Open Access
Asian Australas. J. Anim. Sci.
Vol. 29, No. 4 : 500-508 April 2016
http://dx.doi.org/10.5713/ajas.15.0402
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

Effects of *Momordica charantia* Saponins on *In vitro* Ruminal Fermentation and Microbial Population

Jinhe Kang, Bo Zeng, Shaoxun Tang, Min Wang, Xuefeng Han, Chuanshe Zhou, Qiongxian Yan, Zhixiong He, Jinfu Liu[1], and Zhiliang Tan*

Key Laboratory for Agro-Ecological Processes in Subtropical Region, and
Hunan Research Center of Livestock and Poultry Sciences, and
South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture,
Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, China

ABSTRACT: This study was conducted to investigate the effects of *Momordica charantia* saponin (MCS) on ruminal fermentation of maize stover and abundance of selected microbial populations *in vitro*. Five levels of MCS supplements (0, 0.01, 0.06, 0.30, 0.60 mg/mL) were tested. The pH, NH_3-N, and volatile fatty acid were measured at 6, 24, 48 h of *in vitro* mixed incubation fluids, whilst the selected microbial populations were determined at 6 and 24 h. The high dose of MCS increased the initial fractional rate of degradation at t-value = 0 (FRD_0) and the fractional rate of gas production (k), but decreased the theoretical maximum of gas production (V_F) and the half-life ($t_{0.5}$) compared with the control. The NH_3-N concentration reached the lowest concentration with 0.01 mg MCS/mL at 6 h. The MSC inclusion increased ($p<0.001$) the molar proportion of butyrate, isovalerate at 24 h and 48 h, and the molar proportion of acetate at 24 h, but then decreased ($p<0.05$) them at 48 h. The molar proportion of valerate was increased ($p<0.05$) at 24 h. The acetate to propionate ratio (A/P; linear, $p<0.01$) was increased at 24 h, but reached the least value at the level of 0.30 mg/mL MCS. The MCS inclusion decreased ($p<0.05$) the molar proportion of propionate at 24 h and then increased it at 48 h. The concentration of total volatile fatty acid was decreased ($p<0.001$) at 24 h, but reached the greatest concentration at the level of 0.01 mg/mL and the least concentration at the level of 0.60 mg/mL. The relative abundance of *Ruminococcus albus* was increased at 6 h and 24 h, and the relative abundance of *Fibrobacter succinogenes* was the lowest ($p<0.05$) at 0.60 mg/mL at 6 h and 24 h. The relative abundance of *Butyrivibrio fibrisolvens* and fungus reached the greatest value ($p<0.05$) at low doses of MCS inclusion and the least value ($p<0.05$) at 0.60 mg/mL at 24 h. The present results demonstrates that a high level of MCS quickly inhibits *in vitro* fermentation of maize stover, while MCS at low doses has the ability to modulate the ruminal fermentation pattern by regulating the number of functional rumen microbes including cellulolytic bacteria and fungi populations, and may have potential as a feed additive applied in the diets of ruminants. (**Key Words:** *Momordica charantia* Saponin, *In vitro* Fermentation, Microbial Population, Roughage)

INTRODUCTION

Saponins are the most commonly studied phytochemicals as feed additives. Recent studies have demonstrated that saponins have the potential to modulate rumen fermentation, such as increasing total volatile fatty acid (tVFA) concentration, accelerating degradation of feed substrates, reducing methane production, decreasing the acetate to propionate ratio (A/P) ratio and ammonia concentration in the rumen (Wina et al., 2005; Patra and Saxena, 2009; Patra et al., 2012; Budan et al., 2014). Meanwhile, saponins have been shown to modulate ruminal microbial communities, for example, stimulating the growth of some cellulolytic bacteria, and decreasing the protozoa population (Patra and Saxena, 2009; Patra et al., 2012). However, the reported effects of saponins on rumen fermentation have been inconsistent, depending on different

* Corresponding Author: Zhiliang Tan. Tel: +86-731-4615230, Fax: +86-731-4612685, E-mail: zltan@isa.ac.cn
[1] Department of Food Science, Tianjin Agricultural University, Tianjin 300384, China.
Submitted May 6, 2015; Revised Jun. 15, 2015; Accepted Jul. 20, 2015

types and doses of saponins (Wina et al., 2006; Goel et al., 2008; Guo et al., 2008). *Momordica charantia* (MC), known as bitter melon, is cultivated throughout the world for use as vegetable as well as medicine, and is a rich source of saponin compounds (Tan et al., 2014). However, there is little information on the activity of *Momordica charantia* saponin (MCS) in manipulating rumen fermentation. Additionally, maize stover is an important component in the diets of ruminants. It has been widely used as forage for ruminants in the form of fresh-cut straw after harvesting, hay or silage, and its estimated production has exceeded the amounts of grass silage in many countries (Wilkinson, 2003).

Therefore, the objective of this study was to investigate the effect of different doses of MCS on *in vitro* rumen fermentation characteristics, rumen microbial populations, and correlations between ruminal microbial populations, gas production (GP) parameters and volatile fatty acid (VFA) profiles when maize stover was used as fermented substrates.

