Effects of Lactobacillus plantarum on in vitro fermentation characteristics of corn stover and rice straw (Postprint)

Authors: Chen Liang, Ren Ao, Li Bin, Zhou Chuanshe, Zhiliang Tan

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Abstract

This experiment aimed to investigate the effects of *Lactobacillus plantarum* on the in vitro rumen fermentation characteristics of corn straw and rice straw in dairy cows. A single-factor randomized block design was adopted, using corn straw and rice straw as fermentation substrates respectively, to analyze the effects of *Lactobacillus plantarum* at different addition levels [0 (control), $0.25\$\times10^7, 0.50\times10^7, and 0.75\times10^7 CFU/mL] on in vitro fermentation gas production (at 1, 2, 4, 6, 12, 24, 36, 48h), N) in fermentation fluid, and pH. The results showed that * Lactobacillus plantarum * addition significantly increased the gas production rate and volume during the early fermentation stage (1–24h) of 0.05), with the addition level of $0.75\times10^7 CFU/mL$ being the most effective; * Lactobacillus plantarum * addition significantly increased gas production during the late stage (36–48h) of rice straw in vitro fermentation (1 0.05), with the addition level of $0.25\times10^7 CFU/mL$ being the most effective. With increasing addition levels of * Lactobacillus plantarum *, the $NH3-N$ concentration in the fermentation fluid of both substrates showed a signi 0.05). Different addition levels of * Lactobacillus plantarum * had no significant effects on NDFD, DMD, ferm 0.05). Based on the experimental results, it can be inferred that * Lactobacillus plantarum * addition can promote the in vitro fermentation of corn straw and rice straw and their nitrogen metabolism, while als CFU/mL, respectively.

Full Text

Effects of *Lactobacillus plantarum* on in Vitro Rumen Fermentation Characteristics of Maize Stover and Rice Straw

CHEN Liang[1],[2], REN Ao[1],[2], *LI Bin*[3], *ZHOU Chuanshe*[2],[4], TAN Zhiliang[2]

[1]College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, China
[2]Key Laboratory for Agri-Ecological Processes in Subtropical Region, Hunan

Research Center of Livestock & Poultry Sciences, South Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China
[3]Institute of Animal Science, Tibet Academy of Agricultural and Animal Husbandry Sciences, Lhasa 850000, China
[4]Hunan Co-Innovation Center of Animal Production Safety, Changsha 410128, China

Abstract: This experiment aimed to investigate the effects of *Lactobacillus plantarum* on the in vitro ruminal fermentation characteristics of maize stover and rice straw in dairy cows. Using a single-factor randomized block design, four supplemental levels of *Lactobacillus plantarum* [0 (control), 0.25$×10^{7}, 0.50×10^{7}, and 0.75×10^{7}$ CFU/mL] were evaluated for their influence on in vitro fermentation gas production (at 1, 2, 4, 6, 12, 24, 36, and 48 h), gas production parameters, dry matter degradability (DMD), neutral detergent fiber degradability (NDFD), volatile fatty acid (VFA) concentrations, ammonia nitrogen (NH_3-N) concentration, and pH of fermentation fluid. The results demonstrated that *Lactobacillus plantarum* supplementation significantly increased the initial gas production rate and cumulative gas production of maize stover during early fermentation (1–24 h) ($P < 0.05$), with the 0.75$×10^{7}$ CFU/mL level showing the most pronounced effect. For rice straw, *L. plantarum* significantly enhanced gas production during the later fermentation stage (36–48 h) ($P < 0.05$), with 0.25$×10^{7}$ CFU/mL being the optimal level. As the supplementation level increased, NH_3-N concentration exhibited a significant linear increase for both substrates ($P < 0.05$). However, no significant effects were observed on NDFD, DMD, VFA concentrations (acetic, propionic, isobutyric, butyric, and valeric acids), or pH across different supplementation levels ($P > 0.05$). These findings suggest that *L. plantarum* supplementation can promote the in vitro fermentation and nitrogen metabolism of both maize stover and rice straw while maintaining pH stability, with optimal supplementation levels of 0.75$×10^{7}$ and 0.25$×10^{7}$ CFU/mL, respectively.

