Effects of *Lactobacillus acidophilus* supplementation for improving *in vitro* rumen fermentation characteristics of cereal straws

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\section*{ABSTRACT}

We explored and compared the effects of four doses (0, 0.25 × 10^7, 0.50 × 10^7, and 0.75 × 10^7 cfu/mL) of *Lactobacillus acidophilus* supplementation on ruminal fermentation characteristics of maize stover and rice straw. The maximum gas production (Vf) for maize stover and the rate of gas production during early incubation (FRD\textsubscript{0}) for rice straw were quadratic (p < .05) and linear (p < .01), respectively, and increased when supplemented with *L. acidophilus*. The *L. acidophilus* supplementation increased ruminal NH\textsubscript{3}-N concentration for maize stover (p < .01) and rice straw (p < .05), respectively. The supplementation level of *L. acidophilus* did not affect the production of volatile fatty acids (VFAs) except for valeric acid, nor did it affect CH\textsubscript{4} production, IVDMD, IVNDFD, ruminal pH value, total volatile fatty acids (TVFAs) or the ratio of acetic to propionic acid (A:P). As a fermentation substrate, when compared to rice straw, maize stover increased in *vitro* gas production parameters (e.g. Vf, k, FRD\textsubscript{0}, and t\textsubscript{0.5}) IVDMD, IVNDFD, ruminal NH\textsubscript{3}-N concentration and TVFAs, whereas there was no (p > .05) difference in CH\textsubscript{4} production, ruminal pH value or A:P between the two substrates. The results indicate that *L. acidophilus* increase *in vitro* gas production and ruminal NH\textsubscript{3}-N concentration when using either fermentation substrate, with the greatest increase observed at a dosage of 0.75 × 10^7 cfu/mL. Additionally, maize stover is superior to rice straw as a fermentation substrate or forage for ruminants. The present positive *in vitro* results should be tested using *in vivo* experiments in future.

\section*{Introduction}

European Union had banned the use of antibiotics in animal feed as growth promoters in 2006, and currently, the possibility of using alternative additives is under research. One such alternative are probiotics. Probiotics have been defined as ‘microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host’ (Salminen et al. 1999). Recently, worldwide interest in the use of probiotic bacteria for health promotion and disease prevention has increased significantly among scientists, consumers and food producers. This interest is based on the fact that targeted use of microorganisms with suitable properties may have beneficial effects on feed utilisation efficiency, animal performance and host health.

Microorganism preparations have been used to explore the effects on *in vitro* fermentation characteristics of high fibre content forage, and then evaluate the ability to improve utilisation efficiency of low-quality roughages (Tang et al. 2008). The bacterial strains most commonly used as probiotics are *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Bifidobacterium*, which are lactic acid bacteria (LAB). Weinberg et al. (2007) reported that some LAB inoculants applied at ensiling or added directly to the rumen fluid had the potential to increase the DMD and NDFD. Cao et al. (2011) also reported that LAB increased DM digestibility and decreased ruminal methane production.

*Lactobacillus sp.* are characterised by their production of lactic acid, and they are dominant participants in many industrial and artisanal plant, meat and dairy fermentation processes (Marco et al. 2006). *Lactobacillus* has been used widely as a feed additive in the dairy cattle industry to improve intestinal health, feed conversion efficiency and milk production (Chen et al. 2013). Dietary supplementation of...
Lactobacillus was reported to increase milk production, reduce the count of somatic cells in milk (Jiang et al. 2008) and reduce the probability of mastitis by positively regulating interleukin-1 (IL-1) and IL-8 in the mammary gland (Beecher et al. 2009). However, those studies did not document the specific strain of L. acidophilus, and to our knowledge, there is no research evaluating the supplemental efficacy of L. acidophilus in *in vitro* fermentation experiments.

Our study explores the effects of *L. acidophilus* supplementation on *in vitro* fermentation characteristics of cereal straws (maize stover and rice straw) in a dairy cow model to further understand the behaviour of microorganism preparations in the rumen and to provide information on the practical application of these preparations to ruminants’ diets.

**Materials and methods**

This experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture (ISA), Chinese Academy of Sciences, Changsha, China.