MATERIAL AND METHODS

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

Reagents

The MCS was supplied by Department of Food Science, Tianjin Agricultural University, Tianjin, China. The preparation of MCS was as follows: Dried materials of *Momordica charantia* were powdered and sieved (60 mesh). *Momordica charantia* powder (200 g) was extracted with 2,000 mL of ethanol-water (60:40, v/v) solution in an ultrasonic bath (Kunshan Ultrasonic Instrument, Kunshan, China) for 2 h, repeated three times. The filtered solutions were gathered and concentrated to dryness by removing the ethanol solvent using a rotary evaporator device (RE52AA, Shanghai Huxi Instrument Co., Shanghai, China). The extracts obtained were diluted to the concentration of 15 mg/mL with distilled water as sample solution, and then subjected to an AB-8 macroporous resin column. After reaching adsorptive saturation, the column was first washed by distilled water, and then eluted by ethanol-water (80:20, v/v) solution. The 80% ethanol-eluted fraction was concentrated by drying under vacuum. The purity of MCS was calculated on the basis of the standard curve of ginsenoside Rg1: $y = 0.0026 x+0.0107$ ($R^2 = 0.9994$), and the purity of MCS was 73%. The other components of the MCS were H_2O_2 (8%), protein (10%), ash (2%), pectin, cellulose and so on. The MCS was saturated with anhydrous ethanol before dissolving in deionized water as a stock solution. The stock was diluted to the required concentration immediately before use.

Two kg of mature maize stover were randomly collected after maize grains (Zea mays var. Linao 1) were harvested, and chopped to a length of 2 cm. Then approximately 500 g of maize stover was oven-dried at 60°C for 48 h, and ground through a 1-mm screen (DF-2, Changsha Instrument Factory, Changsha, China) for *in vitro* fermentation. All other reagents were of analytical reagent quality.

Experimental design

Experiments were conducted to measure *in vitro* GP characteristics of maize stover. Inclusive levels of MCS were 0, 0.01, 0.06, 0.30, 0.60 mg/mL *in vitro* incubation fluid, respectively. The MCS was mixed with the substrate before the commencement of the experiment.

In vitro fermentation and chemical analyses

Ruminal fluid was collected from three castrated Xiangdong black goats (a local breed in southern China, mean live weight 25±2 kg) fitted with permanent rumen cannulas, before morning feeding, and immediately transported to laboratory. Each goat was fed 140 g of concentrate and 210 g of maize stover per meal at 0800 and 1900 h daily. The mixed diet contained 10% crude protein (CP), 52.5% neutral detergent fiber (NDF), 31.5% acid detergent fiber (ADF). The maize stover contained 91.6% organic matter, 9.0% CP, 71.4% NDF, 44.8% ADF. Ruminal contents were strained through four layers of cheesecloth under a continuous CO_2 stream. Two-hundred±1 mg of oven-dried maize stover was weighed into a 100 mL serum bottle, which was followed by 10 mL of rumen fluid and 20 mL of McDougall's buffer (Cone and Becker, 2012) at 39°C under anaerobic conditions. All bottles were connected with pressure sensors. The pressures in the bottles were recorded at 0, 1, 2, 3, 6, 12, 24, 36, and 48 h during the processes of *in vitro* fermentation.

After incubation at 39°C for 6, 24, 48 h, fluid was sampled to determine pH, NH_3-N and VFA. The pH value of *in vitro* fermentation liquid was determined using a pH meter (Model PHS-3C, Shanghai Precision &Scientific Instrument Co., LTD, Shanghai, China). NH_3-N content was determined as described by Weatherburn (1967) using a spectrophotometer (8500II, Thermo Electron Corporation, Waltham, MA, USA). VFA contents were determined as described by Tang et al. (2013). Briefly, two milliliters of incubation solution was centrifuged at 10,000×g and 4°C for 15 min, then 1.5 mL of supernatant solution was taken and 0.15 mL metaphosphoric acid was added and homogenized. The mixed solution was centrifuged at 10,000×g and 4°C for 15 min again, and the supernatant solution was used to determine VFA content with a gas

chromatograph (HP5890, Agilent 5890; Agilent Technologies Co. Ltd, Santa Clara, CA, USA). A DBFFAP column (30 m in length with a 0.25 mm i.d.) was used for the separation. The attenuation was set at a nitrogen diffluent ratio of 1:50, hydrogen flow was 30 mL/min, airflow was 365 mL/min, injector temperature was 250°C, column temperature was 150°C and detector temperature was 220°C. The N_2 was used as carrier gas at a flow rate of 0.8 mL/min. The relative response factor, representing the peak of each VFA, was calculated using the standard VFA mixture. Total molar concentration was calculated by taking the sum of individual VFA as 100%. To determine the relative abundance of selected microbes to total bacterial 16S rDNA, the fluid from each bottle was sampled under oxygen-free CO_2 and immediately stored at –80°C.

