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Research Article

In-vitro Evaluation of the Fermentation Characters of Maize Stover and Rice Straw with Different Level from *Bacillus coagulans*

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Abstract

The study was carried out to investigate the impact of *Bacillus coagulans* supplementation with different concenteration (0, 0.25×10^7, 0.50×10^7, and 0.75×10^7 cfu/mL) on *in vitro* parameters as methane (CH_4) parameters, nutrients digestibility and the fermentation character of rumen of fibrous agriculture by-products (maize stover and rice straw) using probiotics preparations in ruminants. The results showed that, maximum gas production (V_f), gas production fraction (k) and the time when half of the maximum gas production was achieved ($t_{0.5}$) of maize stover were significantly increased when compared to group of the rice straw. Additionally, *in vitro* dry matter disappearance; (IVDMD), *in vitro* neutral detergent fiber disappearance (IVNDFD), individual volatile fatty acids (VFAs) and total VFA (TVFA) of maize stover were significantly in related to the rice straw group. Also, the gas production rate at the early incubation stage (FRD_0), pH value of rumine and the acetate: propionate ratio of maize stover is significantly lower than the rice straw. V_f of rice straw was quadratic ($P<0.05$) increased when adding *Bacillus coagulans*. These results indicate that, *in vitro* gas production (IVGP) was numerically increased when maize stover and rice straw were fermented using *Bacillus coagulans* at a level of 0.75×10^7 cfu/mL. Additionally, maize stover (CP 0.053, NDF 0.636, ADF 0.386) can be used as a superior roughage for ruminants compared to rice straw. The present *in vitro* positive results should be further testified using in vivo experiments in future.

Keywords: *Bacillus coagulans, Maize strover, In vitro gas production, CH4, Volatile fatty acids*

Farklı Miktarlarda *Bacillus coagulans*'ın Mısır Hasat Kalıntısı ve Pirinç Samanının Fermantasyon Özelliklerine Etkisinin *In Vitro* Değerlendirilmesi

Öz

Bu çalışma, farklı konsantrasyonlarda (0, 0.25×10^7, 0.50×10^7 ve 0.75×10^7 cfu/mL) *Bacillus coagulans*'ın ruminantlarda metan (CH_4) parametresi, besinlerin sindirilebilirliği ve fibröz yapılı zirai yan ürünlerin (mısır hasat kalıntısı ve pirinç samanı) rumen fermantasyon karakteri üzerine etkisini araştırmak amacıyla yapılmıştır. Elde edilen bulgular, pirinç samanı ile karşılaştırıldığında mısır hasat kalıntısı ile maksimum gaz üretimi (V_f), gaz üretim fraksiyonu (k) ve maksimum gazın yarısının oluştuğu zamanın ($t_{0.5}$) anlamlı oranda arttığını gösterdi. Ayrıca, pirinç samanı ile karşılaştırıldığında mısır hasat kalıntısı grubunda *in vitro* kuru madde kaybolması (IVDMD), *in vitro* nötral deterjan lif kaybolması (IVNDFD), bireysel uçucu yağ asitleri (VFAs) ve total VFA (TVFA) daha yüksek olarak bulundu. Pirinç samanı ile karşılaştırıldığında mısır hasat kalıntısının erken inkübasyon döneminde gaz üretim oranı (FRD_0), rumen pH'sı ve asetat:propiyonat oranı daha düşük olarak gözlemlendi. Pirinç samanının V_f değeri *Bacillus coagulans* ilavesi ile kuadratik ($P<0.05$) artım gösterdi. Elde edilen sonuçlar, mısır hasat kalıntısı ve pirinç samanının 0.75×10^7 cfu/mL oranında *Bacillus coagulans* ile fermente edildiğinde *in vitro* gaz üretimini (IVGP) numerik olarak artırdığını göstermektedir. Mısır hasat kalıntısının (CP 0.053, NDF 0.636, ADF 0.386) pirinç samanı ile karşılaştırıldığında ruminantlar için kaba yem olarak tercihen kullanılabileceği düşünüldü. Bu *in vitro* bulgular *in vivo* çalışmalar ile desteklenerek ileriki çalışmalarla teyit edilmelidir.