**Crop straws, *L. acidophilus* and experimental design**

Two types of crop straws, i.e. maize stover from Kexiangtian 1 (bred by ISA) and rice straw from Xiang 125s (a popular local breed), were selected as *in vitro* fermentation substrates. Both of maize stover and rice straw were harvested by hand from farmland in Institute of Subtropical Agriculture (ISA), Chinese Academy of Sciences, Changsha, China. The maize stover and rice straw samples contained the entire plant but did not contain any grain. They were dried at 65°C for 24 h, ground through a 1 mm sieve and stored in plastic bag until assay. Maize stover and rice straw contained (DM basis): 5.3% and 6.2% crude protein (CP), 63.6% and 63.2% neutral detergent fibre (NDF), 38.6% and 43.4% acid detergent fibre (ADF), 19.5% and 22.2% ash, respectively.

*Lactobacillus acidophilus* (NO. 6241) were purchased from and reactivated by the China Centre of Industrial Culture Collection (CICC). Total viable bacteria were counted in the form of colony-forming units (cfu) using the spread plate method after culture amplification.

The experiment followed a blocked experimental design; *L. acidophilus* was supplemented at four levels (0 × 10⁷ [no addition of *L. acidophilus* or control], 0.25 × 10⁷, 0.50 × 10⁷ and 0.75 × 10⁷ cfu/mL). The number of bottle incubated per treatment (n = 3) and the total number of bottle incubated (n = 108, including blanks). Bottles containing only mixed fluids were incubated together with the treated bottles containing different dose of *L. acidophilus* as blanks.

**In vitro gas production and sampling**

Culture solutions, i.e. macroelement, buffered and reducing solutions used for *in vitro* fermentation, were prepared to form artificial saliva according to the procedures modified by Tang et al. (2006). The artificial saliva was maintained in an anaerobic environment by continuously pumping carbon dioxide around it for 2 h and a mineral solution containing, per litre, g: 8.75 NaHCO₃, 1.00 NH₄CO₃, 1.43 Na₂HPO₄, 1.55 KH₂PO₄, 0.15 MgSO₄·7H₂O, 0.52 Na₂S, 0.017 CaCl₂·2H₂O, 0.015 MnCl₂·4H₂O, 0.002 CoCl₂·6H₂O, 0.012 FeCl₃·6H₂O and 0.125 resazurin. Rumen fluids were collected before the morning feeding, from three rumen-cannulated Holstein dairy cows (fed a rice straw based total mixed ration), and immediately transported to the laboratory. Rumen contents were strained through four layers of cheesecloth under a continuous flow of CO₂. Rumen fluids (5 mL) and artificial saliva (45 mL) were placed in prewarmed (39°C) 100 mL fermentation bottles.

A sample of 500 ± 10 mg of each straw type was placed in the 100 mL fermentation bottles. Each sample was measured three times at each incubation time point. *Lactobacillus acidophilus* was preserved at 4°C after amplification culture and counting. *Lactobacillus acidophilus* was added to the straw substrates, artificial saliva and rumen fluids when the *in vitro* fermentation started.

All fermentation bottles were sealed and connected to pressure sensors (CYG130-12; Sqsensor, Kunshan, China) and incubated at 39°C. Fermentation bottle pressure was recorded at 0, 1, 2, 4, 6, 12, 24, 36 and 48 h during the *in vitro* fermentation process. After 12, 24 or 48 h of incubation, fermentation was interrupted. Undegraded residues were filtered through two layers of nylon cloth (40 μm pore size) (Haiyang Biotechnology co., Ltd, Shanghai, China) and a 5 mL sample of gas was collected into a vacuum flask (Labco Exetainer, UK) with a plastic syringe for CH₄ determination. The incubation solution from each treatment was sampled to determine NH₃-N and VFAs concentrations at 12, 24 and 48 h, respectively.

**Chemical analysis**

The DM (method 930.15) and CP (6.25 × N, method 990.03) were analysed using procedures from the Association of Official Analytical Chemists (AOAC 1990). The NDF and ADF content were determined
using a Fibretherm Fibre Analyser (Gerhardt, Bonn, Germany) following the method by Van Soest et al. (1991) with the addition of sodium sulphite and alpha-amylase in the NDF analysis. The filtered residue was dried at 105°C for 12 h and weighed to determine in vitro dry matter disappearance (IVDMD). The NDF content in the dried residues was determined to calculate in vitro NDF disappearance (IVNDFD).