The *in vitro* fermentation was separately run three times on different days of collecting mixed rumen fluids, so that each treatment was conducted in triplicate. Bottles containing maize stover substrate without MCS were included as controls. Bottles containing buffered ruminal fluid alone or buffered ruminal fluid plus MCS without substrates were also prepared and used to correct for GP and fermentation residues resulting from the inoculum or MCS itself.

Real-time quantitative polymerase chain reaction analysis

Total DNA was extracted from each sample as previously described by Zeng et al. (2012). The primers used for the real-time polymerase chain reaction (PCR) are listed in Table 1. The oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanhai, China). Real-time PCR was performed using the Power SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Then, real-time PCR was carried out in aABI-7900 HT Fast Real Time PCR system (Applied Biosystems, USA) according to the following protocol: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 95°C and 60 s at 60°C. Specificity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification according to the following protocol: 15 s at 95°C; 15 s at 60°C and 15 s at 95°C. Dilutions of samples were used to check the PCR amplification efficiencies for each primer pair. Each DNA extract from *in vitro* incubation fluid of each MCS inclusive level was run in triplicate, and a no-template (sterile distilled water) negative control was loaded on each plate run to screen for possible contamination and dimer formation. The individual relative abundance of ruminal microorganism was expressed as a proportion of total rumen bacterial 16S rDNA according to the following equation:

$$\text{Relative quantification of target} = 2^{-(\text{Ct target} - \text{Ct total bacteria})}$$

Where, Ct represents threshold cycle.

Calculation and statistical analysis

Before the MCS experiment was conducted, the correlation between the pressure in bottle and gas volume was measured at 39°C, and then a regression equation was established:

$$y = (x - 0.816)/0.805 \ (n = 20, R^2 = 0.999, p<0.001)$$

Where, y represents gas volume (mL), x is the pressure in bottle (kPa), 0.816 and 0.805 are constants. A logistic-

Table 1. Primers for real-time quantitative PCR analysis

Microbial species	Primer sequence (5′-3′)	Product size (bp)	Reference
Total bacteria	FP: CGGCAACGAGCGCAACCC	130	Zeng et al., 2012
	RP: CCATTGTAGCACGTGTGTAGCC		
Ruminococcus flavefaciens	FP: CGAACGGAGATAATTTGAGTTTACTTAGG	132	Zeng et al., 2012
	RP: CGGTCTCTGTATGTTATGAGGTATTACC		
Fibrobacter succinogenes	FP: GTTCGGAATTACTGGGCGTAA A	121	Zeng et al., 2012
	RP: CGCCTGCCCCTGAACTATC		
Ruminococcus albus	FP: CCCTAAAAGCAGTCTTAGTTCG	176	Zeng et al., 2012
	RP: CCTCCTTGCGGTTAGAACA		
Butyrivibrio fibrisolvens	FP: GAGGAAGTAAAAGTCGTAACAAGGTTTC	160	Zeng et al., 2012
	RP: CAAATTCACAAAGGGTAGGATGATT		
Methanogen	FP: GGATTAGATACCCSGGTAGT	174	Christophersen, 2007
	RP: GTTGARTCCAATTAAACCGCA		
Fungus	FP: GAGGAAGTAAAAGTCGTAACAAGGTTTC	120	Denman and McSweeney, 2006
	RP: CAAATTCACAAAGGGTAGGATGATT		
Protozoa	FP: GCTTTCGWTGGTAGTGTATT	223	Sylvester et al., 2004
	RP: ACTTGCCCTCYAATCGTWCT		

PCR, polymerase chain reaction.

exponential model with the initial value being zero (LE_0) was employed to fit the kinetics of GP (Wang et al., 2011) using NLREG (Version 5.0) software (Sherrod, 1991), and expressed as:

$$V = \frac{V_F(1-\exp(-kt))}{1+\exp(b-kt)}$$

Where, V is the cumulative GP (mL) at time point t, V_F is the theoretical maximum of GP (mL), k is fractional rate of GP at particular time (1/h), b is a shape parameter.

The half-life ($t_{0.5}$, h; represents the time at which half of the final GP) and initial fractional rate of degradation (FRD_0, /h) were calculated according to the equations provided by Wang et al. (2013), and expressed as:

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

$$\mathrm{FRD}_0 = \frac{k}{1+\exp(b)}$$

SAS 8.02 software was used for all statistical analysis including fermentation factors, microbial population and the correlations between ruminal microorganism population and fermentation factors. *In vitro* GP parameters and measures of pH value, NH_3-N, VFA production and microbial population were subjected to the general linear model (GLM) procedure of the SAS 8.02 program. In the model, MCS was the only fixed effect considered. MCS treatment effects on dependent variables were expressed as least-square-means. Tukey's test was used for pair-wise comparisons of individual treatments from significant GLM models, and comparisons among treatments containing increasing levels of MCS were made using orthogonal polynomial contrasts. The interactive matrix language procedure of SAS was used to correct the contrast coefficients of orthogonal polynomial. The $p<0.05$ was considered statistically significant. Pearson correlation analysis was used to test the correlations between ruminal microorganism population and fermentation factors.

