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Effects of Chitosan on *in vitro* Ruminal Fermentation in Diets with Different Forage to Concentrate Ratios

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Abstract: This study was conducted to investigate the effects of chitosan on ruminal fermentation, methane production and microbial populations in diets with different Forage to Concentrate (F:C) ratios *in vitro*. A two factorial *in vitro* experiment was designed with one factor as three F:C ratios (80:20, 50:50 and 20:80) diets and the other as four levels of chitosan in rumen-buffer fluid (0, 333, 667 and 1000 mg L^{-1}). After 24 h incubation, the results showed that chitosan significantly decreased *In Vitro* Dry Matter Disappearance (IVDMD) ($p<0.001$), total gas production ($p<0.001$), methane production ($p<0.001$) and Acetate:Propionate (A:P) ($p = 0.001$) and significantly increased the rumen pH ($p<0.001$). Chitosan significantly increased the molar proportion of propionate of rumen-buffer fluid in F:C ratios of 80:20 ($p = 0.009$) and 50:50 ($p = 0.007$). In F:C ratios of 20:80, chitosan significantly increased the ammonia-N concentration ($p = 0.013$) and the molar proportion of butyrate ($p<0.001$) and significantly decreased the molar proportion of acetate ($p = 0.001$). In conclusion, chitosan affected the ruminal fermentation and reduced methane production of rumen-buffer fluid *in vitro*.

Key words: Chitosan, ruminal fermentation, methane production, *in vitro*, significantly

INTRODUCTION

Methane is a most important greenhouse gas which has a global warming potential 23 times higher than carbon dioxide (Wuebbles and Hayhoe, 2002). Methane in the rumen represents an energy loss to animal which is estimated to be 2-15% of dietary energy in ruminants (Moss, 1993). Many countries have prohibited the use of antibiotics (such as monensin) as growth promoters in animal feeding (Regulation 1831/2003/EC). For this reason, many research groups have investigated potential strategies to find the new replaced additives such as plant extract (Feng *et al*., 2012; Kim *et al*., 2012) and plant oil (Pilajun and Wanapat, 2011).

Chitosan, a N-acetyl-D-glucosamine polymer derived through the deacetylation of chitin is a major component in the exoskeleton of crustaceans (such as shrimp and crabs) (No and Meyers, 1997). As one of the most abundant natural biopolymers, chitosan has the interesting properties of non-toxicity, good biodegradability carbohydrate polymer and the natural antibacterial characteristics (Chung *et al*., 2004; Aranaz *et al*., 2009). Some studies have shown that chitosan was added to diet to improve feed efficiency in poultry (Suk, 2004; Shi *et al*., 2005). However, few studies have been investigated the effects of chitosan on methane production (Goiri *et al*., 2009a, b). Furthermore, to the knowledge, there were also few studies have been reported to research the effects of the chitosan on ruminal fermentation and microbial population under various Forage to Concentrate (F:C) ratios. Therefore, in the present study, researchers mainly investigated the effects of chitosan with different F:C riatos on methane production of rumen-buffer fluid *in vitro*.

MATERIALS AND METHODS

Four fistulated adult goats (mean body weight 55±3 kg) were fed two equal diets of 500 g alfalfa hay and 500 g commercial concentrate mixture (based on maize and soybean meal, 12% crude protein and 75% TDN, Dobetter-Victory Company, Yangling, China) at 08:00 and 18:00 h daily and water was available in *ad libitum*. The three tested substrates consisted of alfalfa hay, corn grain and soybean meal. The tested diet of alfalfa hay and concentrate was ground through a 1.0 mm screen. And the three diets of low, medium and high forage were designed at 80:20 (High Forage, HF), 50:50 (Medium

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Table 1: Ingredient and chemical composition of diet treatments

	Forage to concentrate ratio		
Items	80:20	50:50	20:80
Ingredient composition (g/kg DM)			
Alfalfa hay	800	500	200
Corn grain	88	338	587
Soybean	112	162	213
Chemical composition (g/kg DM)			
DM (g/kg)	937	933	931
OM	915	931	946
CP	154	154	155
NDF	456	393	347
ADF	243	170	98

