0 | 0.40 |
| Butyrate, mmol/L | 12.3 | 13.5 | 13.0 | 12.7 | 0.20 |
| Isobutyrate, mmol/L | 2.17b | 2.69a | 2.23b | 2.40a | 0.03 |
| Valerate, mmol/L | 2.86 | 3.01 | 2.93 | 3.01 | 0.05 |
| Isovalerate, mmol/L | 4.20b | 4.54* | 4.28ab | 4.37ab | 0.06 |
| Acetate/propionate | 3.56 | 3.72 | 3.58 | 3.74 | 0.09 |

*DM-D: dry matter degradability after 72 h in vitro incubation; GP: 72 h gas production; NDF-D: neutral detergent fibre degradability after 72 h in vitro incubation; NH3-N: Ammonia-N; VFA: volatile fatty acids.

#Cumulative gas production data were fitted to the exponential equation: $Y=b(1-e^{-ct})$, where $Y$ is the gas production at time $t$, $b$ is the asymptotic gas volume (mL), $c$ is the gas production rate constant, $t$ is the incubation time (h).

cSEM: standard error of the mean.

Means with different superscripts (a–c) in each row differ significantly (p < .05).
As shown in Table 4, the activity of carboxymethyl-cellulase had positive correlation ($p < .05$) with $R. \text{albus}$ and $F. \text{succinogenes}$ populations in incubation fluid. The activity of $\beta$-glycosidase had positive ($p < .05$) correlation with populations of $R. \text{albus}$, $F. \text{succinogenes}$ and $B. \text{fibrisolvens}$ and had negative ($p < .05$) correlation with $R. \text{flavefaciens}$ population.

**Discussion**

The main purpose of using LAB inoculants is to improve silage fermentation, such as rapid accumulation of lactic acid, reduction in pH values, inhibition on undesirable microbiological activity and preservation of more nutrients of forage (Kung et al. 1984). In this study, silages inoculated with LAB had better fermentation quality, indicated by significantly lower pH and $\text{NH}_3$-$\text{N}$ contents, and higher lactic acid contents compared with those of the control. This indicated that $L. \text{plantarum}$ and the two enterococci strains effectively improved the fermentation quality of alfalfa silage. During ensiling, ammonia is produced by proteolysis and subsequent amino acids decomposition. Proteolysis is mainly the result of plant enzyme...
activity, but the further degradation of the amino acids is mainly caused by harmful microbial activity, such as clostridium in anaerobic state (McDonald et al. 1991). Therefore, in order to obtain good silage fermentation attributes, it is particularly important to limit the activity of harmful microorganisms during ensiling. Effective inhibition of harmful microorganisms and plant enzyme activities mainly depends on the rapid accumulation of lactic acid and low silage pH (Zhang et al. 2010). E. mundtii and E. faecalis are homofermentative LAB, and L. plantarum is facultative heterofermentative LAB (Buxton et al. 2003). Oliveira et al. (2017) concluded that alfalfa silage inoculated with homofermentative and facultative heterofermentative LAB reduced the pH and NH3-N content, and increased lactic acid content. This study has obtained similar results.

The microflora metabolise WSC to obtain energy for growth. Epiphytic LAB on grass material is often low in number, about $1 \times 10^4$ cfu/g FW in this study. LAB inoculants can become dominant bacteria in silage, and reduce the consumption of WSC by harmful bacteria and retain more WSC (Queiroz et al. 2013; Santos et al. 2013). However, the WSC content did not show a significant difference among all silages in this study. This probably because the WSC content in alfalfa material was too low (only 19.8 g/kg FW) to meet the minimum requirement for successful silage preservation (Zhang et al. 2010), inoculants improved fermentation efficiency by producing more lactic acid instead of preserving more WSC. The nutrition value of silage was also improved by inoculants, especially L. plantarum, which reduced NDF and ADF in inoculated silage when compared to the control.

The main intention of this study was to determine the further effect of LAB inoculants on nutrient digestibility and the ruminal fermentation of alfalfa silage in vitro. Menke and Steingass (1988) found that gas production is an indicator of feed value, which depends on the amount of fermentable OM and the activity of the ruminal microorganisms. Asymptotic gas production was higher for silage inoculated with LAB compared with control silage after 72 h of incubation, which was related to the improvement in nutrition value (Trabi et al. 2017). In LAB inoculated silages, more DM, OM and CP were preserved, which ensured the variety of rumen bacteria proliferation, and increased gas production as well as total VFA production. Blummel et al. (1997) have demonstrated that VFA produced by rumen microorganisms during in vitro fermentation is positively related to gas production. In this study, the concentration of acetate and isobutyrate in all LAB inoculation treatments was higher than control. This was mainly due to the silage inoculated with LAB had higher NDF-D than the control. In rumen, the main products of fibre degradation are acetic acid and butyric acid (Dijkstra et al. 1993).