RESULTS

Kinetics of *in vitro* gas production

Effects of MCS inclusion on *in vitro* GP parameters of maize stover are presented in Table 2. The results showed that inclusion of MCS at increasing amounts affected (linear and quadratic, $p<0.001$) the values of V_F, k, FRD_0, $t_{0.5}$. The value of V_F was increased by 9.89% at the low levels of MCS (0.01, 0.06 mg/mL) compared with the control, although it was not significant. The highest dose of MCS (0.60 mg/mL) decreased ($p<0.05$) the values of V_F and $t_{0.5}$ to 19.72 mL and 4.80 h, respectively, but increased the k and FRD_0 values, while no differences ($p>0.05$) were observed in the 0.01, 0.06, 0.30 mg MCS/mL groups compared with the control.

pH and NH_3-N

Effects of MCS inclusion on pH and NH_3-N concentration at different times of *in vitro* incubation are given in Table 3. The pH values were comparable among five groups at 6 h, and fluctuated (linear, $p<0.001$; quadratic, $p<0.01$) by the inclusion of MCS, ranging within 6.61 to 6.72 at 24 h, and altered (linear, $p<0.001$; quadratic, $p<0.001$; cubic, $p<0.05$) by the inclusion of MCS, ranging within 6.54 to 6.69 at 48 h. The inclusion of MCS at 0.60 mg/mL had a greatest pH at 24 h ($p<0.05$) compared to the other groups.

There was a U-shaped curvilinear relationship between NH_3-N concentrations and MCS at 6 h and 24 h of *in vitro* incubation. The NH_3-N concentration reached the least concentration (28.33 mM) at the level of 0.01 mg/mL at 6 h. The MCS supplementation resulted in a comparable concentration of NH_3-N with the control at 48 h of incubation.

Volatile fatty acid

Effects of MCS inclusion on VFA production at different times of *in vitro* incubation are shown in Table 3. The molar proportions of any individual VFA, the concentration of tVFA and the A/P ratio were not affected by MCS compared with the control for all doses at 6 h. The MSC inclusion increased the molar proportions of acetate

Table 2. Effect of *Momordica charantia* saponin (MCS) inclusion on *in vitro* gas production parameters of maize stover

Item	MCS (mg/mL)					SEM	MCS effect		
	0	0.01	0.06	0.30	0.60		Linear	Quadratic	Cubic
V_F (mL)	44.99 [a]	49.44 [a]	49.44 [a]	44.51 [a]	19.72 [b]	2.199	***	***	NS
k (1/h)	0.07 [b]	0.06 [b]	0.05 [b]	0.06 [b]	0.15 [a]	0.009	***	***	NS
FRD_0 (/h)	0.04 [b]	0.03 [b]	0.04 [b]	0.04 [b]	0.15 [a]	0.004	***	***	*
$t_{0.5}$ (h)	14.68 [a]	16.90 [a]	17.30 [a]	17.95 [a]	4.80 [b]	1.063	***	***	NS

SEM, standard error of the mean; V_F, the theoretical maximum of gas production; NS, not significant; k, the fractional rate of gas production at time; $t_{0.5}$, the time at which half of the final gas production; FRD_0, the initial fractional rate of degradation.

[a,b] Means within a row with different superscripts differ ($p<0.05$).

* $p<0.05$; *** $p<0.001$.

Table 3. Effects of *Momordica charantia* saponin (MCS) inclusion on pH, NH_3-N, and VFA of *in vitro* incubation fluid

