



## Effects of Ruminant Infusion of Garlic Oil on Fermentation Dynamics, Fatty Acid Profile and Abundance of Bacteria Involved in Biohydrogenation in Rumen of Goats

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**ABSTRACT:** This study aimed to investigate the effects of ruminal infusion of garlic oil (GO) on fermentation dynamics, fatty acid (FA) profile, and abundance of bacteria involved in biohydrogenation in the rumen. Six wethers fitted with ruminal fistula were assigned to two groups for cross-over design with a 14-d interval. Each 30-d experimental period consisted of a 27-d adaptation and a 3-d sample collection. Goats were fed a basal diet without (control) or with GO ruminal infusion (0.8 g/d). Ruminal contents collected before (0 h) and at 2, 4, 6, 8, and 10 h after morning feeding were used for fermentation analysis, and 0 h samples were further used for FA determination and DNA extraction. Garlic oil had no influence on dry matter intakes of concentrate and hay. During ruminal fermentation, GO had no effects on total VFA concentration and individual VFA molar proportions, whereas GO increased the concentrations of ammonia nitrogen and microbial crude protein ( $p < 0.05$ ). Compared with control, GO group took a longer time for total VFA concentration and propionate molar proportion to reach their respective maxima after morning feeding. The ratio of acetate to propionate in control reduced sharply after morning feeding, whereas it remained relatively stable in GO group. Fatty acid analysis showed that GO reduced saturated FA proportion ( $p < 0.05$ ), while increasing the proportions of C18, *t*11-18:1 (TVA), *c*9,*t*11-conjugated linoleic acid (*c*9,*t*11-CLA), *t*10,*c*12-CLA, and polyunsaturated FA ( $p < 0.05$ ). The values of  $TVA/(c9,t11-CLA+TVA)$  and  $C18:0/(TVA+C18:0)$  were reduced by GO ( $p < 0.05$ ). Real-time PCR showed that GO tended to reduce *Butyrivibrio proteoclasticus* abundance ( $p = 0.058$ ), whereas GO had no effect on total abundance of the *Butyrivibrio* group bacteria. A low correlation was found between *B. proteoclasticus* abundance and  $C18:0/(TVA+C18:0)$  ( $p = 0.910$ ). The changes of fermentation over time suggested a role of GO in delaying the fermentation process and maintaining a relatively modest change of ruminal environment. The inhibitory effects of GO on the final step of biohydrogenation may be related to its antibacterial activity against *B. proteoclasticus* and other unknown bacteria involved. (**Key Words:** Garlic Oil, Conjugated Linoleic Acid, Biohydrogenation, *Butyrivibrio*, Goat)

### INTRODUCTION

Garlic (*Allium sativum*) has been used extensively in herbalism because of its well-known medicinal properties attributed to a number of active organosulphur compounds (Iciek et al., 2009). Recently, researches have shown the effects of garlic oil (GO) on *in vitro* batch fermentation (Busquet et al., 2005b; Cardozo et al., 2005) and *in vitro* continuous culture fermentation (Cardozo et al., 2004; Busquet et al., 2005a) with ruminal fluid. Busquet et al. (2005a, 2006) consistently showed that GO reduced the molar proportion of acetate and increased the molar proportions of propionate and butyrate. However, Cardozo et al. (2004, 2005) reported that GO had no effects on total

volatile fatty acid (VFA) concentration and molar proportions of individual VFA, or had pH-dependent effects on 24-h ruminal fermentation. To date, only a few studies have investigated the *in vivo* effects of GO on ruminal fermentation, and the limited studies showed that GO had no significant effects on ruminal pH, the concentrations of ammonia and total VFA, as well as the molar proportions of individual VFA in dairy cows (Yang et al., 2007) and lambs (Chaves et al., 2008). Collectively, the different responses to GO supplementation in *in vitro* and *in vivo* studies may be related to the dose and composition of GO used, as well as the rumen microbial environment and the adaptation time. Based on the wide spectrum of antibacterial activity of GO (Reuter et al., 1996), we hypothesized that GO may influence the dynamics of fermentation in the rumen.

Several gram-positive bacteria are involved in ruminal biohydrogenation of unsaturated dietary fatty acid (FA)

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(Harfoot and Hazlewood, 1997). The most active rumen bacterial species involved in C18 biohydrogenation belong to the “*Butyrivibrio*” group, where all bacteria form conjugated linoleic acid (CLA) from linoleic acid (LA), whereas only *Clostridium proteoclasticum* is able to convert *t*11-18:1 (TVA) to C18:0 (Polan et al., 1964; Kemp et al., 1975). *C. proteoclasticum* was recently reclassified as *Butyrivibrio proteoclasticus* (Moon et al., 2008). In the present study, we refer *C. proteoclasticum* to *B. proteoclasticus*. Therefore, selective inhibition of *B. proteoclasticus*, without affecting other bacteria in the “*Butyrivibrio*” group, would provide more unsaturated FA in the rumen. Due to the antibacterial activity (Reuter et al., 1996), GO may affect biohydrogenating bacterial abundance and the resulting FA profile in the rumen. However, to our best of knowledge, no information is available on whether GO could affect FA profile and abundance of bacteria involved in biohydrogenation in the rumen of goats.