Anahtar sözcükler: *Bacillus coagulans, Mısır hasat kalıntısı, In vitro gaz üretimi, CH_4, Uçucu yağ asitleri*

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INTRODUCTION

Fibrous agricultural by-products such as maize stover, and crop straws of rice and wheat are abundantly available in many countries [1]. However, they are rich sources of crude fiber with high lignin contents, but their protein content is low, which decreases the dry matter and nutrient degradations in the animal rumen [2]. Different methods have been examined such as chemical and physical treatments to increase the nutritive value of such by-products. Although these methods were effective to improve feed intake and/or digestibility of these fibrous feedstuffs [1-3], they are expensive and harmful to both users and environment [4]. Furthermore, biological methods such as probiotics (microorganism preparations) were found to be a good economic and safe alternative to increse the digestibility of such fibrous by-products [5]. Probiotics are last recent biological methods by using alive and suitable microorganisms that can beneficial effects to the host health when consumed in appropriate and regular quantities [6]. This microorganisms have been used to improve the *in vitro* fermentation characteristics of the roughages with low-quality [7] The scientific interest to several *bacillus* strains have been screened for their potential probiotic functionalities with special regard to *Bacillus natto* and *Bacillus subtilis* due to their beneficial effects on ruminants [8]. However, these species were firstly used as probiotics product (Enterogermina®) in Italy since 1958 [9]. So, the data is limited regarding the effect of *Bacillus coagulans* on the improvement of low quality feeds in ruminant nutrition manipulation.

Therefore, this study was a first detailed report to identify the effects of *Bacillus coagulans* supplementation to fibrous agricultural by-products (maize stover and rice straw) on *in vitro* rumen fermentation parameters and nutrients degradation using gas production technique for further understanding their mode of action in the rumen, and providing more knowledge about their application to ruminants' nutrition.

MATERIAL and METHODS

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

Fermented Substrates Probiotics and Experimental Design

Maize stover from Kexiangtian 1 (bred by ISA) and rice straw from Xiang 125s (a local popular breed) were selected as the fibrous agricultural by-products. They were oven dried (at 65ºC for 24 h), ground (filterated through a 1 mm sieve) and stored for further analysis. They were analyzed on DM basis for crude protein (CP) (5.3% and 6.2%), NDF (63.6% and 63.2%) and ADF (38.6% and 43.4%) respectively. *Bacillus coagulans* (NO. 20138) was purchased, reactivated and amplified to contain 1×10^{11} cfu/g viable bacteria using the spread plate method by the China Center of Industrial Culture Collection (CICC). *Bacillus coagulans* preserved at 4°C after culture amplification and counting. The study was induced in completely block experimental design, and *Bacillus coagulans* was supplemented at four levels (0×10^{7} cfu/mL, 0.25×10^{7} cfu/mL, 0.50×10^{7} cfu/mL and 0.75×10^{7}cfu/mL, respectively).

In vitro Gas Production and Sampling

The modified anaerobic (continuous CO_2 pumping for 2 h) artificial saliva was used for *in vitro* fermentation according to [10]. Around three Holstein dairy cows were housed individually and fed a rice straw based completely mixed ration with adlibtum water. Rumen content was collected through the ruminal fistula before the morning feeding and placed into thermos flasks pre-heated at 39°C and quickly transferred to the laboratory. After collection, the inoculum was strained through 4 layers of cheesecloth maintained anaerobic condition with CO_2 flux.

Approximately 0.5 g of each feedstuff was transferred into fermentation bottles 100 mL. Every sample was measured in triplicates at each point of incubation time. *Bacillus coagulans* was added with graded levels to the tested feedstuffs altogether with 45 mL artificial saliva and 5 mL rumen content as previously mentioned at the start of *in vitro* fermentation. All bottle required for fermentation process were sealed and incubated at 39°C. During the *in vitro* fermentation process, the pressure in the bottle was recorded at 0, 1, 2, 4, 6, 12, 24, 36 and 48 h. And after 12, 24 or 48 h of incubation the fermentation was interrupted due to presence of undegraded residues which were filtered through two layers of nylon cloth. Around 5 mL from gas sample was collected by plastic syringe into the vacuum flask for detect CH_4. Finally, at 12, 24 and 48 h from the incubation, the incubated sample from each treatment was used to calculate NH_3-N level and VFAs concentrations respectively