Item	Incubation time (h)	MCS (mg/mL)					SEM	MCS effect		
		0	0.01	0.06	0.30	0.60		Linear	Quadratic	Cubic
pH	6	6.72	6.73	6.72	6.73	6.73	0.009	NS	NS	NS
	24	6.61 ^{d}	6.64 ^{bc}	6.62 ^{cd}	6.64 ^{b}	6.72 ^{a}	0.007	***	**	NS
	48	6.54^{bc}	6.56 ^{b}	6.52 ^{c}	6.54^{bc}	6.69 ^{a}	0.009	***	***	*
NH_3-N (mM)	6	33.82 ^{ab}	28.33 ^{b}	30.39 ^{b}	31.43 ^{b}	37.70 ^{a}	1.877	*	*	NS
	24	40.17 ^{ab}	34.62 ^{b}	28.52 ^{c}	39.22 ^{ab}	42.87 ^{a}	1.880	*	**	**
	48	47.18	47.72	47.19	46.81	46.82	2.046	NS	NS	NS
Total VFA (mM)	6	16.90	17.94	15.00	13.90	14.03	1.710	NS	NS	NS
	24	35.49 ^{a}	31.20 ^{a}	30.07 ^{a}	20.21 ^{b}	20.56 ^{b}	2.470	***	NS	NS
	48	44.35 ^{b}	52.63 ^{a}	44.63 ^{b}	32.93 ^{c}	28.72 ^{c}	2.535	***	NS	**
Acetate,	6	38.78	42.85	41.05	39.04	37.99	1.962	NS	NS	NS
(molar % of total VFA)	24	41.23 ^{c}	42.64 ^{bc}	44.10 ^{bc}	45.59 ^{ab}	47.85 ^{a}	1.111	***	NS	NS
	48	49.83 ^{ab}	47.12 ^{ab}	54.45 ^{a}	34.79 ^{c}	42.09 ^{bc}	3.214	*	NS	**
Propionate	6	31.29	28.30	28.88	28.54	32.05	1.328	NS	NS	NS
(molar % of total VFA)	24	28.14 ^{a}	27.25 ^{ab}	24.37 ^{c}	28.26 ^{a}	24.74 ^{bc}	0.810	NS	NS	*
	48	23.66 ^{b}	24.91 ^{ab}	21.75 ^{b}	29.66 ^{a}	26.62 ^{ab}	1.732	NS	NS	*
Isobutyrate	6	3.84	3.91	4.32	6.07	4.67	0.769	NS	NS	NS
(molar % of total VFA)	24	4.20	4.81	5.18	7.50	6.38	1.070	NS	NS	NS
	48	3.25 ^{b}	4.14 ^{ab}	3.80 ^{ab}	5.66 ^{a}	3.42 ^{b}	0.682	NS	*	NS
Butyrate	6	19.89	19.05	18.93	27.78	18.25	3.653	NS	NS	NS
(molar % of total VFA)	24	19.68 ^{b}	28.13 ^{ab}	26.33 ^{ab}	36.10 ^{a}	26.49 ^{ab}	4.361	NS	*	NS
	48	16.34 ^{bc}	15.87 ^{bc}	13.23 ^{c}	19.86 ^{a}	18.29 ^{ab}	1.094	*	NS	**
Isovalerate	6	4.56	4.58	5.06	7.45	5.44	0.918	NS	NS	NS
(molar % of total VFA)	24	6.42^{b}	7.49 ^{ab}	8.27 ^{ab}	11.56 ^{a}	10.11^{ab}	1.441	*	NS	NS
	48	5.30 ^{b}	5.26 ^{b}	4.98 ^{b}	7.25 ^{a}	7.17 ^{a}	0.502	***	NS	*
Valerate	6	1.64 ^{ab}	1.27 ^{b}	1.75 ^{ab}	2.46 ^{a}	1.61 ^{ab}	0.284	NS	NS	NS
(molar % of total VFA)	24	2.08 ^{b}	3.55 ^{ab}	3.17 ^{ab}	4.26 ^{a}	3.20 ^{ab}	0.555	NS	*	NS
	48	1.73	2.70	1.79	2.77	2.91	0.390	NS	NS	NS
A/P	6	1.25	1.56	1.45	1.41	1.19	0.127	NS	NS	NS
	24	1.59 ^{c}	1.61^{c}	1.89 ^{ab}	1.72 ^{bc}	1.95 ^{a}	0.064	**	NS	NS
	48	2.26 ^{ab}	1.93 ^{abc}	2.73 ^{a}	1.21 ^{c}	1.61^{bc}	0.315	*	NS	*

VFA, volatile fatty acid; SEM, standard error of the mean; NS, not significant; A/P, acetate to propionate ratio.
$^{a-d}$ Means within a row with different superscripts differ ($p<0.05$).
* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

(linear, $p<0.001$), butyrate (quadratic, $p<0.01$), isovalerate (linear, $p<0.05$), valerate (quadratic, $p<0.05$) and the A/P ratio (linear, $p<0.01$), but decreased the molar proportion of propionate (cubic, $p<0.05$) and the concentration of tVFA (linear, $p<0.001$) at 24 h. The molar proportion of isobutyrate was not affected by MCS for all doses at 24 h. After 48 h of incubation, MSC inclusion increased the molar proportion of propionate (cubic, $p<0.05$), isobutyrate (quadratic, $p<0.05$), butyrate (linear, $p<0.05$; cubic, $p<0.01$), and isovalerate (linear, $p<0.001$; cubic, $p<0.05$), but decreased the molar proportion of acetate (linear, $p<0.05$; cubic, $p<0.01$). The tVFA concentration reached the greatest concentration (52.63 mM) at the level of 0.01 mg/mL and the least concentration (28.72 mM) at the level of 0.60 mg/mL. The A/P ratio reached the least value (1.21) at the level of 0.30 mg/mL. The molar proportion of valerate was not affected by MCS at all doses.

Microbial population

At 6 h of *in vitro* incubation, the relative abundance of *Ruminococcus albus* (*R. albus*) was increased (cubic, $p<0.01$) and the relative abundance of *Fibrobacter succinogenes* (*F. succinogenes*) was decreased (linear, $p<0.001$; quadratic, $p<0.05$) with the increment of MCS doses. The relative abundance of *Butyrivibrio fibrisolvens* (*B. fibrisolvens*) was affected (linear, $p<0.001$; quadratic, $p<0.05$) in response to MCS supplementation, with the greatest value at 0.01 mg/mL, and the least value at 0.60 mg/mL. Fungi were affected (linear, $p<0.001$; quadratic, $p<0.05$; cubic, $p<0.01$) in response to MCS supplementation, with the greatest value at 0.06 mg/mL, and the least value at 0.60 mg/mL. The relative abundance of protozoa reached the least value (linear, $p<0.05$) at 0.60 mg/mL, although the difference was not significant compared to the control.