Therefore, the objective of this study was to investigate the effects of ruminal infusion of GO on fermentation dynamics, FA profile and abundance of bacteria involved in biohydrogenation in the rumen.

## MATERIALS AND METHODS

### Animals, experimental design, and diets

Six wethers (19.30±1.17 kg body weight (BW), mean±standard deviation), fitted with ruminal fistula, were assigned to two groups for cross-over design with a 14-d interval. Each experimental period lasted 30 d, consisting of 27 d of adaptation and 3 d for sample collection. Goats were fed a basal diet without (control) or with GO infusion. Based on the dose of GO in lamb research (0.01 g/kg BW) (Chaves et al., 2008) and our previous study in Saanen dairy goats (0.01, 0.02 and 0.03 g/kg BW) (Zhu, 2011), the dose 0.04 g/kg BW was used in the present research. Accordingly, the dose of GO for each goat was 0.8 g/d. The basal diet was formulated (Table 1) according to Feeding Standard of Meat-producing Sheep and Goats (NY/Y 816-2004; Ministry of Agriculture, China, 2004) in order to fulfill requirements for the growth of goats. Diets for restricted intake consisted of grass hay (*Leymus chinensis*, 0.38 kg/d dry matter (DM)) and concentrate (0.22 kg/d DM). Once offering concentrate to the goat, GO product was infused directly and immediately by a 1-ml syringe via ruminal fistula. For each goat, the infusing lasted a few seconds. The ingestion of concentrate was completed simultaneously for each goat. Afterwards, hay was offered. Diet and GO infusion were offered in equal portions at 08:00 and 18:00 h. All goats had free access to fresh water throughout the experiment. The commercial GO (stored at 4°C; Henan Yuanhua Biotechnology Co., Ltd., China) used

**Table 1.** Ingredient and chemical analysis of the experimental diets

Composition	Diet	
	Concentrate	Grass hay
Ingredient (g/100 g DM <sup>1</sup> )		
Grass hay	-	63.85
Corn meal	29.39	-
Soybean meal	4.08	-
Wheat bran	1.75	-
Limestone	0.45	-
Salt	0.48	-
Chemical analysis (g/100 g DM)		
Dry matter (%)	90.68	89.00
Crude protein	11.73	7.30
Ether extract	3.43	3.40
Neutral detergent fiber	10.54	57.10
Acid detergent fiber	4.58	36.00
Crude ash	9.04	9.56
Calcium	0.50	0.37
Phosphorus	0.35	0.18

<sup>1</sup> DM = Dry matter.

in the experiment was produced by steam distillation (150°C) which was used to collect the products that evaporate with steam. The main components of GO were sulfur compounds, such as the predominant diallyl trisulfide (29.3%) and diallyl disulfide (31.3%). No FA was detected in GO used in this study. Goats were fed individually in pens and managed in accordance with guidelines regarding animal care provided by the College of Animal Science and Technology in Nanjing Agricultural University.

### Sampling and chemical analysis

Feed intake for individual goats was recorded on the last 3 d of each experimental period. Feed samples were dried at 65°C for 24 h in a forced-air oven, ground through a 1 mm screen, and analyzed for DM, crude ash content, ether extract and crude protein using standard procedures (AOAC, 1990). The concentrations of acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined by sequential procedures (pretreatment with  $\alpha$ -amylase for NDF) (Van Soest et al., 1991), and the results were expressed inclusive of residual ash.

Ruminal contents from multiple sites within the rumen of each animal were collected by suction via ruminal fistula before (0 h) and at 2, 4, 6, 8, and 10 h after morning feeding on the last 3 d of each period. The pH value was measured immediately using a portable pH meter (HI 8424; Hanna, Singapore). Then ruminal contents were collected into tubes. The 0-h samples were collected in triplicate. The tubes were capped and placed in an ice bucket and immediately transferred to the laboratory. Duplicates of 0-h samples

were immediately stored at  $-25^{\circ}\text{C}$  for latter DNA extraction and FA analysis. The others were vortexed for 1 min, and then centrifuged at  $1,000\times g$  for 10 min. A 1-ml sample of the supernatant was acidified with 1 ml of 0.2 mol/L HCl and frozen at  $-25^{\circ}\text{C}$  for latter ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) analysis by spectrophotometer (UV-2450; Shimadzu, Japan) (Broderick and Kang, 1980). One ml of the supernatant was mixed with 0.2 ml 25% (w/v) metaphosphoric acid including crotonic acid (64.64%, w/v) as an internal standard and frozen at  $-25^{\circ}\text{C}$  for latter VFA analysis by gas chromatograph (GC-14B; Shimadzu, Japan) equipped with capillary column (Supelco 41491-03B,  $30\text{ m}\times 0.32\text{ mm}\times 0.25\text{ }\mu\text{m}$  film thickness; Bellefonte, USA) (Qin, 1982). One ml of the supernatant was stored at  $-25^{\circ}\text{C}$  for microbial crude protein (MCP) analysis by spectrophotometer (UV-2450; Shimadzu, Japan) (Makkar et al., 1982).