Chemical Analysis

Dry matter (DM) (method 930.15) of the tested substrate and filtered residues were analyzed by drying at 105°C for 12 h and weighed for *in vitro* DM degradation (IVDMD) and they ground for further chemical analysis. CP was detected according to [11]. NDF and ADF values were estimated as described by [12] and all triplicate samples were analyzed. Alpha amylase (Sigma A-3306, Sigma, Aldrich, China), and sodium sulphite were added for NDF determination. The NDF of the dried residues was analyzed for *in vitro* NDF digestibility (IVNDFD). 2 mL of the fermented solutions were centrifuged at 10000 × g and 4°C for 15 min, then 1.5 mL of the supernatant were mixed with 0.15 mL meta-phosphoric acid. Then another centrifugation at 10.000 × g and 4°C for 15 min, and the supernatant was taken to

analyze VFA content with a gas chromatograph (HP5890, Agilent 5890; Agilent Technologies Co. Ltd, USA). The peak of VFA was calculated using their standard concentration curve, which was prepared using 10 samples for each treatment. Total molar concentration was determined by the sum of individual VFA as 100% [13]. pH meter was used to mesure pH of the fermented fluids immediately. For ammonia level determination, around 5 mL of fermented solutions were centrifuged at 4000 ×g at 4°C for 10 min, and 2 mL of the supernatant were mixed 8 ml 0.2 M HCl. 0.4 mL from mixed solution was subsequently mixed with 2 mL of sodium nitroprusside solutions (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), then homogenized at room temperature for 10 min. The absorption was determined at 700 nm using spectrophotometer. The NH_4Cl standard solutions were prepared as follows: 0.382 g of NH_4Cl was diluted with 0.2 M HCl to 100 mL as the preservation solutions kept at 4°C. After that, 10 mL of preservation solutions were diluted to 100 mL with distilled water as the working solution in which the concentration of N was 10 mg/dL. Subsequently, 0, 1, 2, 4, and 6 mL of working solutions were separately mixed with 10, 9, 8, 6, and 4 mL of distilled water and then all diluted with 0.2 M HCl to 50 mL as the NH_4Cl standard solutions in which the concentrations of N were 0, 0.2, 0.4, 0.8 and 1.2 mg/dL, respectively. Finally, 0.4 mL of the NH_4Cl standard solution of each concentration was treated to obtain a standard curve as previously mentioned. CH_4 was analyzed using gas chromatography equipped with a Hayesep Q packing column (2.44 M × 1/8 in. × 2.0 mm ID). The microbial crude protein production in rumen liquor was analyzed using the trichloroacetic acid. Firstly, 0.5 g ground dry sample was weighted into a 125 Erlenmeyer flask then around 50 mL from distilled water was added and wait for 30 min. 10 mL from 10% trichloroacetic acid was add to the mix and incubate the solution for 20-30 min then was filtrated on whatman #54 or 541 paper by gravity. Finally, the filter paper was washed twice with trichloroacetic acid solution followed by transfer the paper to Kjeldahl apparatus to calculate nitrogen percent. NPN was calculated by subtracting residual nitrogen from total nitrogen. NPN value may be expressed as crude protein value which equal to (N X 6.25) or percent of total feed nitrogen [14].

Statistical Analysis

The experimental data were analyzed separately using the PROC MIXED procedure of SAS (SAS Institute, 2001) Orthogonal polynomial contrasts was used for detect linear and quadratic effect. Cubic effects of dose were not analyzed due to the inexplicability in biology. The significance was detected at $P<0.05$ and the least squares means are reported throughout the text.