Table 4. Effects of *Momordica charantia* saponin (MCS) on abundance of fungus, methanogen, protozoa and major fibrolytic bacteriain *in vitro* incubation fluid (as relative % of total bacterial 16S rDNA)

Item	Incubation time (h)	MCS (mg/mL)					SEM	MCS effect		
		0	0.01	0.06	0.30	0.60		Linear	Quadratic	Cubic
Fibrobacter succinogenes	6	0.82 ^{a}	0.54 ^{b}	0.55 ^{b}	0.02 ^{c}	0.01 ^{c}	0.001	***	*	NS
	24	0.88 ^{a}	0.63 ^{a}	1.08 ^{a}	1.23 ^{a}	0.02 ^{b}	0.002	*	**	NS
Ruminococcus albus	6	0.23 ^{b}	0.22 ^{b}	0.46 ^{a}	0.21 ^{b}	0.39 ^{a}	0.001	NS	NS	**
	24	0.63 ^{b}	0.90 ^{b}	1.71 ^{a}	1.01 ^{b}	1.22 ^{ab}	0.002	NS	*	*
Butyrivibrio fibrisolvens (10^{-3})	6	0.34 ^{b}	0.60 ^{a}	0.50 ^{ab}	0.29 ^{b}	0.04 ^{c}	0.007	***	*	NS
	24	17.78 ^{bc}	34.81 ^{ab}	52.43 ^{a}	22.80 ^{abc}	0.08 ^{c}	0.096	*	*	NS
Ruminococcus flavefaciens (10^{-3})	6	2.07 ^{ab}	0.66 ^{b}	3.19 ^{a}	1.70 ^{ab}	2.85 ^{ab}	0.007	NS	NS	NS
	24	1.75 ^{ab}	0.93 ^{b}	2.99 ^{a}	0.86 ^{b}	0.96 ^{b}	0.006	NS	NS	NS
Fungus (10^{-3})	6	0.32 ^{b}	0.54 ^{a}	0.57 ^{a}	0.21 ^{bc}	0.007 ^{c}	0.001	***	*	**
	24	14.48 ^{b}	33.52 ^{a}	41.87 ^{a}	15.64 ^{b}	0.09 ^{c}	0.037	***	**	**
Methanogen	6	0.14	0.13	0.15	0.12	0.15	0.000	NS	NS	NS
	24	0.22	0.24	0.48	0.22	0.20	0.001	NS	NS	NS
Protozoa	6	0.40 ^{ab}	0.65 ^{a}	0.42 ^{ab}	0.26 ^{b}	0.15 ^{b}	0.001	*	NS	NS
	24	0.25	0.25	0.26	0.17	0.07	0.001	*	NS	NS

SEM, standard error of the mean; NS, not significant.
$^{a-c}$ Means within a row with different superscripts differ ($p<0.05$).
* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In general, the relative abundance of each microorganism or class of microorganisms measured by real-time PCR was greatest at 0.06 mg MCS/mL of incubation fluid at 24 h (Table 4). The relative abundance of *R. albus* was increased (quadratic and cubic, $p<0.05$) with the increment of MCS doses. The relative abundances of *F. succinogenes*, *B. fibrisolvens* and fungi were affected both linearly and quadratically in response to MCS supplementation, with the greatest value at 0.06 mg/mL, and the least value at 0.60 mg/mL. The relative abundance of protozoa reached the least value (linear, $p<0.05$) at 0.60 mg/mL, but no significant changes were observed among the five groups. The MCS inclusion did not affect the relative abundances of *Ruminococcus flavefaciens* (*R. flavefaciens*) and methanogens at all times of incubation (Table 4).

Correlation analysisof ruminal microbial population, GP parameters and VFA

F. succinogenes displayed a significant positive correlation with $t_{0.5}$ (R = 0.905, $p<0.05$) (Table 5). Protozoa had a significant positive correlation with V_F (R = 0.942, $p<0.05$), but a negative correlation with FRD_0 (R = –0.901, $p<0.05$). There were no significant correlations between the other monitored microbial species and GP parameters. *R. albus* displayed positive correlations with the molar proportion of acetate (R = 0.801, $p<0.01$), the concentration of tVFA (R = 0.732, $p<0.05$) and the A/P ratio (R = 0.845, $p<0.01$), but a negative correlation with the molar proportion of propionate (R = 0.837, $p<0.01$). Both *B. fibrisolvens* (R = 0.842, $p<0.01$) and methanogen (R = 0.742, $p<0.05$) showed positive correlations with the concentration of tVFA. Protozoa displayed negative correlations with the molar proportion of isovalerate (R = 0.688, $p<0.05$) and the concentration of tVFA (R = 0.314, $p<0.05$) (Table 5). As well, *R. flavefaciens* and fungi