Ruminal contents collected at 0 h on d 28, 29, and 30 were used for DNA extraction. Total DNA was extracted from 1.5 ml homogenized ruminal contents by bead-beating for 3 min using a mini-bead beater (Biospec Products, USA), followed by phenol-chloroform extraction (Zoetendal et al., 1998). DNA was then precipitated with ethanol and pellet was resuspended in  $70\text{ }\mu\text{l}$  of Tris-HCl/EDTA (TE) buffer.

#### Fatty acid analysis

Ruminal contents collected at 0 h on d 28, 29, and 30 were used for FA determination. Lipid extraction was performed as described by Folch et al. (1957) with some modifications. Briefly, the homogenized ruminal contents (10 g) were mixed with 20 ml chloroform/methanol (2/1, v/v). After dispersion, the whole mixture was agitated for 20 min in an orbital shaker at room temperature. The homogenate was centrifuged ( $1,200\times g$  for 10 min) to recover the liquid phase. Then the liquid phase was washed with 5 ml 0.9% NaCl solution. After vortexing 2 min, the mixture was centrifuged at  $1,200\times g$  for 10 min to separate the two phases. The upper phase was removed by siphoning, the lower chloroform phase containing lipid was evaporated at  $50^{\circ}\text{C}$  under nitrogen. Methylation of FA was performed with 2 ml 0.5 mol/L methanolic KOH, followed by rocking (about 3 min) in a water bath at  $50^{\circ}\text{C}$  under nitrogen until the oil beads disappeared. The flasks were cooled and then 2 ml 14% boron trifluoride/methanol was added. Afterwards, the flasks were incubated in a water bath at  $50^{\circ}\text{C}$  under nitrogen for 3 min. After cooling, one ml n-heptane was added followed by vortexing. The upper organic phase was rinsed with saturated NaCl solution, and then transferred to 2-ml centrifuge tube containing anhydrous sodium sulfate.

Fatty acid methyl esters were analyzed according to the reported methods (Lu et al., 2005; Lou et al., 2010) with

some modifications using a gas chromatography (6890N series; Agilent Technology, USA) equipped with mass spectrometry (5973 series; Agilent Technology, USA). Fatty acid separation was performed on a fused silica capillary column (HP-5MS,  $100\text{ m}\times 0.25\text{ mm}\times 0.20\text{ }\mu\text{m}$ ; Supelco, Bellefonte, USA). Ultrapure helium was used as carrier gas, the injector pressure was held constant at 43.9 kPa. Analysis of FA required injection of  $1\text{ }\mu\text{l}$  with split ratio of 10:1 at a constant flow rate of 1 ml/min by carrier gas. Injector temperature was maintained at  $280^{\circ}\text{C}$ . Initial column temperature was  $80^{\circ}\text{C}$ , increased to  $220^{\circ}\text{C}$  at a rate of  $6^{\circ}\text{C}/\text{min}$  (held for 13 min), and then raised at  $10^{\circ}\text{C}/\text{min}$  to a final temperature of  $280^{\circ}\text{C}$  (held for 15 min). The electron-impact ionization mass spectrometer was operated as follows: ionization voltage, 70 eV; ion source temperature,  $230^{\circ}\text{C}$ ; mass scan range, 29 to 450. Mass spectral identification of FA was carried out by comparing spectra with those in the NIST98 (National Institute of Standards and Technology, USA). Results for each FA were expressed as a percentage of the sum of all identified FA.

#### Real-time PCR

Real-time PCR was performed on an ABI 7300 system (Applied Biosystems, USA) associated with 7300 Sequence Detection Systems Software (version 2.2.1; Applied Biosystems, USA). The primers described below were synthesized and the PCR reagents were provided by Sangon (Sangon Biotech Co., Ltd., China).

According to the method from Boeckeaert et al. (2008), 16S rRNA gene of the *Butyrivibrio* group of bacteria was quantified by *Butyrivibrio*-specific forward and reverse primers B395f (5'-GYG AAG AAG TAT TTC GGT AT-3') and B812r (5'-CCA ACA CCT AGT ATT CAT C-3'), respectively. Quantitative PCR (qPCR) reaction mixture ( $20\text{ }\mu\text{l}$ ) consisted of  $10\text{ }\mu\text{l}$  of 2 $\times$ FastStart Universal SYBR Green Master (Roche Applied Science, Germany),  $0.6\text{ }\mu\text{l}$  forward primer ( $10\text{ }\mu\text{mol}/\text{L}$ ),  $0.6\text{ }\mu\text{l}$  reverse primer ( $10\text{ }\mu\text{mol}/\text{L}$ ),  $2\text{ }\mu\text{l}$  DNA and  $6.8\text{ }\mu\text{l}$  PCR-grade  $\text{H}_2\text{O}$ . Quantitative PCR amplification was performed using the following conditions: 1 cycle of 2 min at  $50^{\circ}\text{C}$ , 10 min at  $95^{\circ}\text{C}$ , 40 cycles of 15 s at  $95^{\circ}\text{C}$ , 30 s at  $55^{\circ}\text{C}$ , and 45 s at  $72^{\circ}\text{C}$ . Quantification of 16S rRNA gene copies in each sample was performed in triplicate, and the mean value was calculated. The *Butyrivibrio* group bacterial 418-bp PCR product amplified by specific primers (Boeckeaert et al., 2008) from the strain *B. fibrisolvens* DSM 3071 was used as a template for standard curves. Standard curves were constructed using at least five different 10-fold serial dilutions of PCR product ranging from 9.82 copies to  $9.82\times 10^7$  copies of product per  $\mu\text{l}$ . The efficiency of amplification was calculated as follows: efficiency =  $(10^{(-1/\text{slope})}-1)$ . The bacterial 16S rRNA gene copies were