RESULTS

Effects of *Bacillus coagulans* supplementation levels on gas production parameters of fibrous agriculture byproducts are shown in *Table 1*. The supplementation of *Bacillus coagulans* had significantly lowered ($P<0.01$) the maximum gas production (V_f) of rice straw, while it had no ($P>0.05$) effect among the other three supplemental treatments. The supplementation levels of *Bacillus coagulans* had no significant effect ($P>0.05$) V_f on maize stover, gas production fraction (k), the initial fractional rate of degradation (FRD_0) and the time when half of the maximum gas production was achieved ($t_{0.5}$) for both fermentation substrates. The value of V_f, k and $t_{0.5}$ of maize stover was significant ($P<0.05$) higher than that of rice straw, which was increased by 20.76, 7.86 and 35.48%, respectively. While FRD_0 of maize stover was significant ($P<0.0001$) lower than that of rice straw, which was decreased by 117.16%. Both the fermentation substrate and supplementation level had no significant effect ($P>0.05$) onCH_4 production. The combination between fibrous - by product and *bacillus coagulans* supplementation level had significant ($P<0.05$) effects on gas fermentation characteristics except V_f and CH_4 for both maize stover and rice straw.

Effect of *Bacillus coagulans* adding with different levels on IVDMD, IVNDFD and MCP production of both maize stover and rice straw are shown in *Table 2*. IVDMD, IVNDFD and MCP production of maize stover were significant ($P<0.05$) higher than that of rice straw, which was higher by 16.75, 40.04 and 1.50%, respectively while, this combination had no significant effect on IVDMD, IVNDFD and MCP production for the two fermentation substrates.

The impact of *Bacillus coagulans* supplementation at different levels on ruminal acidity and ammonia level of fermented substrates are recorded in *Table 3*. The pH value of maize stover was significantly ($P<0.05$) decreased when compared to that of rice straw, which was decreased by 0.89%. While the supplementation levels and the combination between substrate level have no statically affect ($P>0.05$) of ruminal NH_3-N concentration of maize stover and rice straw.

The effects of different supplementation levels of *Bacillus coagulans* on *in vitro* ruminal VFA contents of maize stover and rice straw are shown in *Table 4*. VFA of maize stover was significantly ($P<0.01$) elevated in compared to the rice straw, which was increased by 28.86, 31.61, 60.79, 52.50, 89.23 and 76.28%, respectively. While A:P of maize stover was significant ($P<0.05$) lower than that of rice straw, which was decreased by 25.45%. The TVFA content was not significantly affected ($P>0.05$) by substrates, supplementation levels and the interaction between substrate and supplementation level. The supplementation levels and the combination between substrate and *bacillus coagulans* supplementation level not significantly effect ($P>0.05$) on ruminal individual

Table 1. *Effects of different supplementation levels of Bacillus coagulans on in vitro gas production kinetic parameters and CH_4 production of maize stover and rice straw*

Item	Substrate	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Mean[1]	0	0.25	0.50	0.75		Substrate	Level	S×L
[1]V_f (mL)	Maize stover	67.43[e]	68.72	51.53	72.63	76.84	6.63	<0.05	NS	NS
	Rice straw	55.84[f]	55.89[a]	53.93[b]	56.86[b]	56.68[b]			Q (P<0.01)	
	SEM[4]	3.32								
[2]$k(10^{-2})$	Maize stover	9.47[e]	9.31	9.34	9.38	9.84	0.87	<0.01	NS	<0.05
	Rice straw	8.78[f]	8.58	11.20	8.05	7.29			NS	
	SEM[4]	0.44								
[3]FRD_0 (10^{-2}) (mL/h)	Maize stover	1.34[e]	1.50	1.29	1.25	1.32	0.10	<0.0001	NS	<0.05
	Rice straw	2.91[f]	2.75	2.65	3.08	3.16			NS	
	SEM[4]	0.05								
[4]$t_{0.5}$ (h)	Maize stover	22.11[e]	21.25	22.61	22.82	21.76	0.34	<0.0001	Q(P<0.01)	<0.05
	Rice straw	16.32[f]	16.53	16.46	15.99	16.32			NS	
	SEM[4]	0.18								
[5]CH_4 (mL/g)	Maize stover	9.02	8.98	8.93	11.47	6.69	1.50	NS	NS	NS
	Rice straw	8.86	10.12	9.69	6.66	8.99			NS	
	SEM[4]	0.76								