Keywords: *Lactobacillus plantarum*; in vitro fermentation; rumen; dairy cows; maize stover; rice straw

Introduction

Growing public concern regarding livestock product safety and environmental protection has heightened interest in microecological agents as green, safe, and efficient feed additives for livestock and poultry production. Current research on microecological agents primarily focuses on lactic acid bacteria, fungi and yeasts, bacilli, and photosynthetic bacteria. Among these, lactic acid bacteria, yeasts, and bacilli are most widely applied in ruminant nutrition and feed, with lactic acid bacteria being particularly utilized in silage fermentation. *Lactobacillus plantarum*, a species of lactic acid bacteria, is commonly employed in

silage fermentation. Studies by Contreras-Govea et al. demonstrated that *L. plantarum* significantly promoted microbial growth in alfalfa and maize silage, while other research confirmed its ability to enhance fermentation progression and aerobic stability of corn silage, although some reports indicate no significant effect on aerobic stability.

Maize stover and rice straw represent major agricultural residues in China, constituting a substantial proportion of annual crop straw production. Utilizing these residues for livestock feeding holds significant importance for improving resource efficiency. Compared with forage grasses, maize and rice straw are of lower nutritional quality, making processing technologies critical for enhancing their feeding value. While numerous studies have investigated various additives for straw-based animal production, few have examined whether *L. plantarum* supplementation can improve the in vitro ruminal fermentation characteristics of maize stover and rice straw. This study employed real-time in vitro fermentation monitoring technology to evaluate the effects of *L. plantarum* on ruminal fermentation characteristics of these two substrates, providing theoretical and technical support for its practical application in dairy production.

Materials and Methods

1.1.1 Test Strain

The *Lactobacillus plantarum* strain (strain number: 22696) was purchased from the China Center of Industrial Culture Collection and preserved by vacuum freeze-drying in ampoules.

1.1.2 MRS Medium

The MRS medium contained (per liter): casein peptone 10.0 g, beef extract 10.0 g, yeast powder 5.0 g, glucose 5.0 g, sodium acetate 5.0 g, diammonium citrate 2.0 g, Tween 80 1.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $MnSO_4 \cdot H_2O$ 0.05 g, $CaCO_3$ 20.0 g, and agar 15.0 g, dissolved in distilled water and adjusted to pH 6.8.

1.1.3 Buffer Solution

Rumen in vitro fermentation anaerobic buffer was prepared according to the method of Menke et al.

1.1.4 Fermentation Substrate

Maize stover (Kexiang Tianyu No. 1, Changsha, Hunan) and rice straw (Liuyang Xiang 125s, Hunan) were used as fermentation substrates. Both straw types were dried at 65 °C for 24 h, ground to pass through a 1 mm sieve, and stored for later use. Crude fiber content was determined according to GB/T 18868-2002. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were measured using a Fibretherm FT12 automatic fiber analyzer (Gerhardt

Analytical Systems, Germany) following the method of Hall et al. Dry matter (DM), organic matter (OM), crude protein (CP), and neutral detergent solubles (NDS) were determined using conventional methods described by Yang Sheng. The main nutrient compositions of maize stover and rice straw are presented in Table 1 .

1.1.5 Experimental Animals and Diet

Three healthy Holstein dairy cows fitted with permanent rumen fistulas (body weight: 500 ± 50 kg) served as rumen fluid donors. The cows were provided by Shenghe Dairy Farm in Wangcheng County, Changsha, Hunan Province. During the experimental period, the basal diet was formulated according to NRC (2001) standards, consisting of roughage (rice straw) and concentrate at a 40:60 forage-to-concentrate ratio. Nutrient levels of the basal diet were determined using the same methods as for the substrates. Calcium (Ca) and phosphorus (P) contents were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). The composition and nutrient levels of the basal diet are shown in Table 2 .