plotted against the  $C_T$  value used to estimate the number of copies in each sample. PCR reaction mixture (50  $\mu$ l) consisted of 1  $\mu$ l DNA extract, 1  $\mu$ l of each primer (10  $\mu$ mol/L), 1  $\mu$ l deoxynucleotide triphosphate mixture (10 mmol/L each), 5  $\mu$ l 10 $\times$ PCR amplification buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.5, 0.1% Triton X-100), 6  $\mu$ l MgCl<sub>2</sub> (25 mmol/L), 0.25  $\mu$ l Taq polymerase (5 U/ $\mu$ l) and lastly PCR-grade H<sub>2</sub>O. PCR amplification was carried out in Eppendorf Mastercycler<sup>®</sup> Gradient (Eppendorf, Germany), with an initial DNA denaturation step at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s, and final extension at 72°C for 7 min. The amplicons were measured in triplicate using a NanoDrop-1000 spectrophotometer (Labtech International, UK) and analyzed by electrophoresis on a 1.5% agarose gel (w/v) containing ethidium bromide.

Quantification of *B. proteoclasticus* was conducted according to Paillard et al. (2007) using forward (SA-FW, 5'-TCC GGT GGT ATG AGA TGG GC-3') and reverse (SA-RV, 5'-GTC GCT GCA TCA GAG TTT CCT-3') primers. Reaction mixture (20  $\mu$ l) consisted of 10  $\mu$ l of 2 $\times$ FastStart Universal Probe Master (Roche Applied Science, Germany), 0.8  $\mu$ l forward primer SA-FW (10  $\mu$ mol/L), 1.6  $\mu$ l reverse primer SA-RV (10  $\mu$ mol/L), 0.5  $\mu$ l molecular beacon probe (5'-FAM-CCG CTT GGC CGT CCG ACC TCT CAG TCC GAG CGG-DABCYL-3') (Invitrogen, China) (10  $\mu$ mol/L), 2  $\mu$ l DNA and 5.1  $\mu$ l PCR-grade H<sub>2</sub>O. The qPCR amplification program was the same as for the *Butyrivibrio* group bacteria. Dilutions of PCR product (1,506 bp) amplified by forward 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse 1510r (5'-GGC TAC CTT GTT ACG A-3') primers (Ding and Yokota, 2004) from 16S rRNA gene clone A23 (accession No. HQ326602) were used for constructing specific standard curves. The 10-fold serial dilutions of PCR product ranged from 6.06 copies to 6.06 $\times$ 10<sup>7</sup> copies of product per  $\mu$ l. The PCR amplification mixture of clone A23 was the same as

for *B. fibrisolvans* DSM 3071. Amplification conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 30 s, 57°C for 30 s and 68°C for 1.5 min, and a final extension of 68°C for 7 min. The amplicons were measured and analyzed as above.

### Statistical analysis

Data were analyzed using PROC MIXED of SAS (version 8.01; SAS Institute, Inc., USA) (SAS, 2000) according to the model  $Y_{ijk} = \mu + G_i + P_j + T_k + e_{ijk}$ , where  $Y_{ijk}$  was the response variable,  $\mu$  was the overall mean,  $G_i$  was the random effect of goat  $i$ ,  $P_j$  was the effect of period  $j$ ,  $T_k$  was the effect of treatment  $k$ , and  $e_{ijk}$  was the random residual error. For the statistical analysis of ruminal fermentation characteristics (pH, NH<sub>3</sub>-N, MCP, and VFA), sampling time ( $H_t$ ) and interaction of treatment with sampling time ( $T \times H$ )<sub>kr</sub> were added to the model, and analyzed using repeated measures of PROC MIXED with the covariance type auto-regressive order 1 (AR (1)). The effect of sampling time was repeated measures. Means were separated by using the PDIF option in the LSMEANS statement. Pearson correlation was analyzed using PROC CORR procedure to investigate the relationship between microbial abundance and FA proportion in the rumen. Significances were determined at  $p < 0.05$ ; differences of  $p < 0.1$  were discussed as trend.

## RESULTS

### Feed intake

No refusals of concentrate in the two groups were observed. The DM intake of concentrate for each goat was 0.22 kg/d. Garlic oil had no influence on the intake of hay (0.33 kg/d for control and 0.32 kg/d for GO group).

### Ruminal fermentation

The effects of GO on ruminal fermentation are shown in Table 2. A slight reduction ( $p < 0.05$ ) in ruminal pH was found in GO group (6.49) as compared with control (6.55).