DM: Dry Matter; OM: Organic Matter; CP: Crude Protein; NDF: Neutral Detergent Fibre; ADF: Acid Detergent Fibre

Forage, MF) and 20:80 (Low Forage, LF) F:C ratios. The ingredient and chemical composition in diets is shown in Table 1. Chitosan is derived from shrimp shells, degree of deacetylation ≥75% (Sigma-Aldrich, USA). Each diet of 1,200 mg was accurately weighed into bottles. The experiment was designed by 3×4 two factors with one as three different F:C ratios (80:20, 50:50 and 20:80) and the other as the four different doses of chitosan (0, 333, 667 and 1000 mg L^{-1} incubation fluid). All treatments were tested in four replicates.

Rumen fluid was obtained from the four fistulated adult goats before morning feeding. The collected rumen fluid was transported immediately to laboratory in vacuum flasks, mixed and strained through four layers of cheesecloth. Then, the particle-free rumen fluid was mixed with two volumes of buffer (Menke and Steingass, 1988) and the 120 mL incubation medium was dispensed into each bottle under CO_2 flushing. All the bottles were sealed with rubber stoppers and moved into a constant temperature shaker for incubation at 39°C.

After 24 h incubation, all the bottles were cooled with ice-water to terminate fermentation. The total gas production of each bottle was measured using a calibrated syringe with a needle through the rubber stopper. A sample gas of each bottle was collected and stored with the vacuum tube for analysis of methane concentration. Then, the rubber stopper of each bottle was removed and the final pH after 24 h of fermentation was measured with a pH meter (Sartorius PB-10). The prepared solution contains metaphosphoric acid (100 g L^{-1}) for deproteinise and crotonic acid (0.4 g L^{-1}) as an internal standard. The 1 mL fermentation fluid was added to 1 mL prepared solution and stored at -40°C for determination of VFA. Then, 1 mL fermentation fluid was added to 1 mL 0.5M HCl and stored for ammonia-N analysis.

Methane was measured by a gas chromatograph (Agilent Technologies 7820A, USA) using methods as described by Hu *et al.* (2005). The frozen samples for VFA were thawed and centrifuged at 12,000×g for 15 min to thoroughly remove protein. The supernatant was filtered through a 0.45 μm filter membrane. The VFA was determined by a gas chromatography (Agilent Technologies 7820A, USA) equipped with FFAP column (30 m×0.25 mm×0.33 μm, LanZhou Atech, China), the method as described by Eun *et al.* (2007). Ammonia-N concentration was analyzed as described by Weatherburn (1967). The Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF) were determined by the method of Van Soest *et al.* (1991). Heat-stable α-amylase (Sigma A3306, Sigma-Aldrich, Shanghai, China) and sodium sulfite were used for NDF determination. The DM (method ID number 930.15), OM (method ID number 942.05), CP (method ID number 984.13) were analyzed as described by AOAC (1995). *In Vitro* Dry Matter Disappearance (IVDMD) was calculated by the method of Elwakeel *et al.* (2007). Carboxymethylcellulase (CMCase) was analyzed as described by Agarwal *et al.* (2000).

The total DNA was isolated according to Murray and Thompson (1980) and Zhou *et al.* (1996). The relative quantification of different microbial groups was done with real time PCR as a proportion of total bacterial 16S rDNA according to the equation:

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

where, Ct represents threshold cycle. The primer sets used for real time PCR were forward primer (5'-3') CGGCAACGAGCGCAACCC, reverse primer (5'-3') CCATTGTAGCACGTGTGTAGCC targeting *16S rRNA* gene for rumen bacteria, forward primer (5'-3') GTTCGGAATTACTGGGCGTAAA, reverse primer (5'-3') CGCCTGCCCCTGAACTATC for *Fibrobacter succinogenes* and forward primer (5'-3') GAGGAAGTAAAAGTCGTAACAAGGTTTC, reverse primer (5'-3') CAAATTCACAAAGGGTAGGATGATT for General anaerobic fungi (Denman and McSweeney, 2006), forward primer (5'-3') TTCGGTGGA TCDCARAGRGC, reverse primer (5'-3') GBARGTCGWAWCCGTAGAATCC targeting *mcrA* gene for Methanogens (Denman *et al.*, 2007), forward primer (5'-3') GCTTTCGWTGGTAGTGTATT, reverse primer (5'-3') CTTGCCCTCYAATCGTWCT for protozoa (Sylvester *et al.*, 2004).