1.2.1 Experimental Design

A single-factor randomized block design was employed with four supplementation levels of *Lactobacillus plantarum* [0 (control), 0.25$×10^{7}, 0.50×10^{7}, and 0.75×10^{7}$ CFU/mL]. Each supplementation level included three sampling time points (12, 24, and 48 h) with three replicates per time point.

1.2.2 Strain Cultivation

The ampoule tip was sterilized by flame, then 1–2 drops of sterile water were added and the tip was gently tapped to break it. Subsequently, 0.5–1.0 mL of sterile liquid MRS medium (without agar) was added to dissolve the lyophilized strain, which was then transferred using a sterile 1 mL syringe into a 50 mL conical flask containing 20 mL of liquid MRS medium. After static cultivation at 37 °C for 48 h, continuous subculturing was performed. At the fourth generation, plate counting was conducted. When the required concentration of 1$×10^{7}$ CFU/mL was achieved, the culture was stored at 4 °C for later use. All procedures for strain activation, subculturing, and plate counting were performed under aseptic conditions.

1.2.3 In Vitro Fermentation Fluid Preparation

Rumen digesta were collected from the three fistulated cows before morning feeding, filtered through eight layers of cheesecloth, and equal volumes of filtrate were mixed and transferred into a pre-warmed (39.5 °C) thermos flask filled with CO_2. The mixture was immediately transported to the laboratory and combined with anaerobic buffer solution preheated to 39.5 °C in a constant-temperature

water bath (buffer:rumen fluid = 9:1, v/v) while continuously flushing with CO_2.

1.2.4 In Vitro Cultivation

The in vitro fermentation system was independently developed and consisted of a constant-temperature shaking incubator (6$×$6 grid), computer host, and monitor. Each grid cell represented one unit housing a single fermentation bottle. Each cell was equipped with an air pressure sensor connected to the fermentation bottle, which was linked to the computer host for real-time monitoring of pressure changes.

Approximately (0.5000 ± 0.0003) g of fermentation substrate was weighed into each fermentation bottle, and 0, 0.25, 0.50, or 0.75 mL of bacterial suspension was added according to the experimental design. The prepared fermentation bottles were preheated in a 39.5 °C incubator, flushed with CO_2, then 50 mL of fermentation fluid was added while continuing CO_2 flushing. The bottles were immediately sealed, vented with a needle to equalize internal and external pressure, and rapidly returned to the incubator for 48 h at 39.5 °C.

1.2.5 Measurement of Total In Vitro Gas Production

Gas pressure inside the fermentation bottles was measured at 1, 2, 4, 6, 12, 24, 36, and 48 h using a pressure sensor (CYG130-12, Kunshan Shuangqiao Sensor Measurement and Control Technology Co., Ltd.). Pressure values were converted to gas volume at standard room temperature and pressure using the formula y = 1.506x, where 1.506 is the conversion coefficient between measured pressure and volume, x represents measured pressure, and y represents gas production.

Cumulative gas production data were fitted using the LE model proposed by Wang et al.:

$$V(t) = V_f \cdot \exp\{-\exp[-k(t-d)]\}$$

where $V(t)$ is gas production at time t (mL), V_f is theoretical maximum gas production (mL), k is fractional gas production rate (%/h), and d is a curve shape parameter. The parameter b is used to characterize the curve shape ($b > 0$ indicates S-shaped curve, $b < 0$ indicates non-S-shaped curve). The initial fermentation rate (FRD_0, < 12 h) and time to reach half of theoretical maximum gas production ($t_{0.5}$) were calculated using the following formulas:

$$FRD_0 = k \cdot \exp(b)$$

$$t_{0.5} = \frac{\ln(\ln 2) + d}{k}$$

1.2.6 Determination of DMD, NDFD, NH_3-N, VFA, and pH

At 12, 24, and 48 h of fermentation, bottles were removed and fermentation fluid was filtered through 400-mesh nylon cloth. Filtrate pH was immediately measured using a pH meter (REX PHS-3C, Shanghai Instrument Equipment Factory). The filtrate was then aliquoted into centrifuge tubes for NH_3-N and VFA analysis. NH_3-N concentration was determined using the modified colorimetric method of Feng Zongci et al., while VFA concentration was measured according to the method of Vanzant et al.