Table 5. Correlations between ruminal microbial population and gas production parameters, VFA

Item	V_F	FRD_0	$t_{0.5}$	Acetate (% of tVFA)	Propionate (% of tVFA)	Isobutyrate (% of tVFA)	Butyrate (% of tVFA)	Isovalerate (% of tVFA)	Valerate (% of tVFA)	tVFA	A/P
F. succinogenes	0.826	–0.878	0.905*	0.131	–0.200	–0.489	–0.333	–0.210	0.352	0.567	0.162
R. albus	–0.078	–0.153	–0.037	0.801**	–0.837**	–0.552	–0.479	0.326	0.631	0.732*	0.845**
B. fibrisolvens	0.820	–0.749	0.783	0.427	–0.509	–0.426	–0.254	0.122	0.591	0.842**	0.462
R. flavefaciens	0.390	–0.323	0.293	0.228	–0.072	–0.488	–0.341	–0.399	0.468	0.529	0.157
Fungus	0.814	–0.729	0.750	–0.081	0.249	–0.367	0.223	–0.849	0.172	–0.271	–0.271
Methanogen	0.456	–0.372	0.411	0.590	–0.590	–0.497	–0.374	0.126	0.558	0.742*	0.597
Protozoa	0.942*	–0.901*	0.832	–0.177	0.379	–0.077	0.073	–0.688*	–0.628	–0.314*	–0.358

F. succinogenes, *Fibrobacter succinogenes*; *R. albus*, *Ruminococcus albus*; *B. fibrisolvens, Butyrivibrio fibrisolvens*; *R. flavefaciens*, *Ruminococcus flavefaciens*; VFA, volatile fatty acid; V_F, the theoretical maximum of gas production; FRD_0, the initial fractional rate of degradation; $t_{0.5}$, the time at which half of the final gas production; tVFA, total VFA; A/P, acetate to propionate ratio.
* $p<0.05$; ** $p<0.01$.

showed no significant correlations with molar proportions of any individual VFA, or the concentration of tVFA.

DISCUSSION

Results in the current study showed that a high level of MCS inclusion (0.60 mg/mL) significantly decreased the V_F. This finding was consistent with the result of a previous *in vivo* experiment, where *Sapindus rarak* saponins was poisonous to the ruminal bacteria of goats, which further reduced the degradation of substrate by bacteria and resulted in the reduction of GP (Wina et al., 2006). In contrast, Rodriguez and Fondevila (2012) reported that the *Enterolobium cyclocarpum* saponins increased the *in vitro* GP. This GP persistence in response to saponins addition *in vitro* or *in vivo* could be due to the different source or dose of saponins. The increment in FRD_0 and k value, and the decline in V_F and $t_{0.5}$ with 0.60 mg/mL MCS addition in the current study indicated that the *in vitro* fermentation of maize stover could be inhibited quickly (at the initial stage) by a high dose of MCS.

The pH, and VFA and NH_3-N concentrations are important parameters reflecting the ruminal fermentation environment. In the current study, pH values were increased with MCS addition and within the normal range (>6.3) at 24 and 48 h of incubation. A change in pH value was also observed by Santoso et al. (2007), who observed that ruminal pH value was increased linearly in goats fed with saponins. NH_3-N is a main source of nitrogen for the synthesis of ruminal bacteria, and the ruminal NH_3-N concentration is a crude predictor of the efficiency of dietary N conversion to microbial N (Salter et al., 1979). In the present study, ruminal NH_3-N concentration was decreased in response to MCS at low doses, and this was consistent with earlier *in vivo* reports (Santoso et al., 2007; Wang et al., 2009) that ruminal NH_3-N concentrations decreased with saponins inclusion from *Biophytum petersianum Klotzsch* or *Yucca schidigera*. Therefore, the results of current study indicate that MCS at low doses may increase the efficiency of dietary N conversion to microbial N under the conditions of *in vitro* fermentation.

VFA production in the rumen depends highly on the degree and rate of fermentation (Dijkstra et al., 2005; Cone and Becker, 2012). With regard to VFA production, Wang et al. (2009) reported that addition of dietary saponin increased tVFA concentration and had no effect on any individual VFA *in vivo*. Malik and Singhal (2008) found that lucerne saponin supplementation decreased acetate, increased propionate, and did not alter tVFA concentration. In present study, it could be clearly noted that MCS inclusion decreased the molar proportion of propionate and the concentration of tVFA, but increased the proportion of acetate, which led to a higher A/P ratio at 24 h of incubation. On the contrary, MCS inclusion increased the molar proportion of propionate and decreased the proportion of acetate, which led to a lower A/P ratio at 48 h of incubation. This implied that MCS inclusion might inhibit the activities of some ruminal microbes, and change the fermentation pattern. Furthermore, increment of V_F, and reduction of NH_3-N and tVFA concentration synergistically reflected the enhancement of ruminal microbes' activity, because the growth of microbes required NH_3-N and tVFA as nitrogen and energy sources. Meanwhile, the current study showed that MSC inclusion increased the molar proportions of butyrate, valerate and isovalerate at 24 h, and the molar proportions of propionate, butyrate, isobutyrate and isovalerate at 48 h, suggesting that deamination activity of *in vitro* incubation fluid may have been stimulated by MSC inclusion, because branch-chain VFAs are derived from deamination of amino acids in the rumen (Hino and Russell, 1985). And it was inconsistent with the result of an earlier *in vitro* report that tea saponin decreased molar proportions of butyrate, valerate, isobutyrate, and isovalerate (Wang et al., 2012). The experimental differences among these studies may be related to the chemical structure and dosage of saponin, diet composition, microbial community, and adaptation of microbia to saponin (Patra and Saxena, 2009).