**Table 2.** Effects of garlic oil on ruminal fermentation

Item	Treatment		SEM	p		
	Control	Garlic		Treatment	Time	Treatment $\times$ time
pH	6.55	6.49	0.011	0.012	<0.001	0.673
NH <sub>3</sub> -N (mmol/L)	6.49	8.02	0.177	<0.001	<0.001	0.219
MCP ( $\mu$ g/ $\mu$ l)	1.54	1.91	0.035	<0.001	<0.001	0.510
Total VFA (mmol/L)	73.18	69.93	0.529	0.054	<0.001	0.017
VFA (mol/100 mol)						
Acetate	66.64	67.46	0.210	0.140	0.059	0.068
Propionate	21.54	20.99	0.304	0.547	<0.001	0.014
Butyrate	11.82	11.56	0.160	0.622	0.001	0.503
Acetate/propionate	3.31	3.29	0.049	0.911	<0.001	0.008

NH<sub>3</sub>-N = Ammonia nitrogen; MCP = Microbial crude protein; Total VFA = Acetate+propionate+butyrate; SEM = Standard error of mean; p = Probability.

There were increases ( $p < 0.05$ ) in the concentrations of  $\text{NH}_3\text{-N}$  and MCP upon GO infusion. Garlic oil had no influence on total VFA concentration, molar proportions of individual VFA, or ratio of acetate to propionate. There were interactions between treatment and sampling time for total VFA concentration, propionate molar proportion and ratio of acetate to propionate ( $p < 0.05$ ). The temporal changes showed that total VFA concentration and propionate molar proportion reached maxima at 2 h after morning feeding in control, whereas the maxima occurred at 6 h in GO group (Figure 1). The ratio of acetate to propionate in control reduced sharply after morning feeding,

whereas it remained relatively stable in GO group (Figure 1).

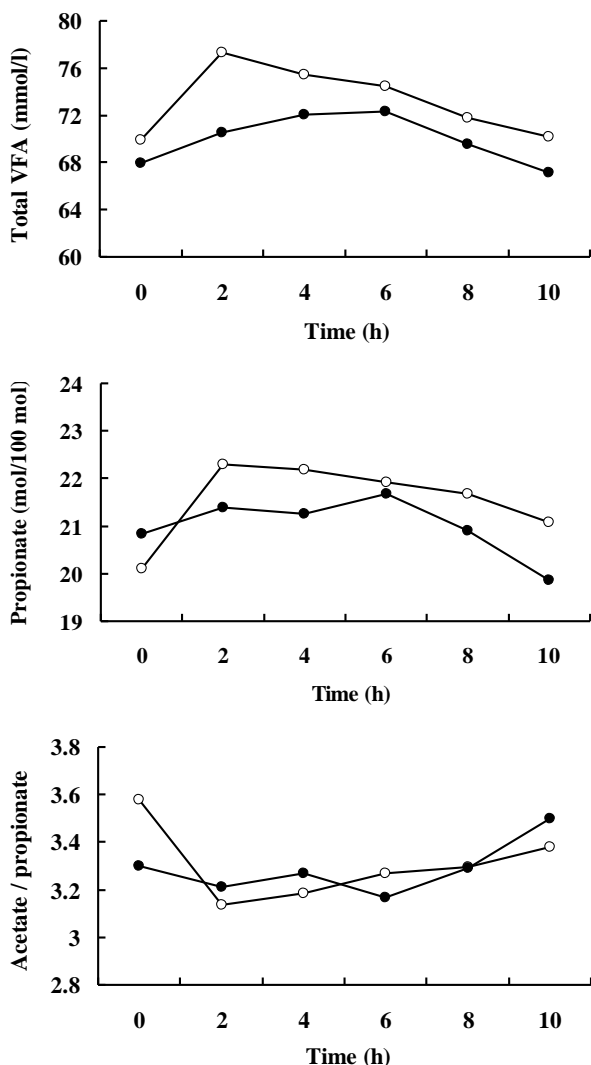
### Fatty acid proportion

Details regarding FA proportion are summarized in Table 3. Generally, GO had no effects on C4 to C14 FA proportions, while reducing C15 and C16 FA proportions ( $p < 0.05$ ) and increasing the proportions of C18 FA, TVA,

**Table 3.** Effects of garlic oil on fatty acid proportion in ruminal contents

Fatty acid (%)	Treatment		SEM	p
	Control	Garlic		
C4:0	2.64	2.59	0.017	0.185
C6:0	1.73	1.70	0.013	0.225
C8:0	1.14	1.11	0.015	0.246
C10:0	1.20	1.18	0.017	0.253
C12:0	3.00	2.97	0.018	0.279
C14:0	7.27	7.24	0.018	0.333
C15:0	1.13	1.06	0.014	<0.001
C15:1	0.77	0.75	0.006	0.008
C16:0	32.97	32.60	0.080	<0.001
C16:1	1.63	1.54	0.014	<0.001
C17:0	0.38	0.37	0.003	0.052
C17:1	0.26	0.26	0.003	0.553
C18:0	13.27	13.50	0.032	<0.001
C18:1	22.12	22.44	0.096	0.033
TVA	0.32	0.33	0.001	<0.001
C18:2	1.24	1.32	0.010	<0.001
C18:3	0.70	0.76	0.007	<0.001
<i>c9,t11</i> -CLA	0.14	0.15	0.002	<0.001
<i>t10,c12</i> -CLA	0.02	0.03	0.001	0.035
C20:0	0.14	0.15	0.002	<0.001
C20:1 (n-9)	0.25	0.28	0.004	<0.001
C20:2 (n-6)	0.15	0.16	0.002	<0.001
C20:4 (n-6)	0.08	0.10	0.003	<0.001
C20:5 (n-3)	0.06	0.08	0.003	<0.001
C22:1 (n-9)	0.14	0.15	0.002	0.019
C22:3 (n-3)	0.02	0.03	0.001	0.005
C22:5 (n-3)	0.02	0.03	0.001	<0.001
C22:6 (n-3)	0.07	0.09	0.002	<0.001
C24:1 (n-9)	0.01	0.02	0.001	0.001
Total other	7.13	7.03	0.034	0.052
SFA	64.86	64.48	0.104	0.004
MUFA	25.50	25.75	0.099	0.076
PUFA	2.51	2.74	0.023	<0.001
TVA/( <i>c9,t11</i> -CLA+TVA)	70.11	69.24	0.237	0.021
C18:0/(TVA+C18:0)	97.64	97.61	0.006	0.040