[a,b] Means within a row for supplementation levels do not have a common superscript differ (P<0.05); [e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; Q = quadratic effect of supplementation levels; [4] SEM for pooled mean of substrate including the level of 0; During the initial stages of this work, the correlativity between the pressure in bottle and gas volume was measured at 39°C, and the regression equation was then established: y = 1.506x (n = 20, R^2 = 0.999, P<0.0001); Where y represents gas volume (mL), x is the pressure in bottle (kPa), 1.506 is a constant; Measured pressure was then converted to gas production (mL). In vitro gas production at 0, 1, 2, 4, 6, 12, 24 and 48 h were fitted to Logistic-Exponential [31]: GP = Vf (1 - exp (d - t × k))/(1 + exp(b - k × t)) (2); Where GP represents gas production at t time, Vf means the maximum gas production (mL), k represents gas production fraction (/h), b and d represent the shapes of the gas production curve. The following equation: $t_{0.5}$ = ln (exp (b) + 2 exp (d))/k [34] was used to calculate the time ($t_{0.5}$, h) when half of the maximum gas production was achieved. FRD_0 = k/(1 + exp(b)) was used to calculate the initial fractional rate of degradation (/h)

Table 2. *Effects of different supplementation levels of Bacillus Coagulans on IVDMD, IVNDFD and MCP production of maize stover and rice straw*

Item	Substrate	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Means[1]	0.00	0.25	0.50	0.75		Substrate	Level	S×L
IVDMD (%)	Maize stover	50.18[e]	49.64	52.11	49.58	49.38	3.82	<0.05	NS	NS
	Rice straw	42.98[f]	42.91	43.34	42.30	43.36			NS	
	SEM[4]	1.91								
IVNDFD (%)	Maize stover	38.58[e]	35.90	45.78	37.31	35.32	5.87	<0.05	NS	NS
	Rice straw	27.55[f]	28.14	27.32	27.84	26.91			NS	
	SEM[4]	2.98								
MCP (mg/mL)	Maize stover	3.39[e]	3.38	3.41	3.39	3.38	0.02	<0.001	NS	NS
	Rice straw	3.33[f]	3.33	3.33	3.34	3.31			NS	
	SEM[4]	0.01								

[e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; [4] SEM for pooled mean of substrate including the level of 0

VFA content and A:P for both fermentation substrates.

DISCUSSION

The *in vitro* fermentation cumulative gas production technique widely used [15]. It is an important technique which used to evaluate rumen fermentation for ruminants, which provide valuable information for the kinetics of feed digestion in rumen, reflect the utilization efficiency of fermentation substrates [16]. The results showed that, V_f of maize stover was significantly elevated than the rice straw under different supplementation levels of *Bacillus*

Table 3. *Effects of different supplementation levels of Bacillus coagulans on in vitro ruminal pH value and NH_3-N concentration of maize stover and rice straw*

Items	Substrates	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Means[1]	0.00	0.25	0.50	0.75		Substrate	Level	S×L
pH	Maize stover	6.75[f]	6.78	6.74	6.75	6.73	0.04	<0.05	NS	NS
	Rice straw	6.81[e]	6.82	6.84	6.82	6.77			NS	
	SEM[4]	0.02								
NH_3-N (mg/dL)	Maize stover	6.12	6.28	6.13	6.08	6.01	0.55	NS	NS	NS
	Rice straw	6.39	6.16	6.36	6.53	6.53			NS	
	SEM[4]	0.28								

[e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus Coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; [4] SEM for pooled mean of substrate including the level of 0

Table 4. *Effects of different supplementation levels of Bacillus coagulans on in vitro ruminal VFA contents of maize stover and rice straw*