PCR reaction was carried out in real time PCR machine (BIO-Rad iCycler iQ 5, Inc., Hercules, CA, USA) using SYBR green (SYBR Premix Ex Taq™ II, TaKaRa Biotechnology (Dalian) Co., Ltd. China). The PCR reaction program was: predenature at 95°C for 2 min, followed by 40 cycles of 5 sec for denaturation at 95°C, annealing at 60°C for 30 sec and extension at 72°C for 1 min. Program melting curve as shown below: 95°C, 2 min; 72°C, 1 min; 95°C, 30 sec, step 0.5°C sec^{-1}; 30°C, 1 min.

Statistical analysis: Data were analyzed using the General Linear Model (GLM) of SPSS 18.0. When a significant

effect of treatment ($p<0.05$) was detected, differences between means were assessed by the Least Significant Differences (LSD).

RESULTS AND DISCUSSION

Effect of chitosn on methane production: As shown in Table 2, reducing the F:C ratio led to a significant increase in methane production ($p<0.001$). Chitosan significantly reduced methane production ($p<0.001$). No significant interaction was observed for methane production ($p = 0.316$) between F:C and chitosan.

Effects of chitosan on ruminal fermentation parameters and IVDMD: The effects of chitosan on fermentation characteristics and IVDMD in diets with different F:C ratios were shown in Table 2. The incubated F:C ratios diets significantly affected total gas production ($p<0.001$), pH ($p<0.001$), IVDMD ($p<0.001$), CMCase ($p<0.001$) and CH_4/IVDMD ($p = 0.008$). The chitosan significantly reduced total gas production ($p<0.001$), IVDMD ($p<0.001$), CMCase ($p<0.001$) and CH_4/IVDMD ($p = 0.008$) and significantly increased rumen pH ($p<0.001$). There were significant interaction between F:C ratios and chitosan for total gas production ($p<0.001$), rumen pH ($p = 0.018$), IVDMD ($p = 0.012$), CMCase ($p<0.001$), CH_4/IVDMD ($p = 0.007$) and ammonia-N ($p = 0.026$).

Effect of chitosan on VFA: As shown in Table 3, the incubated F:C ratios diets significantly impacted the total VFA ($p<0.001$), A:P ($p<0.001$) and the molar proportion of individual VFA ($p<0.001$). Chitosan significantly increased the molar proportion of propionate ($p = 0.002$) and decreased A:P ($p = 0.001$) but had no significant effect on the concentration of total VFA ($p = 0.121$), the molar proportion of butyrate ($p = 0.105$) and isobutyrate ($p = 0.090$). The interactions between F:C ratios and chitosan were found for A:P ($p = 0.121$), the molar proportion of propionate ($p = 0.121$), butyrate ($p = 0.121$), isovalerate ($p = 0.121$), valerate ($p = 0.121$) and Branched Chain Volatile Fatty Acid (BCVFA) ($p = 0.121$). And no interactions were determined for the concentration of total VFA ($p = 0.213$), the molar proportion of acetate ($p = 0.073$) and isobutyrate ($p = 0.056$).

Effect of chitosan on microbial population: As shown in Table 4, chitosan increased the number of *F. succinogenes* ($p<0.001$) but had no significant effects on the number of total bacteria, protozoa and general anaerobic fungi. Chitosan did not significantly influence the methanogens ($p>0.05$).

In the current study, researchers investigated the effects of chitosan and F:C ratios on methane production and also researched other fermentation parameters, IVDMD and microbial population.