Numerous studies (Wang et al., 2012) have reported the positive effect of saponin on ruminal microbial activities. Supplementation with *Samanea saman* saponin to diets of cows resulted in greater *F. succinogenes* numbers (Anantasook et al., 2014). *Quillaja* saponin at high dose increased numbers of *F. succinogenes* and *R. flavefaciens* (Patra and Yu, 2013). Results of this study showed that MCS could stimulate the growth of *R. albus*, *B. fibrisolvens* at low doses of MCS, but inhibited the growth of *F. succinogenes*, *B. fibrisolvens* at the high doses of MCS. Similarly, previous studies also reported that saponin increased cellulolytic bacteria significantly (Goel et al., 2008).

Anaerobic fungi are important in the rumen for fibre degradation, although they only comprise a small proportion of the total mass of rumen micro-flora. The present study found that MCS accelerated the growth of fungi at low concentrations, but inhibited it at the highest level (0.60 mg/mL). This observation was consistent with that of Wina et al. (2006), in which *S. rarak* saponins inclusion at low levels had a positive effect on fungi in goats. Saponin displayed strong anti-protozoa activity and may serve as an effective defaunating agent for ruminants in some studies (Goel et al., 2008; Jayanegara et al., 2014). However, MCS showed no significant effect on the protozoa in this study. It was consistent with that of Benchaar et al. (2008) who reported that *Yucca schidigera* saponin had no the effectiveness of anti-protozoa in dairy cattle. The inconsistent effects of saponins on protozoa

might be due to different doses and types of saponins used.

Due to the presence of a lipid-soluble aglycone and water soluble sugar chains in their amphiphilic structure, saponins are surface active compounds with detergent, wetting, emulsifying and foaming properties (Sarnthein-Graf and La Mesa, 2004). Present results showed that rumen microbes have different responses to the MCS. Our previous results have indicated that alkyl polyglycoside, a type of non-ionic surfactant, reduced the relative abundance of *R. albus*, but had no effect on *B. fibrisolvens* or *R. flavefaciens* in both rumen fluid and ruminal particulate fractions of goats (Zeng et al., 2012). Another literature also indicated that the surfactant, Tween 80, increased the growth of non-cellulolytic bacteria but not of cellulolytic bacteria (Ha et al., 2002). Usually, the effects of surfactants on ruminal microbes have been attributed to at least two causes: i) action on the cell membrane causing increased permeability, promotion of the release of bound enzymes (Reese and Maguire, 1969); ii) decrease in growth rate due to reduced oxygen supply in aerobic condition (Hulme and Stranks, 1970). It might be possible that the MCS inclusion affected the cell membrane lipid balance, fluidity, and permeability of rumen microbes, and consequent substance trasportation via the membrane, which would have influenced microbial survival. The possible reason for the different responses of rumen microbes to the MCS might be the difference in structure and chemical composition of the cell membrane among microbial strains.

Simultaneously, the examined relationships between ruminal microbial population and the GP parameters, VFA concentrations indicated that the change of the abundances of *F. succinogenes*, *R. albus*, *B. fibrisolvens*, methanogens and protozoa in rumen liquor would affect the *in vitro* ruminal fermentation. Certainly, the effectiveness of MCS for the improvement of *in vitro* ruminal fermentation of roughages at low doses needed to be further validated *in vivo*.

CONCLUSIONS

In summary, it could be concluded that a high level of MCS inclusion quickly inhibited the *in vitro* fermentation of maize stover, while MCS at low doses had the ability to modulate the rumen fermentation pattern and improve the ruminal degradability of roughages by directly stimulating the number of functional rumen microbes including cellulolytic bacteria and fungi populations, and had potential as a feed additive in the diets of ruminants. In addition, it is necessary to further explore the impact of saponins originating from different plants on ruminal fermentation and microbial community in ruminants' diets.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support received from the National Natural Science Foundation of China (Grant No. 31201826, 31320103917), "Strategic Priority Research Program - Climate Change: Carbon Budget and Relevant Issues" (Grant No. XDA05020700), Hunan Provincial Creation Development Project (2013TF3006) and Tianjin Science and Technology Support Plan Project (13ZCZDNC01800).

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