Items	Substrates	Supplementation Levels (× 10^7 cfu/mL)					SEM[2]	Significance[3]		
		Mean[1]	0.00	0.25	0.50	0.75		Substrates	Level	S×L
Acetate (mmol/L)	Maize stover	21.21[e]	21.0	22.28	21.16	20.33	2.14	<0.01	NS	NS
	Rice straw	16.46[f]	16.1	16.54	16.48	16.66			NS	
	SEM[4]	1.08								
Propionate (mmol/L)	Maize stover	7.87[e]	7.68	8.19	7.71	7.88	0.87	<0.01	NS	NS
	Rice straw	5.98[f]	5.84	6.03	5.98	6.04			NS	
	SEM[4]	0.44								
Isobutyrate (mmol/L) (10^{-2})	Maize stover	23.99[e]	24.00	25.16	23.94	22.88	2.82	<0.0001	NS	NS
	Rice straw	14.92[f]	14.51	14.86	14.95	15.35			NS	
	SEM[4]	1.42								
Butyrate (mmol/L)	Maize stover	2.44[e]	2.42	2.54	2.45	2.36	0.19	<0.0001	NS	NS
	Rice straw	1.60[f]	1.56	1.61	1.59	1.62			NS	
	SEM[4]	0.10								
Isovalerate (mmol/L) (10^{-2})	Maize stover	38.64[e]	38.82	39.94	37.96	37.82	6.14	<0.0001	NS	NS
	Rice straw	20.42[f]	21.91	18.97	20.08	20.72			NS	
	SEM[4]	3.11								
Valerate (mmol/L) (10^{-2})	Maize stover	26.83[e]	26.77	27.74	26.79	26.01	2.26	<0.0001	NS	NS
	Rice straw	15.22[f]	14.94	15.18	14.87	15.89			NS	
	SEM[4]	1.14								
TVFA (mmol/L)	Maize stover	32.61[e]	32.07	33.94	32.20	32.22	3.34	<0.01	NS	NS
	Rice straw	24.54[f]	24.09	24.67	24.56	24.85			NS	
	SEM[4]	1.68								
A:P	Maize stover	2.75[f]	2.75	2.73	2.77	2.75	0.07	<0.05	NS	NS
	Rice straw	2.85[e]	2.87	2.83	2.85	2.86			NS	
	SEM[4]	0.04								

[e,f] Means within a column for Bacillus coagulans do not have a common superscript differ (P<0.05); [1] Mean = mean for individual Bacillus coagulans across supplementation levels including the level of 0; [2] SEM for supplementation level×substrate; [3] NS = not significant (P>0.05); S×L = interaction between substrate and supplementation level; [4] SEM for pooled mean of substrate including the level of 0

coagulans, it might result from the outcome that IVDMD of maize stover was significantly higher than that of rice straw *(Table 2)*. The maximum gas production was positively related to readily fermentable substrates [17], hemicellulose and crude protein (CP) contents, and negatively related to the ADF and NDF contents, while other studies observed an adverse relationship between the production of gas and nitrogen content [18,19].

The indices of FRD_0 and $t_{0.5}$ generally means the rate of deterioration in early incubation stages "<12 h" and the incubation time to reach half of the maximum gas

production, respectively. In general, the faster FRD_0 the shorter $t_{0.5}$ become [20]. FRD_0 value of maize stover was significantly higher than that of rice straw, while it was reverse for $t_{0.5}$. It specified that the rate of degradation at early incubation period of maize stover was significantly higher than that of rice straw because of the supplementation of *Bacillus coagulans*. The reason might cause by the difference between maize stover and rice straw, because maize stover was C4 plant while rice straw was C3 plant, C4 plant could synthesize more carbohydrates than C3 plant during the process of photosynthesis and then resulted in faster fermentation rate for maize stover.

CH_4 is an inevitable product generated from dietary carbohydrates during anaerobic fermentation in the rumen, and methanogenesis possesses specific biological regulatory mechanism. Many researchers focused on ruminant CH_4 formation in recent years, due to its contribution to global climatic change [21]. During the ruminant metabolism process, Methane generation in the rumen is the main reason of energy loss in the rumen fermentation, about 6%-15% of the feed energy is loss by the form of methane [22]. CH_4 production may be affected by the composition of fermented carbohydrates, such as cellulose, hemicellulose, soluble residues and digestible ADF in the diets are also important fiber fractions enhancing CH_4 production [23]. CH_4 production had a stronger relationship with digestible NDF, ADF and cellulose intake [24,25]. However, in the current study, CH_4 production was not affected when supplemented with *Bacillus coagulans*, it might be caused by differences between *in vitro* and *in vivo* experiments, and different fermentation substrates (single fermented cell wall substrates VS total mixed ration substrates).