TVA = *t11-18:1*; CLA = Conjugated linoleic acid; SFA = C4:0+C6:0+C8:0+C10:0+C12:0+C14:0+C15:0+C16:0+C17:0+C18:0+C20:0; MUFA = C15:1+C16:1+C17:1+C18:1+*t11-18:1*+C20:1 (n-9)+C22:1 (n-9)+C24:1 (n-9); PUFA = C18:2+C18:3+*c9,t11*-CLA+*t10,c12*-CLA+C20:2 (n-6)+C20:4 (n-6)+C20:5 (n-3)+C22:3 (n-3)+C22:5 (n-3)+C22:6 (n-3); SEM = Standard error of mean; p = Probability.



**Figure 1.** Temporal changes in total VFA concentration, propionate molar proportion, and ratio of acetate to propionate in goats fed basal diet (corn-soybean meal concentrate and *Leymus chinensis*) without ( $\circ$ ) or with garlic oil infusion ( $\bullet$ , 0.8 g/d) via ruminal fistula. Values represent the means from 6 goats for 3-d sample collection. Standard error of mean = 0.529 mmol/L, 0.304 mol/100 mol, and 0.049 for total VFA concentration, propionate molar proportion, and ratio of acetate to propionate, respectively. Total VFA = Acetate+propionate+butyrate.

*c9,t11*-CLA and *t10,c12*-CLA ( $p < 0.05$ ) in the rumen. Garlic oil reduced saturated FA (SFA) proportion ( $p < 0.05$ ), whereas GO increased polyunsaturated FA (PUFA) proportion ( $p < 0.05$ ) and tended to increase monounsaturated FA (MUFA) proportion ( $p = 0.076$ ). The values of TVA/ (*c9,t11*-CLA+TVA) and C18:0/(TVA+C18:0) were reduced by GO ( $p < 0.05$ ).

### Bacterial abundance

The abundance of *Butyrivibrio* group bacteria and *B. proteoclasticus* in ruminal contents is listed in Table 4. Quantitative PCR efficiency for all assays was between 90% and 110% (data not shown). Correlations of DNA standards for all real time PCRs were  $>0.97$  (data not shown). Results showed that GO had no influence on the *Butyrivibrio* group bacterial abundance, whereas GO tended to reduce *B. proteoclasticus* abundance ( $p = 0.058$ ). Correlation analysis showed that there was a low correlation between *B. proteoclasticus* abundance and C18:0/(TVA+C18:0) ( $r = 0.02$ ,  $p = 0.910$ ).

## DISCUSSION

Since garlic has been shown to exhibit a wide spectrum of antibacterial activity (Reuter et al., 1996), a high dose of GO could have detrimental effects on ruminal fermentation as suggested by Yang et al. (2007). In this study, the dose of GO per body weight was higher than those investigated in growing lambs (Chaves et al., 2008) and lactating cows (Yang et al., 2007). In the current research, the DM intake in goat was not affected by GO infusion. This result suggested that the dose of GO used in this study had no detrimental effects on digestion of feed. Meanwhile, a longer adaptation of rumen to the presence of GO may partly explain the lack of effects on intake of diets.

Previous researches have shown that rumen fermentation could be adapted to the addition of GO. Cardozo et al. (2004) found that GO modified the molar proportions of individual VFA between d 2 to 6 of fermentation in a dual-flow continuous-culture fermentor system, but all these effects disappeared after 6-d adaptation. In *in vitro* continuous culture trials, total VFA concentration, individual VFA molar proportions, and  $\text{NH}_3\text{-N}$  concentration in fermentation changed during the first 2 d after the addition of GO, but remained stable thereafter

(Busquet et al., 2005a, b). The different adaptation time may be due to the different concentration of GO used in experiments. In addition, Castillejos et al. (2007) demonstrated that VFA concentration and proportions only required a short adaptation time, longer than 24 h but shorter than 6 d, whereas N metabolism required an adaptation time of up to 4 wks, which may suggest that the microbial populations associated with N metabolism changed relatively slowly. In the current study, ruminal contents collected after 27-d adaptation were feasible for analyzing dynamics of fermentation.