A 5 ml sample from each incubation solution was centrifuged at 10,000 × g at 4°C for 15 min for pH determination using a pH metre (MP512-03, Shanghai precision instrument co., Ltd, China).

A 2 ml sample from each incubation solution was centrifuged at 10,000 × g and 4°C for 15 min, then 1.5 mL of the supernatant was taken and homogenised with 0.15 mL of metaphosphoric acid. The mixed solution was again centrifuged at 10,000 × g and 4°C for 15 min, and the supernatant used to determine VFAs (including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid) content with a gas chromatograph (HP5890, Agilent 5890; Agilent, Santa Clara, CA). A DB-FFAP column (30 m in length with a 0.25 mm i.d.) was used for separation. Attenuation was set at a nitrogen diffuent ratio of 1:50, hydrogen flow 30 ml/min, airflow 365 mL/min, injector temperature 250°C, column temperature 150°C and detector temperature 220°C. N2 was used as carrier gas at a flow rate of 0.8 mL/min. The relative response factor, representing the peak for each VFAs, was calculated using the standard VFAs mixture, which was chromatographed with each group of 10 samples. Total molar concentration was calculated by taking the sum of individual VFAs as 1 (Wang et al. 2014).

To determine NH4-N concentration, 5 mL of each incubation solution was centrifuged at 4000 × g and 4°C for 10 min, then 2 mL of the supernatant was taken and homogenised with 8 mL 0.2 M HCl. Subsequently, 2 mL of sodium nitroprusside (0.08 g sodium nitroprusside dissolved in 100 mL of 0.14 natrium salicylicum) and 2 mL of prepared solutions (2 mL sodium hypochlorite solution mixed with 100 mL 0.3 M sodium hydroxide solution) were homogenised with 0.4 mL of the mixed solution and equilibrated at room temperature for 10 min. The ultraviolet absorption value was recorded at 700 nm using a spectrophotometer (UV-2300, Shimadzu, Japan). The NH4Cl standard solutions were prepared as follows: 0.382 g of NH4Cl was diluted with 0.2 M HCl to 100 mL and kept at 4°C. To create a working solution in which the concentration of N was 10 mg/dL, 10 mL of preservation solution were diluted with distilled water to 100 mL. Subsequently, 0, 1, 2, 4 and 6 mL of working solution were mixed with 10, 9, 8, 6 and 4 mL of distilled water, respectively, and then diluted with 0.2 M HCl to 50 mL to make NH4Cl standard solutions in which N concentration was 0, 0.2, 0.4, 0.8 and 1.2 mg/dL, respectively. Finally, 0.4 mL of each concentration of the NH4Cl standard solution were used to obtain a standard curve according to previously mentioned procedures (Wang et al. 2016).

The CH4 analysis was performed by GC-flame ionisation detection (FID) using a gas chromatograph (GC7890A; Agilent, Santa Clara, CA) equipped with a HayeSep Q packing column (2.44 M × 1/8 in. × 2.0 mm ID). The temperature of column and injector were set at 60°C and 100°C, respectively, and held for 3 min. N2 was used as carrier gas at a flow rate of 21 mL/min (Wang et al. 2014).

**Calculation and statistical analysis**

The correlation between fermentation bottle pressure and gas volume was measured at 39°C, 20 bottles were used to determine the parameters in the equation, and the following regression equation was established:

\[
y = 1.506x \left( n = 20, R^2 = .999, p < .0001 \right)
\]

where y represents gas volume (ml), x is bottle pressure (kPa), and 1.506 is a constant. Pressure measurements were then converted to gas production (ml). The following logistic-exponential equation (Wang et al. 2011) was fitted to in vitro gas production at 0, 1, 2, 4, 6, 12, 24 and 48 hours:

\[
GP = \frac{VF \times \frac{1 - \exp \left( d - t \times k \right)}{1 + \exp \left( b - k \times t \right)}}{k}
\]

where GP represents gas production at t time, VF is the maximum gas production (ml), k is the gas production fraction (/h), and b and d is the shape of the gas production curve. The following equation was used to calculate the elapsed time (T0.5, h) until half of the maximum gas production was achieved (Wang et al. 2011).