Filtered residues were transferred to quartz crucibles, rinsed repeatedly with hot distilled water, and dried at 105 °C for 8 h to determine residual dry matter content and calculate DMD. Dried residues were sealed in sample bags for subsequent NDFD determination.

1.3 Data Analysis

Experimental data were analyzed using the MIXED procedure of SAS 8.2. Differences among supplementation levels were compared using contrast statements. Statistical significance was defined as $P < 0.05$.

Results

2.1 Effects of Different *Lactobacillus plantarum* Supplementation Levels on In Vitro Gas Production

The effects of different *L. plantarum* supplementation levels on in vitro gas production from maize stover are presented in Table 3 . When maize stover served as the fermentation substrate, gas production at 1, 2, and 4 h was significantly higher with 0.75$×10^{7}$ CFU/mL supplementation compared to the control, $0.25\$\times10^{\{7\},and0.50\times10}\{7\}\$$ CFU/mL groups ($P < 0.05$), while no significant differences existed among the latter three groups ($P > 0.05$). At 6 h, the 0.75$×10^{7}$ CFU/mL group exhibited significantly higher gas production than the 0.50$×10^{7}$ CFU/mL group ($P < 0.05$), but did not differ from the control or 0.25$×10^{7}$ CFU/mL groups ($P > 0.05$). At 12 h, the 0.75$×10^{7}$ CFU/mL group showed significantly higher gas production than all other groups ($P < 0.05$), while the 0.50$×10^{7}$ CFU/mL group was also significantly higher than the control and 0.25$×10^{7}$ CFU/mL groups ($P < 0.05$). At 24 h, both the 0.75$×10^{7}$ and 0.50$×10^{7}$ CFU/mL groups demonstrated significantly higher gas production than the control and 0.25$×10^{7}$ CFU/mL groups ($P < 0.05$), with no significant differences between the two high-dose groups or between the control and 0.25$×10^{7}$ CFU/mL group ($P > 0.05$). No significant differences were observed among any groups at 36 and 48 h ($P > 0.05$). These results indicate that *L. plantarum* supplementation significantly promoted early-stage fermentation of maize stover, with 0.75$×10^{7}$ CFU/mL being the most effective dose.

When rice straw served as the substrate, the control group exhibited sig-

nificantly higher gas production at 1 h than all treatment groups ($P < 0.05$), with the 0.25$×10^{7}$ CFU/mL group being significantly higher than the 0.75$×10^{7}$ CFU/mL group ($P < 0.05$) but not differing from the 0.50$×10^{7}$ CFU/mL group ($P > 0.05$). This may be attributed to an adaptation period for *L. plantarum* at fermentation initiation, which temporarily inhibited normal fermentation. At 36 and 48 h, the 0.25$×10^{7}$ CFU/mL group showed significantly higher gas production than the control and 0.75$×10^{7}$ CFU/mL groups ($P < 0.05$), but did not differ from the 0.50$×10^{7}$ CFU/mL group ($P > 0.05$). No significant differences were detected among the control, $0.50\$\times10^{\{7\},and0.75\times10}\{7\}\$$ CFU/mL groups ($P > 0.05$).