Studies of plant essential oil to ruminant nutrition have been focused on their potentials to improve nitrogen and energy utilization in the rumen (Benchaar et al., 2008). In the present research, GO increased  $\text{NH}_3\text{-N}$  concentration in the rumen. However, the *in vivo* studies showed that GO just numerically reduced (Chaves et al., 2008) or increased (Yang et al., 2007)  $\text{NH}_3\text{-N}$  concentration in the rumen of growing lambs and lactating cows, respectively. Interestingly, in 24-h batch culture fermentation, Busquet et al. (2005b) found that some doses (e.g. 3 to 300 mg/L) of GO and its compounds increased  $\text{NH}_3\text{-N}$  concentration, whereas the high dose (3,000 mg/L) of compounds reduced  $\text{NH}_3\text{-N}$  concentration. On the contrary, Cardozo et al. (2005) found that GO (3 to 300 mg/L) reduced  $\text{NH}_3\text{-N}$  concentration of the 24-h fermentation culture with different initial pH (7.0 and 5.5). Therefore, the discrepancy among these studies is probably due to the actual concentration and chemical composition of GO, as well as experimental conditions (including animal condition, diets/substrate and adaptation time) used in studies. It was reported that  $\text{NH}_3\text{-N}$  formation is affected only at the last step of the breakdown sequence of protein, that is the deamination of amino acids (Wallace, 2004). In this study, the increase of  $\text{NH}_3\text{-N}$  concentration during fermentation suggested that GO seemed to stimulate the deamination. Meanwhile, reduction in  $\text{NH}_3\text{-N}$  absorption by rumen epithelium could also cause the increase of  $\text{NH}_3\text{-N}$  concentration in the rumen. It is known that  $\text{NH}_3\text{-N}$  is regarded to be the most important nitrogen source for microbial protein synthesis in the rumen (Wanapat et al., 2008). The concentrations of  $\text{NH}_3\text{-N}$  obtained in the present study (201.19 and 248.62 mg/L) were close to the optimal ruminal  $\text{NH}_3\text{-N}$  (150 to 300 mg/L) (Wanapat et al., 2011). Increased  $\text{NH}_3\text{-N}$  concentration in GO group may benefit microbial protein synthesis, as observed in the increased level of MCP. Nevertheless, further study is needed to investigate the mechanism of increased  $\text{NH}_3\text{-N}$  and MCP concentrations by GO.

Although reduction in pH was found in GO group, this difference between the treatments was marginal (6.55 and 6.49). Such a small change of pH could be considered similar in the rumen. Yang et al. (2007) stated that a high

**Table 4.** Effects of garlic oil on 16S rRNA gene copies of ruminal bacteria (log<sub>10</sub> copies/ml ruminal contents)

Bacteria	Treatment		SEM	p
	Control	Garlic		
<i>Butyrivibrio</i> group bacteria	11.78	11.52	0.195	0.427
<i>Butyrivibrio proteoclasticus</i>	9.81	9.43	0.106	0.058

p = Probability.

dose of GO could have detrimental effects on ruminal fermentation. At present, no influence on the intake and total VFA concentration were found, suggesting that the dose of GO used may not be harmful to the rumen after adaptation. Owing to the antibacterial activity (Reuter et al., 1996), the sensitive bacteria are readily inhibited by GO. From a long-term continuous culture study, Busquet et al. (2005b) suggested that after longer adaptation, the microbial population inhibited by GO may be replaced by other resistant bacterial groups, leading to the lack of detrimental effects reported in *in vitro* continuous culture systems (Cardozo et al., 2004; Busquet et al., 2005a, b). Thus, in the present study, the lack of effects of GO on total VFA concentration and individual VFA molar proportions may be due to the long adaptation time (27 d) for the ruminal microflora.

An interesting finding of this study is that the effectiveness of GO on ruminal fermentation related to the time after infusion. Garlic oil supplementation seemed to delay the fermentation process as evidenced by a delayed occurrence of maximal total VFA concentration and molar proportion of propionate as compared with control. While the ratio of acetate to propionate in control reduced sharply after morning feeding, this ratio in GO group remained relatively stable, suggesting that GO could maintain a modest change of ruminal environment during fermentation. These interesting findings may be due to the antibacterial activity of GO, which might affect the abundance and activity of microorganisms after feeding. Rapid fermentation usually takes place after feeding, especially when diets contain high proportions of concentrates. Thus, GO may have the potential for its use as additives to prevent the rapid change of ruminal environment during fermentation.

To our knowledge, no information is available on the effects of GO on FA profile in the rumen. This study, for the first time, investigated GO effects on FA proportion and abundance of bacteria involved in biohydrogenation, and then explored the possible mechanism of GO effects on biohydrogenation. In the rumen, dietary lipids are subject to hydrolysis by microbial lipases (Jenkins et al., 2008; Lourenço et al., 2010). Here, the higher proportions of C18:1, C18:2 and C18:3 in GO group compared with control may be related to a GO effect on lipolysis in the rumen. Whether GO inhibits lipolysis or not requires further investigation. The free FA from lipolysis, both saturated and unsaturated, can be sequestered into microbial cells for lipid synthesis (Harfoot and Hazlewood, 1997). Thus, the modifications of FA profile by GO in the current study may be in part attributed to its effects on lipid synthesis in microorganisms. The major microbial transformations in the rumen are *de novo* synthesis of odd- and branched-chain FA and biohydrogenation of unsaturated FA (Kim et al., 2005).