Table 2: Effects of chitosan on total gas, rumen pH, IVDMD, Carboxymethylcellulase (CMCase), methane, ammonia-N *in vitro* with different Forage to Concentrate (F:C) ratios diets

	Treatment															
Items	F:C (80:20)				F:C (50:50)				F:C (20:80)					Significance		
Chitosan (mg L^{-1})	0	333	667	1000	0	333	667	1000	0	333	667	1000	SEM	Chitosan	F:C	Chitosan×F:C
Total gas (mmol)	10.28^{a}	8.82^{b}	9.40^{b}	9.10^{b}	10.90^{a}	10.32^{b}	9.84^{c}	10.39^{b}	14.21^{a}	13.22^{b}	12.96^{b}	12.04^{c}	0.248	<0.001	<0.001	<0.001
pH	6.15^{c}	6.18^{b}	6.20^{a}	6.21^{a}	6.08^{d}	6.10^{c}	6.15^{a}	6.14^{b}	5.90^{c}	5.92^{b}	5.98^{a}	5.97^{a}	0.015	<0.001	<0.001	0.018
IVDMD (%)	60.71^{a}	61.37^{a}	55.59^{b}	54.67^{b}	69.90^{a}	67.12^{ab}	66.94^{b}	63.64^{b}	81.55^{a}	75.64^{b}	71.24^{c}	73.01^{bc}	1.155	<0.001	<0.001	0.012
CMCase (U mL^{-1})	0.39^{a}	0.30^{b}	0.32^{b}	0.33^{b}	0.53^{ab}	0.54^{a}	0.49^{bc}	0.47^{c}	0.76^{a}	0.74^{a}	0.60^{b}	0.58^{b}	0.022	<0.001	<0.001	<0.001
Methane (mmol)	2.21^{a}	1.84^{b}	1.82^{b}	1.77^{b}	2.38^{a}	2.11^{b}	2.15^{b}	2.11^{b}	3.02^{a}	2.83^{b}	2.88^{b}	2.70^{c}	0.062	<0.001	<0.001	0.316
CH_4/IVDMD	3.65^{a}	2.99^{b}	3.22^{b}	3.25^{b}	3.41	3.16	3.22	3.33	3.71^{b}	3.74^{b}	4.05^{a}	3.67^{b}	0.052	0.008	<0.001	0.007
N-NH_3 (mg/100 mL)	29.54	26.28	28.45	28.93	24.37	25.35	23.87	25.53	21.45^{b}	21.24^{b}	23.16^{a}	23.17^{a}	0.424	0.053	<0.001	0.026

Table 3: Effects of chitosan on *in vitro* VFA with different Forage to Concentrate (F:C) ratios diets

	Treatment															
Items	F:C (80:20)				F:C (50:50)				F:C (20:80)					Significance		
Chitosan (mg L^{-1})	0	333	667	1000	0	333	667	1000	0	333	667	1000	SEM	Chitosan	F:C	Chitosan×F:C
Total VFA (mmol L^{-1})	76.52	75.74	76.23	76.55	78.18	80.26	79.85	80.09	82.31^{b}	84.21^{a}	83.35^{ab}	83.15^{ab}	0.451	0.121	<0.001	0.213
A:P	3.85^{a}	3.74^{b}	3.77^{b}	3.76^{b}	4.06^{a}	3.98^{ab}	3.84^{bc}	3.79^{c}	3.82	3.78	3.74	3.82	0.016	0.001	<0.001	0.003
Individual VFA (mmol/100mmol)																
Acetate	61.16	60.86	61.22	61.38	61.81	61.07	60.80	60.37	60.71^{a}	59.93^{b}	59.48^{b}	59.54^{b}	0.125	0.005	<0.001	0.073
Propionate	16.03^{b}	16.29^{a}	16.26^{a}	16.31^{a}	15.23^{b}	15.35^{b}	15.83^{a}	15.95^{a}	15.89	15.85	15.91	15.58	0.054	0.002	<0.001	<0.001
Butyrate	13.75	13.72	13.55	13.45	15.53	15.90	15.54	15.66	16.58^{c}	17.13^{b}	17.31^{ab}	17.65^{a}	0.219	0.105	<0.001	0.008
Isobutyrate	2.06^{a}	2.06^{a}	2.04^{ab}	2.02^{b}	1.73	1.78	1.81	1.84	1.61^{b}	1.65^{ab}	1.69^{a}	1.68^{a}	0.025	0.090	<0.001	0.056
Isovalerate	4.30	4.34	4.25	4.19	3.41	3.55	3.65	3.74	3.08^{c}	3.23^{b}	3.35^{a}	3.31^{ab}	0.066	0.010	<0.001	0.012
Valerate	2.70	2.74	2.68	2.64	2.29	2.35	2.39	2.45	2.13^{b}	2.21^{a}	2.26^{a}	2.25^{a}	0.031	0.026	<0.001	0.019
BCVFA	9.07	9.13	8.97	8.85	7.43	7.68	7.84	8.02	6.82^{a}	7.10^{a}	7.30^{a}	7.23^{a}	0.121	0.020	<0.001	0.017