\[
T_{0.5} = \frac{\ln (\exp (b) + 2 \exp (d))}{k}
\]

FRD0 was used to calculate the initial fractional rate of degradation (/h) as follows (Wang et al. 2013):

\[
FRD_0 = \frac{k}{1 + \exp (b)}
\]

Gas production (GP), IVDMD and IVNDFD were corrected by subtracting values obtained for the blanks. Data were analysed by straw substrate using the PROC MIXED procedure in SAS (SAS Institute 2001). For the
statistical analyses of gas production parameters, the model included substrates, level and their interaction as fixed effects. For the analyses of pH, NH₃-N, CH₄ production, VFAs, IVDMD and IVNDFD, the model included substrates, level and their interaction as fixed effects with incubation time as a repeated factor. Linear and quadratic effects of supplementation level were analysed using orthogonal polynomial contrasts. Cubic effects of supplementation level were not examined due to inexplicability in a biological context. Least squares means are reported throughout the text, and significance was declared at $p < .05$.

**Results**

*In vitro gas production parameters and CH₄ production*

The effect of *L. acidophilus* supplementation on *in vitro* gas production parameters and CH₄ production of maize stover and rice straw is shown in Table 1. *In vitro* gas production parameters were affected ($p < .01$ or $p < .05$) in the presence of *L. acidophilus*. For maize stover, $V_f$ and FRD₀ were average 15.27% and 50.31% greater ($p < .01$) than those of rice straw, while $K$ and $t_{0.5}$ decreased ($p < .05$ and $p < .01$) by 14.41% and 12.36%, respectively, compared to rice straw.

There were no interactive effects ($p > .05$) for *in vitro* gas production parameters for either maize stover or rice straw. Considering the *in vitro* gas production and rates we obtained, the greatest increase observed at a dosage of *L. acidophilus* is $0.75 \times 10^7$ cfu/mL.

The CH₄ production were not affected ($p > .05$) by supplementation of *L. acidophilus*, nor were there interactive effects ($p > .05$) on these parameters for maize stover or rice straw.

**In vitro dry matter and neutral detergent fiber disappearance**

The effect of *L. acidophilus* supplementation on IVDMD and IVNDFD of maize stover and rice straw is shown in Table 2. In *in vitro* IVDMD and IVNDFD were not affected by *L. acidophilus* supplementation for both fermentation substrates ($p > .05$). For maize stover, IVDMD and IVNDFD were higher by average 12.77% and 15.42% compared to rice straw, respectively ($p < .01$). However, IVDMD and IVNDFD were not affected ($p > .05$) in the presence of *L. acidophilus*, and there were no interactive effects ($p > .05$) on IVDMD or IVNDFD for either fermentation substrate.

**pH, NH₃-N concentration**

The effect of *L. acidophilus* supplementation on pH, NH₃-N concentration for maize stover and rice straw is shown in Table 3. The pH value was not affected ($p > .05$) with the addition of *L. acidophilus*, nor were there interactive effects ($p > .05$) on these parameters for maize stover or rice straw. NH₃-N concentration was higher by 19.58% in maize stover compared to rice straw ($p < .01$). The addition of *L. acidophilus* had

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**Table 1.** Effect of different supplementation levels of *L. acidophilus* on kinetic parameters of *in vitro* gas production and CH₄ production for the fermentation of maize stover and rice straw.