2.2 Effects of Different *Lactobacillus plantarum* Supplementation Levels on In Vitro Gas Production Parameters

The effects of different *L. plantarum* supplementation levels on gas production parameters of maize stover are shown in Table 4 . The mean values of theoretical maximum gas production, initial fermentation rate, and time to reach half of theoretical maximum gas production for maize stover were all significantly higher than those for rice straw ($P < 0.05$).

For maize stover substrate, the initial fermentation rate with 0.75$×10^{7}$ CFU/mL supplementation was significantly higher than the control, $0.25\$\times10^{\{7\},and0.50\times10}\{7\}\$$ CFU/mL groups ($P < 0.05$). The 0.50$×10^{7}$ CFU/mL group also showed a significantly higher initial fermentation rate than the control ($P < 0.05$), but did not differ from the 0.25$×10^{7}$ CFU/mL group ($P > 0.05$), which in turn did not differ from the control ($P > 0.05$). The time to reach half of theoretical maximum gas production with 0.75$×10^{7}$ CFU/mL supplementation was significantly lower than the control and 0.25$×10^{7}$ CFU/mL groups ($P < 0.05$), but did not differ from the 0.50$×10^{7}$ CFU/mL group ($P > 0.05$). No significant differences were observed among supplementation levels for theoretical maximum gas production ($P > 0.05$).

For rice straw substrate, no significant effects of supplementation level were observed on theoretical maximum gas production or time to reach half of theoretical maximum gas production ($P > 0.05$). The initial fermentation rate with 0.75$×10^{7}$ CFU/mL supplementation was significantly higher than the control ($P < 0.05$), and a significant linear increase in initial fermentation rate was observed with increasing supplementation levels ($P < 0.05$). A significant interaction between substrate and supplementation level was detected for initial fermentation rate ($P < 0.05$), but not for theoretical maximum gas production or time to reach half of theoretical maximum gas production ($P > 0.05$).

2.3 Effects of Different *Lactobacillus plantarum* Supplementation Levels on In Vitro VFA Concentration

The effects of different *L. plantarum* supplementation levels on VFA concentrations from maize stover and rice straw fermentation are presented in Table 5 . Mean concentrations of all individual VFAs and the acetate/propionate ratio were significantly higher for maize stover than for rice straw ($P < 0.05$).

No significant effects of supplementation level were observed on acetic, propionic, isobutyric, butyric, valeric, or total VFA concentrations, nor on the acetate/propionate ratio for either substrate ($P > 0.05$). Additionally, no significant interaction between supplementation level and substrate was detected for any VFA parameter ($P > 0.05$).

2.4 Effects of Different *Lactobacillus plantarum* Supplementation Levels on In Vitro NDFD and DMD

The effects of different *L. plantarum* supplementation levels on NDFD and DMD of maize stover and rice straw are shown in Table 6 . No significant differences were observed in mean NDFD and DMD values between the two substrates across supplementation levels ($P > 0.05$). Furthermore, *L. plantarum* supplementation level did not significantly affect NDFD or DMD for either substrate ($P > 0.05$), and no significant interaction between substrate and supplementation level was detected for these parameters ($P > 0.05$).

2.5 Effects of Different *Lactobacillus plantarum* Supplementation Levels on In Vitro NH_3-N Concentration and pH

The effects of different *L. plantarum* supplementation levels on NH_3-N concentration and pH are presented in Table 7 . A significant linear increase in NH_3-N concentration was observed with increasing supplementation levels for both maize stover and rice straw ($P < 0.05$). For maize stover, the 0.75$×10^{7}$ CFU/mL group showed significantly higher NH_3-N concentration than the control and 0.25$×10^{7}$ CFU/mL groups ($P < 0.05$), while the 0.50$×10^{7}$ CFU/mL group was also significantly higher than the control ($P < 0.01$). For rice straw, the 0.75$×10^{7}$ CFU/mL group exhibited significantly higher NH_3-N concentration than all other groups ($P < 0.05$). No significant effects of substrate, supplementation level, or their interaction were observed on fermentation pH ($P > 0.05$), which remained within the normal range for ruminal fluid.