F:C: Forage to Concentration Ratios; SEM: Standard Error of Mean; $^{a-d}$Means in the same row within F:C = 80:20, 50:50 and 20:80 subgroups with different superscripts differ based on single degree of freedom contrasts ($p<0.05$)

Table 4: Effects of chitosan on microorganism *in vitro* in three different Forage to Concentrate (F:C) ratios diets

Items	Treatment															
	F:C (80:20)				F:C (50:50)				F:C (20:80)					Significance		
Chitosan (mg L^{-1})	0	333	667	1000	0	333	667	1000	0	333	667	1000	SEM	Chitosan	F:C	Chitosan×F:C
Total bacterial	1.03	0.95	0.89	1.06	1.03	1.07	1.18	1.08	0.89	0.94	0.86	0.88	0.033	0.991	0.088	0.884
Protozoa ($\times10^{-2}$)	2.56^{b}	4.32^{a}	3.42^{ab}	2.20^{b}	4.42	7.33	4.92	5.83	6.02	5.43	5.18	4.04	0.298	0.058	<0.001	0.239
Fibrobacter																
Succinogenes ($\times10^{-3}$)	4.51	5.18	11.42	7.83	1.20^{b}	3.14^{a}	3.58^{a}	4.73^{a}	0.89^{b}	1.89^{b}	10.16^{a}	9.71^{a}	0.673	<0.001	0.001	0.059
Methanogens ($\times10^{-3}$)	5.43	5.07	7.62	6.81	3.92	2.94	2.98	4.41	5.26	2.22	5.66	10.58	0.558	0.080	0.078	0.420
General anaerobic fungi ($\times10^{-3}$)	3.73	3.10	4.04	2.97	1.00	1.61	1.48	1.76	0.62	0.87	0.80	0.42	0.227	0.675	<0.001	0.316

F:C: Forage to Concentration Ratios; SEM: Standard Error of Mean; $^{a-d}$Means in the same row within F:C = 80:20, 50:50 and 20:80 subgroups with different superscripts differ based on single degree of freedom contrasts ($p<0.05$)

Effects of chitosan on methane production: Enteric methane emission is a major contributor to greenhouse gas and also a loss of feed energy during ruminal fermentation process (Boadi *et al*., 2004). Earlier studies reported that low F:C ratios diet increased the methane production (Van Nevel and Demeyer, 1995; Lee *et al*., 2009). Similarly, in the present study, after 24 h *in vitro* incubation, the low F:C ratios diet increased the methane production. In the lower F:C ratio diets, the increasing of methane production may be due to the increase of protozoal population.

It was reported that chitosan could reduce the methane production (Goiri *et al*., 2009a, 2010). In the present study, chitosan reduced the methane production irrespective of the F:C ratios diets. This is because the chitosan inhibit the activity of H_2-producing microorganism such as fibrolytic bacterial (Wang *et al*., 2001) and chitosan could decrease in organic matter degradability (Goiri *et al*., 2009b).

Effects of chitosan on total gas production, *In Vitro* Dry Matter Disappearance (IVDMD) and CMCase: Gas production has a strong correlation with organic matter digestibility (Menke and Steingass, 1988). Eun and Beauchemin (2007) reported there was a positive correlation between Organic Matter (OM) digestibility and gas production.

When the dairy cows were fed lower F:C ratios diets, the digestibility of dry matter increased (Arriola *et al*., 2011). In the current experiment, with the F:C ratios decreased, the total gas production and the IVDMD also increased.