| Item      | Substrate    | Supplementation levels ($\times 10^7$), cfu/mL | SEM* | Significance** |
|-----------|--------------|-----------------------------------------------|------|---------------|
| $V_f$, mL/g | Maize stover | 78.72a                                          | 6.82a| $p < .01$     |
|           | Rice straw   | 68.29                                          | 6.62 | Q             |
|           | SEM          | 0.57                                           |      | NS            |
| $k$ (10⁻²) | Maize stover | 9.09a                                          | 1.40b| $p < .05$     |
|           | Rice straw   | 10.40b                                         | 1.17 | NS            |
|           | SEM          | 0.37                                           |      | NS            |
| FRD₀ (10⁻²), mL/h | Maize stover | 2.39a                                          | 1.59a| $p < .01$     |
|           | Rice straw   | 1.34b                                          | 1.49a| L             |
|           | SEM          | 0.06                                           |      | L             |
| $t_{0.5}$, h | Maize stover | 17.32a                                         | 19.46| $p < .01$     |
|           | Rice straw   | 17.45b                                         | 19.97| NS            |
|           | SEM          | 0.22                                           |      | NS            |
| CH₄, mL/g | Maize stover | 18.63                                          | 16.34| NS            |
|           | Rice straw   | 16.66                                          | 16.26| NS            |

*Means within a row for supplementation levels without a common superscript differ ($p < .05$).
*aMeans within a column for *L. acidophilus* without a common superscript differ ($p < .05$).
*bMean = mean for individual *L. acidophilus* across supplementation levels including control.
*cSEM for supplementation level × substrate.
*dNS = not significant ($p > .05$); S × L = interaction between substrate and supplementation level; L = linear effect of supplementation level; Q = quadratic effect of supplementation level.
*eSEM for pooled mean of substrate including control.
a linear \( (p < .01) \) effect on NH\(_3\)-N concentration for both the substrate incubated.

**Volatile fatty acid concentration**

The effect of *L. acidophilus* supplementation on VFAs concentration for maize stover and rice straw is shown in Table 4. Substrates affected VFAs concentration except that of propionate and A/P \( (p < .05 \text{ or } p < .01) \). The average values of acetic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and total VFAs concentration for maize stover were higher by 21.81%, 36.02%, 38.34%, 48.27%, 37.29% and 24.31% compared to those of rice straw \( (p < .05 \text{ or } p < .01) \), respectively. There were no interactive effects \( (p > .05) \) on volatile fatty acid concentration for maize stover or rice straw.

**Discussion**

**In vitro gas production characteristics and CH\(_4\) production**

The *in vitro* gas production technique has been used widely to study feed degradation (Rymer et al. 2005); it can provide valuable information on the kinetics of feed digestion in rumen and reflect the utilisation efficiency of fermentation substrates (Metzler-Zebeli et al. 2012). Previous studies have also reported that LAB could survive in rumen, affected rumen microflora, and then changed the *in vitro* ruminal fermentation mode (Gollop et al. 2005; Weinberg et al. 2004). Our results indicate that supplementation of *L. acidophilus* affects the process of *in vitro* fermentation for maize stover and rice straw, which may occur by changing the activity of relevant enzymes and/or improving the activity or amount of microbes. We found that \( V_i \) for maize stover was higher than for rice straw after 48 h of *in vitro* incubation when supplemented with *L. acidophilus*, which may be caused by higher DM degradation of the maize stover (Table 2).

FRD\(_0\) and \( T_{0.5} \) indexes reflect the rate of degradation during early incubation \( (<12 \text{ h}) \) and the required incubation time to reach half of the maximum gas production, respectively. Generally speaking, the faster FRD\(_0\) is, the shorter \( T_{0.5} \) becomes (Wang et al. 2013). We found that FRD\(_0\) for maize stover was significantly higher than for rice straw, i.e. the rate of degradation during early incubation was significantly faster for maize stover than for rice straw with the addition of *L. acidophilus*. This difference may be more directly related to the different chemical composition of two kinds of substrate incubated, besides, maize stover is a

### Table 2. Effect of different supplementation levels of *L. acidophilus* on IVDMD and IVNDFD for the *in vitro* fermentation of maize stover and rice straw.

| Item        | Substrate       | Supplementation levels \( (\times 10^7) \), cfu/mL | Significance\(^3\) |
|-------------|-----------------|-----------------------------------------------|-------------------|
|             |                 | Mean\(^1\) 0.00 0.25 0.50 0.75 SEM\(^2\)        | Substrate Level S × L |
| IVDMD, %    | Maize stover    | 51.04\(^a\) 52.74 51.56 50.18 49.69 2.38     | p < .01 NS NS      |
|             | Rice straw      | 45.26\(^b\) 45.28 46.07 44.76 44.93           | NS                 |
| IVNDFD, %   | Maize stover    | 41.77\(^a\) 43.35 43.39 42.05 38.31 1.37      | p < .01 NS NS      |
|             | Rice straw      | 36.19\(^b\) 34.51 38.04 35.84 36.35           | NS                 |

\(^a\)Means within a column for *L. acidophilus* without a common superscript differ \( (p < .05) \).
\(^b\)Mean = mean for individual *L. acidophilus* across supplementation levels including control.
\(^2\)SEM for supplementation level × substrate.
\(^3\)NS = not significant \( (p > .05) \); \( S × L \) = interaction between substrate and supplementation level.