It is interesting to note that silage inoculated with and L. plantarum and E. mundtii reduced the percentage of CH4 in gas production, but E. faecium did not, although it increased silage DM-D and the ruminal VFA production. These results were related to rumen microbial community. In the rumen, large amounts of hydrogen are mainly produced by cellulolytic Ruminococci, while the cellulolytic bacterium F. succinogenes is the non-hydrogen producing bacteria (Holdeman et al. 1977; Mitsumori et al. 2012). Hydrogen is the major precursor for methanogens to produce CH4 (Hobson and Stewart 1997). Although the highest ruminal F. succinogenes population was found in silage inoculated with E. faecium, the enhanced ruminal R. albus might result in large amounts of hydrogen production, and thus resulted in no significant difference in percentage of CH4 in gas production compared with that of control. However, L. plantarum and E. mundtii inoculants had lower ruminal R. albus population and similar ruminal F. succinogenes population. Some research found that the increased of

| Cellulase activity       | Rumen cellulolytic bacteria         | Pearson coefficient | Significant* |
|--------------------------|-------------------------------------|---------------------|--------------|
| Carboxymethyl-cellulase  | Ruminococcus albus                   | 0.6391              | *            |
|                          | Ruminococcus flavefaciens           | −0.3820             | *            |
|                          | Fibrobacter succinogenes            | 0.5850              | *            |
|                          | Butyrivibrio fibrisolvens           | 0.4740              | *            |
| β-glycosidase            | Ruminococcus albus                   | 0.7156              | **           |
|                          | Ruminococcus flavefaciens           | −0.6027             | *            |
|                          | Fibrobacter succinogenes            | 0.7371              | **           |
|                          | Butyrivibrio fibrisolvens           | 0.8124              | **           |
| Xylanase                 | Ruminococcus albus                   | 0.0627              |              |
|                          | Ruminococcus flavefaciens           | −0.4447             |              |
|                          | Fibrobacter succinogenes            | 0.7537              | **           |
|                          | Butyrivibrio fibrisolvens           | 0.2760              |              |

*Significant at 0.05; **Significant at 0.01.
DM-D and the ruminal VFA production was beneficial to enhance energy efficiency of alfalfa silage by inoculants, whether they decreased (Cao et al. 2010, 2011) or did not change (Contreras-Govea et al. 2011) the ruminal CH₄ production.

This study showed that the higher ruminal *F. succinogenes* and *R. albus* populations were observed in alfalfa silage inoculated with *E. faecium* and *L. plantarum*. Of the selected cellulolytic bacteria, *F. succinogenes* has a strong ability to degrade the structure of tough cellulose by carboxymethyl-cellulase and β-glycosidase (Wanapat et al. 2014). The results of this study confirmed that ruminal carboxymethyl-cellulase and β-glycosidase activity had positive correlation with *F. succinogenes* population. The high DM-D and NDF-D were observed in *E. faecium* and *L. plantarum* groups, which were consistent with the results reported by Weinberg et al. (2007). Furthermore, unlike the proliferation of ruminal *R. albus*, the population of ruminal *R. flavefaciens* decreased in LAB-treated silage. On the contrary, the maximum ruminal *R. flavefaciens* population and the least ruminal *R. albus* population were observed in the control group. This may be due to some strains of *R. albus* can produce bacteriocins and thus inhibit *R. flavefaciens*, and *R. albus* is usually more numerous than *R. flavefaciens* in the rumen (Odenyo et al. 1994; Hobson and Stewart 1997). In this study, with the increase of CP content in silage, the relative proportions of ruminal major proteolytic bacteria *B. fibrisolvens* and *R. amylophilus* slightly increased. In addition, slightly lower *P. ruminicola* proportion was observed in alfalfa silage inoculated with *L. plantarum* compared to the enterococci. Although it is unable to reveal the mechanism of inoculants effect on the rumen microbiota, the increased proportions of ruminal cellulolytic bacteria and their activities by silage inoculated with LAB were identified. In some reports, inoculants could promote the ruminal microbial biomass (Muck et al. 2007; Contreras-Govea et al. 2011). In the future, it is worthwhile to investigate the interactions between different types of LAB and selected rumen bacterial strains.

**Conclusions**

All LAB inoculants could improve fermentation quality of alfalfa silage and did not show significant difference among them. However, *L. plantarum* inoculants were more effective in enhancing alfalfa silage utilisation than the two enterococci inoculants. *L. plantarum* not only increased in vitro digestibility of silage, but also reduced the percentage of CH₄ in the ruminal gas emissions. Therefore, it is suggested that *L. plantarum* be used as inoculants for alfalfa silage making.

**Disclosure statement**

No potential conflict of interest was reported by the authors. The authors alone are responsible for the content and writing of this paper.

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