As no FA was detected in GO product and no GO effect on diet intake was observed, the total amount of FA in ruminal contents could not be affected by GO infusion. Hence, reductions in the proportions of C15:0 and C15:1 indicated that GO might reduce their *de novo* synthesis in the rumen. Presently, the increased proportions of C18:1, C18:2, C18:3, TVA, *c9,t11*-CLA, and *t10,c12*-CLA in GO group, at least in part, may suggest that GO partially inhibited them from microbial biohydrogenation.

Results of the current study showed that GO increased the proportion of *c9,t11*-CLA in the rumen. It is known that *c9,t11*-CLA is transformed from LA not linolenic acid (LNA) (Harfoot and Hazlewood, 1997). The formation of *c9,t11*-CLA is transient as it is biohydrogenated to TVA (Shingfield et al., 2010). Therefore, the increased proportion of *c9,t11*-CLA in GO group may be due to the reduced extent of biohydrogenation of *c9,t11*-CLA, as indicated by the decrease of  $TVA/(c9,t11-CLA+TVA)$ . Bacteria falling within the *Butyrivibrio* group form *c9,t11*-CLA and TVA (Jenkins et al., 2008). Here, we investigated GO effect on the abundance of *Butyrivibrio* group bacteria. Although GO has a wide spectrum of antibacterial activity (Reuter et al., 1996), GO did not influence the *Butyrivibrio* group bacterial abundance in the rumen. Thus, GO increased *c9,t11*-CLA proportion probably not through its effect on total abundance of the *Butyrivibrio* group bacteria.

In the rumen, TVA can be further transformed to C18:0 (Harfoot and Hazlewood, 1997), and this transformation is considered rate limiting in the biohydrogenation process (Shingfield et al., 2010). Depression of this final step of the biohydrogenation process could cause the accumulation of TVA in the rumen. In the present study, the lower  $C18:0/(TVA+C18:0)$  in GO group compared with control indicated that GO had the capacity to inhibit the final step of biohydrogenation. It is known that *B. proteoclasticus* is involved in the specific biohydrogenation of TVA to C18:0 (Harfoot and Hazlewood, 1997). Here, we found that GO tended to reduce the abundance of *B. proteoclasticus*. Therefore, the inhibitory effect of GO on the final step of biohydrogenation may be related to its antibacterial activity against *B. proteoclasticus*. However, there was a low correlation between *B. proteoclasticus* abundance and  $C18:0/(TVA+C18:0)$  in the present study. A similar relationship has been observed in previous trials (Kim et al., 2008; Huws et al., 2010). These results could indicate that the induction of  $C18:0/(TVA+C18:0)$  was not only through the antibacterial activity of GO against *B. proteoclasticus*. Recent reports showed that other unknown bacteria may play a more predominant role in the final step of biohydrogenation in the rumen (Boeckert et al., 2008; Kim et al., 2008; Huws et al., 2011). Thus, the inhibitory effect of GO on the final step of biohydrogenation may also be related to GO's antibacterial activity against other unknown

biohydrogenating bacteria. A further understanding of the GO effect on these unknown bacteria is required to explore the inhibitory mechanism of GO in the final biohydrogenation process. Unexpectedly, the higher proportion of C18:0 in GO group compared with control was also observed in this study. In addition to TVA, *c*15-C18:1, *t*15-C18:1 (biohydrogenation intermediates of LNA) and *c*9-C18:1 can also be transformed to C18:0 in the rumen (Jenkins et al., 2008), which may in part explain the increase of C18:0 proportion in GO group. Collectively, GO has an inhibitory effect on the final step of biohydrogenation in the rumen, which may be related to its antibacterial activity against *B. proteoclasticus* and other unknown bacteria involved.

In this paper, very long-chain FA ( $\geq$ C20) mainly PUFA increased upon GO infusion. It is known that lipids are hydrolyzed into non-esterified FA (NEFA). These unsaturated NEFA then undergo biohydrogenation by microorganisms in the rumen (Jenkins et al., 2008). Thus, the increase in the proportions of very long-chain unsaturated FA may result from the decreased extent of biohydrogenation by GO. Maia et al. (2010) suggested that biohydrogenation occurred to enable the biohydrogenating bacteria to survive the bacteriostatic effects of PUFA. The sensitivity of different isolates in *Butyrivibrio*-related phylogenetic tree to unsaturated FA was highly variable, with *B. proteoclasticus* being much more sensitive than others (Jenkins et al., 2008). The accumulation of NEFA in the rumen can inhibit the whole process of biohydrogenation (Lourenço et al., 2010). Thus, the increased PUFA in the rumen contributed to the inhibition of the complete biohydrogenation of unsaturated FA in the diet.

## CONCLUSION

In conclusion, GO infusion (0.8 g/d) did not affect ruminal fermentation except NH<sub>3</sub>-N and MCP, but delayed the fermentation process, suggesting its potential to maintain the ruminal environment. Garlic oil altered ruminal FA profile, with TVA and *c*9,*t*11-CLA proportions increased, but reduced TVA/(*c*9,*t*11-CLA+TVA) and C18:0/(TVA+C18:0). Garlic oil inhibited the final step of biohydrogenation, and this may be related to its antibacterial activity against *B. proteoclasticus* and other unknown bacteria involved.

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