### Table 3. Effect of different supplementation levels of *L. acidophilus* on pH and NH\(_3\)-N concentration for the *in vitro* fermentation of maize stover and rice straw.

| Item       | Substrate       | Supplementation levels \( (\times 10^9) \), cfu/mL | Significance\(^3\) |
|------------|-----------------|-----------------------------------------------|-------------------|
|            |                 | Mean\(^1\) 0.00 0.25 0.50 0.75 SEM\(^2\)        | Substrate Level S × L |
| pH         | Maize stover    | 6.72 6.73 6.70 6.75 6.70 0.06                  | NS NS NS          |
|            | Rice straw      | 6.75 6.79 6.73 6.72 6.78                       | NS                 |
| NH\(_3\)-N, mg/Dl | Maize stover | 8.49\(^a\) 6.29\(^b\) 7.99\(^ab\) 9.43\(^ab\) 10.23\(^ab\) 0.62 | p < .01 L \( (p < .01) \) L \( (p < .05) \) NS |
|            | Rice straw      | 7.10\(^b\) 5.91 7.25 7.49 7.75                 | NS                 |

\(^a\)Means within a row for supplementation levels without a common superscript differ \( (p < .05) \).
\(^b\)Means within a column for *L. acidophilus* without a common superscript differ \( (p < .05) \).
\(^1\)Mean = mean for individual *L. acidophilus* across supplementation levels including control.
\(^2\)SEM for supplementation level × substrate.
\(^3\)NS = not significant \( (p > .05) \); \( S × L \) = interaction between substrate and supplementation level; \( L \) = linear effect of supplementation level.
\(^4\)SEM for pooled mean of substrate including control.
Table 4. Effect of different supplementation levels of *L. acidophilus* on VFA concentration for the *in vitro* fermentation of maize stover and rice straw.

| Item                | Substrate       | Supplementation levels (× 10^3), cfu/mL | Significance^3 | SEM^4 |
|---------------------|-----------------|----------------------------------------|----------------|-------|
|                     |                 | Mean^1 0.00 0.25 0.50 0.75             |                 |       |
| Acetic acid, mmol/L | Maize stover    | 22.40^a 20.17 21.73 24.47 23.24       | 2.49 L (>0.05)  |       |
|                     | Rice straw      | 18.39^b 16.59 16.04 18.03 22.29       | 2.49 L (>0.05)  |       |
|                     | SEM^4           | 1.25                                   |                |       |
| Propionic acid, mmol/L | Maize stover | 8.22 7.93 7.73 8.80 8.04               | 1.05 NS NS NS   |       |
|                     | Rice straw      | 6.88 6.39 6.11 6.65 8.34               | 1.05 NS NS NS   |       |
|                     | SEM^4           | 0.54                                   |                |       |
| Isobutyric acid (10^-2), mmol/L | Maize stover | 24.17^a 20.62 22.53 25.81 27.73       | 3.73 P < 0.05 NS NS NS |       |
|                     | Rice straw      | 17.77^a 14.73 14.56 16.84 24.95       | 3.73 P < 0.05 NS NS NS |       |
|                     | SEM^4           | 1.96                                   |                |       |
| Butyric acid, mmol/L | Maize stover   | 2.67^a 2.61 2.51 2.82 2.74             | 0.28 p < 0.01 NS NS NS |       |
|                     | Rice straw      | 1.93^a 1.86 1.73 1.86 2.28             | 0.28 p < 0.01 NS NS NS |       |
|                     | SEM^4           | 0.14                                   |                |       |
| Isovaleric acid (10^-2), mmol/L | Maize stover | 47.95^a 33.38 55.83 47.79 54.81       | 5.31 p < 0.01 NS NS NS |       |
|                     | Rice straw      | 32.34^a 30.02 29.00 30.47 39.87       | 5.31 p < 0.01 NS NS NS |       |
|                     | SEM^4           | 1.27                                   |                |       |
| Valeric acid (10^-2), mmol/L | Maize stover | 34.41^a 33.19 32.60 36.51 35.57       | 4.06 p < 0.01 NS NS NS |       |
|                     | Rice straw      | 27.68^a 25.44 24.55 26.95 33.75       | 4.06 p < 0.01 NS NS NS |       |
|                     | SEM^4           | 2.10                                   |                |       |
| Total VFA, mmol/L   | Maize stover    | 2.74 2.75 2.77 2.77 2.74               | 0.06 NS NS NS   |       |
|                     | Rice straw      | 2.69 2.65 2.66 2.66 2.73               | 0.06 NS NS NS   |       |
|                     | SEM^4           | 0.03                                   |                |       |