Discussion

Low-level *L. plantarum* supplementation promoted mid-to-late stage fermentation of rice straw to some extent, while high-level supplementation showed limited promotional effects. Ruminal gas primarily originates from metabolic byproducts including short-chain fatty acids, methane, hydrogen, and carbon

dioxide produced by rumen microorganisms consuming soluble carbohydrates and other nutrients. The differential effects of *L. plantarum* on early-stage fermentation of maize stover versus mid-to-late stage fermentation of rice straw may be related to differences in plant cell wall structure and nutrient release patterns between the two substrates.

In vitro gas production partially reflects the degree of substrate utilization by rumen microorganisms and can effectively predict in vivo dry matter digestibility and metabolizable energy. Muck et al. reported that 65–70% of total gas production occurs within the initial 9–10 h of fermentation. In contrast, the current study found that maize stover and rice straw required approximately 15 and 19 h, respectively, to reach half of theoretical maximum gas production. This longer duration may be attributed to differences in cultivation methodologies. Contreras-Govea et al. observed no significant changes in VFA concentration when using *L. plantarum*-inoculated silage for in vitro fermentation, consistent with our findings. While microbial inoculation of silage has been shown to affect VFA concentrations, and ruminal inoculation with lactic acid bacteria can influence VFA composition, our results showed no significant effects of *L. plantarum* supplementation on individual VFA concentrations or acetate/propionate ratio, except for differences between substrates. These substrate-related differences likely stem from variations in chemical composition and cell wall structure, as different organic matter and mineral contents can lead to different VFA concentrations. Zhang et al. reported significant differences in total VFA production and individual VFA components (except butyrate) among six different plant cell wall sources.

Previous studies using *L. plantarum*-inoculated silage for in vitro fermentation reported no significant differences in NDFD and DMD compared to controls, consistent with our results. However, some research has shown that *L. plantarum* can improve in vitro DMD of silage, with discrepancies likely arising from substrate differences. In our study, DMD tended to increase with higher supplementation levels, correlating positively with gas production, which aligns with established relationships between these parameters. The higher average DMD and NDFD observed for maize stover compared to rice straw may be attributed not only to chemical composition differences but also to microbial adhesion capacity and substrate structure. Bacterial adhesion to substrate is a critical factor affecting digestibility, and differences in plant tissue structure and microbial colonization patterns may contribute to variations in fiber degradation among substrates.

The consistent increase in NH_3-N concentration with fermentation time and gas production suggests that *L. plantarum* influences ruminal nitrogen metabolism. Meng et al. reported a high correlation ($r > 0.99$) between NH_3-N concentration and gas production in vitro. Hu et al. found that *L. plantarum* significantly reduced NH_3-N concentration in corn silage of varying dry matter content, likely due to substrate nutritional differences. Ruminal pH serves as an important biochemical indicator of nutrient fermentation, with stability directly affecting

rumen ecosystem diversity and animal health. The pH values of 6.40–6.47 observed in this study fall within the normal range for ruminants and remained stable across supplementation levels, consistent with VFA concentration patterns. In contrast, some studies have reported pH values below 4 in *L. plantarum*-fermented silage, possibly due to high microbial and organic acid content in silage materials.

Conclusion

1. *Lactobacillus plantarum* supplementation significantly improved the initial gas production rate and cumulative gas production of maize stover during early fermentation (1–24 h), with 0.75$×10^{7}$ CFU/mL being the optimal level.
2. *Lactobacillus plantarum* supplementation significantly enhanced gas production from rice straw during the later fermentation stage (36–48 h), with 0.25$×10^{7}$ CFU/mL being the optimal level.
3. Increasing *L. plantarum* supplementation levels produced a significant linear increase in NH_3-N concentration for both substrates, indicating enhanced nitrogen metabolism while maintaining pH stability.

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Note: Figure translations are in progress. See original paper for figures.