This study showed that IVDMD, IVNDFD and MCP of maize stover were significant higher than those of rice straw, this result might be associated with differences of CP contents and components (especially for rumen degradable protein) between two fermentation substrates. The ruminal microbial population might be another key reason caused this difference, because rumen is a very complex ecosystem, in which numerous microorganisms and factors play an important role and *Bacillus coagulans* do not possess any enzymatic capability to hydrolyze cell-wall constituents, and the activity of cellulolytic bacteria might be not affected by their supplementation administered [26]. Further investigations are needed to evaluate the mechanism of *Bacillus coagulans* supplementation on the activity of amylolytic, proteolytic and cellulolytic microorganisms in *in vitro* rumen fermentation. Our results also showed that IVDMD and IVNDFD were not affected by the supplementation of *Bacillus coagulans*, which was in line with the previous reported results [27].

As acidity is an important indicator for ruminal homeostasis, therefore maintenance of ruminal pH within a physiological range (about 5.5-7.0) is a key factor for efficient fermentation [28]. The results of the study showed that *in vitro* ruminal pH value was kept at 6.73-6.84, which was suitable for fermentation, microbial activity, and fiber digestion in the rumen [29]. Our results also showed that the pH value of maize stover was significantly lower than that of rice straw under different supplementation levels of *Bacillus coagulans*, it might result from the outcome that TVFA contents of maize stover was significantly increased than rice straw *(Table 4)*. There was no significant effect on *in vitro* ruminal pH value for both fermentation substrates after adding *Bacillus coagulans*, it indicated that the different supplementation levels of *Bacillus coagulans* possessed positive significance for ruminal stability manipulation.

Ruminal NH_3-N concentration reflects the equilibrium state for protein degradation and synthesis under specific dietary condition in a certain extent. It is consider an important nitrogen source for microbial growth and protein synthesis, ruminal NH_3-N had a low efficiency for milk protein synthesis partially due to NH_3-N losses in the rumen [30,31] stated that the optimum level of ruminal ammonia concentration should be above 5 mg/dL in order to maintain the microbial growth as well as the microbial protein synthesis, but excessive ammonia could adversely affect its microbial utilization [32]. The results showed that NH_3-N concentration was not affected across four supplemented levels of *Bacillus coagulans*, and it ranged from 6.01 to 6.53 $mg \cdot dL^{-1}$, indicating that the microbial activity was not affected when supplemented with *Bacillus coagulans*.

Ruminal VFAs are the main source of energy for ruminants, both its content and composition are important physiological indexes to reflect rumen digestion and metabolism. Ruminal microorganisms could transform carbohydrates (e.g. crude fiber, starch and soluble sugar) to pyruvic acid, which could be transferred into different VFAs by metabolic pathways. Many researches have been conducted to verify that VFAs produced from the rumen could provide 50-80% energy needed by ruminants [33]. Our result showed that individual VFA and TVFA contents of maize stover were significantly higher than that of rice straw, it might result from the differences of fiber content and its continents, starch content and other carbohydrates between two fermentation substrates, and then affected the microorganism activities or the activation of microbial enzyme to alter fermentation model. Wang et al.[34] reported that dietary supplementation of *B. subtillis natto* to lactating cows trended to decrease ruminal A:P. In this study, the A:P was not affected by different supplementation levels of *Bacillus coagulans* for both fermentation substrates, it might cause by the utilization of various bacterial stain and differences between *in vivo* and *in vitro* experiments. Additionally, the significant differences of A:P between two fermentation substrates when supplemented with *Bacillus coagulans* might result form or FROM difference in composition of carbohydrate between maize stover and rice straw.

The results concluded that, *Bacillus coagulans* numerically increased IVGP when crop straws were used as fermented substrates, and the optimal dose might be 0.75×10^7 cfu·mL^{-1}. Additionally, maize stover increased IVGP, the rate of gas production at early incubation stage, *in vitro* dry matter digestibility, *in vitro* neutral detergent fiber degradation and TVFA were increased when maize stover was supplemented with *Bacillus coagulans* compared to the results of rice straw. Finally, the present *in vitro* study should be repeated using *in vivo* experiments with different period in the future study.

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