^a,bMeans within a row for supplementation levels without a common superscript differ (p < 0.05).

^cMeans within a column for *L. acidophilus* without a common superscript differ (p < 0.05).

^dMean = mean for individual *L. acidophilus* supplementation levels including control.

^eSEM for supplementation level × substrate.

^fSEM for pooled mean of substrate including control.

C4 plant whereas rice straw is a C3 plant; C4 plants can synthesise more carbohydrates than C3 plants during photosynthesis (Zhou et al. 2016), resulting in a faster fermentation rate for maize stover. Our results also showed that FRD0 increased linearly with the addition of *L. acidophilus*, indicating that the optimum supplementation level of *L. acidophilus* was 0.75 × 10^7 cfu/mL for both substrates, due to the improvement in microbial activity in the *in vitro* fermentation system during the initial phases of fermentation.

CH4 is an inevitable product generated from the anaerobic fermentation of dietary carbohydrates in the rumen, and methanogenesis possesses a specific biological regulatory mechanism. Recently, many researchers have focussed on ruminant CH4 formation because of its contribution to global climate change, which has become a major global environment concern (Martin et al. 2010). During the ruminant metabolic process, the generation of methane in the rumen is the main source of energy loss in rumen fermentation; approximately 6–15% of feed energy is lost in the formation of methane (Johnson & Johnson 1995). Santoso et al. (2003) reported that CH4 production could be affected by the nature of the carbohydrates being fermented, such as cellulose, hemicellulose, soluble residues and digestible ADF in the diet; cellulose and hemicellulose are also important fibre fractions that influence CH4 production. CH4 production has a stronger relationship with digestible NDF intake (Estermann et al. 2002), and ADF and cellulose contents in forage also has a positive correlation with CH4 production (Singh et al. 2012). However, we found that CH4 production was not affected with the addition of *L. acidophilus*, which may be because of differences between *in vitro* and *in vivo* experiments, and/or fermentation substrates (single fermented cell wall substrates compared to total mixed ration substrates).

In vitro DM and NDF degradation

*In vitro* DM degradation (IVDMD) reflects the degree of degradation of substrates by microorganisms in fermentation systems. Reich and Kung (2010) reported that a combination of *L. buchneri* 40788 with *L. plantarum* or *Pediococcus acidilactici* tended to increase *in vitro* NDF degradation (IVNDFD) in treated compared to untreated silage, and other researchers have reported similar effects on IVDMD and IVNDFD (Contreras-Govea et al. 2011). However, there is little information available about the effects of *L. acidophilus* supplementation on the *in vitro* fermentation characteristics of maize stover and rice straw. Our results...
showed that the IVDMD of maize stover was higher than that of rice straw, this may be much more related to fibre composition and proportion between different compounds, silicates, lignification, etc. The ruminal microbial population may be another key reason causing this difference. The rumen is a very complex ecosystem, in which numerous microorganisms and factors play an important role, LAB do not possess the enzymatic ability to hydrolyse cell-wall constituents (Rooke & Hatfield 2003), and the activity of cellulolytic bacteria may not have been affected with the addition of *L. acidophilus*. Further study is needed to investigate the effect of *L. acidophilus* supplementation on the activity of amylolytic, proteolytic and cellulolytic bacteria in *in vitro* rumen fermentation.