Chitosan has the antimicrobial activity of being against different groups of microorganisms (Goiri *et al*., 2009b; Benhabiles *et al*., 2012) which produce enzymes to hydrolyze the forage and that results in the reduction of the total gas production and the decrease of IVDMD. Chitosan was reported that it could decrease total gas production and IVDMD (Goiri *et al*., 2009a, b). In the present study, the similar results were found that chitosan reduced total gas production and IVDMD (Table 2). There were interactions between F:C ratios and chitosan for total gas production and IVDMD and the treatment of F:C ratio 8:20 and the level of chitosan 1000 mg L^{-1} had the lowest IVDMD.

In accordance with the results reported by Benhabiles *et al*. (2012) that chitoasn inhibited the growth of microorganism producing CMCase, chitosan also decreased CMCase in the present trail (Table 2).

Effects of chitosan on rumen pH: A diet with increasing of the F:C ratios could increase the pH (Aguerre *et al*., 2011). In the present study, the high F:C ratios had higher rumen pH than low F:C ratios diet.

Aranaz *et al*. (2009) thought that chitosan increases pH maybe because of the physical hydrogel and gaseous ammonia which neutralizes H^+ in solution. Goiri *et al*. (2009c, 2010) found that chitosan also increased the rumen pH in *in vtiro* ruminal fermentation experiment. Similarly, in the present study, it was found that chitosan increased the final rumen pH.

Effects of chitosan on VFA, molar proportion of acteate, propionate and A:P: Feed ingested by ruminants is fermented by microbes in the rumen, the Volatile Fatty Acids (VFA) acetate, propionate and butyrate are the mainly products of fermentation. The acetate, propionate and butyrate form an important part of the ruminant's energy. Eun and Beauchemin (2005) and Arriola *et al*. (2011) reported cows fed high F:C ratio diets produced low concentration of total VFA. It was found that high F:C ratios diets also decreased the concentration of the molar proportion of propionate (Arriola *et al*., 2011). In the present study, researchers also found that high F:C ratios diet decreased the concentration of total VFA and molar proportion of propionate.

Goiri *et al*. (2009c) reported that chitosan did not significantly affect the concentration of total VFA but increased molar proportion of propionate and decreased A:P regardless of the F:C ratios and did not significantly

affect molar proportion of acetate except the low F:C ratio diet in which the molar proportion of acetate was significantly decreased. In the present study, in high and median F:C ratios diets (80:20 and 50:50), chitosan also did not significantly affect the concentration of total VFA and molar proportion of acetate and significantly increased molar proportion of propionate and A:P (Table 3). However, researchers also found that in the low F:C ratio diet of 20:80, chitosan significantly increased total VFA and butyrate molar proportion, decreased acetate molar proportion but had no significantly effect on propionate molar proportion and A:P indicating that the change of VFA depended on F:C ratios in diets, though Goiri *et al.* (2009c) reported there was no significant effect on A:P regardless of the F:C ratios.

Effects of chitosan on microbial profile: The rumen is an anaerobic fermentation chamber, it's microbial populations have symbiotic relationships in which metabolites are exchanged that promote or compensate each others growth (Wolin *et al.*, 1997). Methane is formed in the ruminant rumen by methanogens, thus methanogens is very important to the loss of feed energy during ruminal fermentation process. In the present study, after 24 h incubation, although chitosan did not significantly decreased the number of methanogens (Table 4) which resulted in decrease of the methane production (Table 2). Pilajun and Wanapat (2011) also found that supplementation of coconut oil and Mangosteen Peel Powder significantly reduced methane production, however did not significantly decrease the number of methanogens and suggested that the methane inhibitor likely affect on CO_2 or H_2, the precursor of methane synthesis or methanogenesis process. Kim *et al.* (2012) observed that extracts of wormwood, garlic, mandarin orange and honeysuckle increased the numbers of *F. succinogens* and decreased methane production. In the present study, chitosan was also found to increase the number of *F. succinogenes* (Table 4).

CONCLUSION

In this study, it was found that chitosan significantly reduced the methane production. Chitosan in high F:C ratio and low F:C ratio (80:20 and 50:50) diets did not significantly affect the concentration of VFA and increased molar proportion of propionate, decreased A:P. Chitosan in low F:C ratio (20:80) diet significantly increased the concentration of VFA did not affect molar proportion of propionate and decreased tendency for A:P.

In conclusion, the effects of chitosan on concentration of VFA, molar proportion of individual VFA depend on the incubated nature of F:C ratio diet.

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