**In vitro fermentation parameters**

As pH is the main index reflecting internal homeostasis of the rumen environment, maintaining a relatively stable ruminal pH is vital to ensuring efficient rumen fermentation. Ruminants usually possess highly developed systems to maintain ruminal pH within a physiological range of approximately 5.5–7.0 (Krause & Oetzel 2006). We maintained the range of *in vitro* ruminal pH between 6.72–6.79, which provided suitable conditions for fermentation, growth of microorganisms and fibre degradation in the rumen (Stewart et al. 1997). *In vitro* ruminal pH was not affected by the addition of *L. acidophilus*; different supplementation levels of *L. acidophilus* did not interfere with ruminal stability.

Although it is an important nitrogen source for microbial growth and protein synthesis, ruminal NH$_3$-N has been shown to have a low efficiency for milk protein synthesis partially due to ammonia N losses in the rumen (Hristov & Ropp 2003). Satter and Slyter (1974) suggested that the lowest concentration of NH$_3$-N in rumen liquor should not be less than 5 mg/dL to maintain high bacterial growth rate. Deficiency of NH$_3$-N restricts microbial protein synthesis, while high concentrations of NH$_3$-N also inhibit microbial utilisation of this compound (Hristov et al. 2002). To a certain extent, ruminal NH$_3$-N concentration reflects the equilibrium state for protein degradation and synthesis under specific dietary conditions. We found that the ruminal NH$_3$-N concentration of maize stover was higher than that of rice straw with the addition of *L. acidophilus*, which may result from a difference in protein content and components between these two substrates, subsequently affecting fermentation end-products and the amount of protein synthesis or degradation by rumen microbes. Additionally, our results showed that the concentration of NH$_3$-N across different supplementation levels of *L. acidophilus* ranged from 5.91 to 10.23 mg/dL, indicating that growth and protein synthesis of microorganisms was not restricted. We also found that ruminal NH$_3$-N concentration increased linearly with the addition of *L. acidophilus*. Adding *L. acidophilus* may promote growth of rumen protozoa, which could phagocytise rumen bacteria resulting in the invalid microcirculation of microbial protein (Jouany 1996). Additionally, some rumen bacteria also play a role in NH$_3$-N generation. Many studies have demonstrated that protein decomposing microbes (e.g. *Prevotella* sp.) play an important role in the degradation of protein to NH$_3$-N (Wallace 1994). Therefore, addition of *L. acidophilus* may have some effect on microbial protein synthesis and cellulose decomposition efficiency.

Ruminal volatile fatty acids (VFAs) are major energy sources for ruminants; their content and composition are important physiological indexes that reflect rumen digestion and metabolism. Ruminal microorganisms can transform carbohydrates (e.g. crude fibre, starch and soluble sugar) into pyruvic acid, which can be used to synthesise different VFAs via metabolic pathways. Many studies have indicated that rumen VFAs could provide 50–80% of the energy needed by ruminants (Sutton 1985). We found that, with or without *L. acidophilus*, ruminal VFAs content of maize stover was higher than for rice straw, except for propionic acid and A:P, which may be caused by differences in the carbohydrate content (e.g. fibre content and its composition, starch content, etc.) between the two fermentation substrates (Wallace 1996). Additionally, our results showed that different *L. acidophilus* supplementation levels did not affect VFAs content except for valeric acid. Differences in propionic acid and A:P between maize stover and rice straw and the differences in valeric acid at different levels of *L. acidophilus* supplementation require further research.

**Conclusions**

*Lactobacillus acidophilus* supplementation increased *in vitro* gas production and *in vitro* fermentation NH$_3$-N concentration of maize stover and rice straw, and the greatest increase observed at a dosage of $0.75 \times 10^7$ cfu/mL. Additionally, *in vitro* gas production, the rate of gas production during early incubation, IVDMD, IVNDFD and total VFAs in maize stover were higher...
than those of rice straw in in vitro fermentation. The present in vitro results should be tested further using in vivo experiments to explore the effects of *L. acidophilus* on milk production in dairy cows in the future.

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**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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