



Diversity of *Butyrivibrio* Group Bacteria in the Rumen of Goats and Its Response to the Supplementation of Garlic Oil

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ABSTRACT: This study aimed to investigate the diversity of the *Butyrivibrio* group bacteria in goat rumen and its response to garlic oil (GO) supplementation as revealed by molecular analysis of cloned 16S rRNA genes. Six wethers fitted with ruminal fistulas were assigned to two groups for a cross-over design with 28-d experimental period and 14-d interval. Goats were fed a basal diet without (control) or with GO ruminal infusion (0.8 g/d). Ruminal contents were used for DNA extraction collected before morning feeding on d 28. A total bacterial clone library was firstly constructed by nearly full-length 16S rRNA gene cloned sequences using universal primers. The resulting plasmids selected by *Butyrivibrio*-specific primers were used to construct a *Butyrivibrio* group-specific bacterial clone library. *Butyrivibrio* group represented 12.98% and 10.95% of total bacteria in control and GO group, respectively. In libraries, clones were classified to the genus *Pseudobutyrvibrio*, *Butyrivibrio* and others within the family Lachnospiraceae. Additionally, some specific clones were observed in GO group, being classified to the genus *Ruminococcus* and others within the family Ruminococcaceae. Based on the criterion that the similarity was 97% or greater with database sequences, there were 29.73% and 18.42% of clones identified as known isolates (i.e. *B. proteoclasticus* and *Ps. ruminis*) in control and GO groups, respectively. Further clones identified as *B. fibrisolvans* (5.41%) and *R. flavefaciens* (7.89%) were specifically found in control and GO groups, respectively. The majority of clones resembled *Ps. ruminis* (98% to 99% similarity), except for Lachnospiraceae bacteria (87% to 92% similarity) in the two libraries. The two clone libraries also appeared different in Shannon diversity index (control 2.47 and GO group 2.91). Our results indicated that the *Butyrivibrio* group bacteria had a complex community with considerable unknown species in the goat rumen. (**Key Words:** *Butyrivibrio*, 16S rRNA Gene, Diversity, Garlic Oil, Goat Rumen)

INTRODUCTION

In the rumen, bacteria are largely responsible for biohydrogenation of dietary unsaturated fatty acid (FA) (Harfoot and Hazlewood, 1997). The most active bacterial species involved in the biohydrogenation of C18 unsaturated FA belong to the *Butyrivibrio* group (Paillard et al., 2007; Boeckeaert et al., 2008; Durmic et al., 2008), including the genus *Butyrivibrio* and *Pseudobutyrvibrio*, and the species *B. proteoclasticus* (previously referred as *Clostridium proteoclasticum*) (Paillard et al., 2007; Moon et al., 2008). In this group, all bacteria are capable of

hydrogenating linoleic acid and forming *cis*₉, *trans*₁₁-conjugated linoleic acid (*c*₉, *t*₁₁-CLA), and *t*₁₁-C18:1 (TVA) as well, whereas only *B. proteoclasticus* convert TVA to C18:0 (Polan et al., 1964; Kepler et al., 1966). Nevertheless, within the complex ecosystem of rumen, there were bacteria other than *B. proteoclasticus* may play a predominant role in the final step of biohydrogenation (Boeckeaert et al., 2008; Kim et al., 2008; Huws et al., 2010). Sequencing of bands from denaturing gradient gel electrophoresis (DGGE) also revealed that as yet uncultured bacteria classified as *Prevotella*, *Anaerovoax* (member of the Lachnospiraceae incertae sedis family), and unclassified Clostridiales and Ruminococcaceae may play a role in biohydrogenation (Huws et al., 2011). These results suggested that bacteria involved in the biohydrogenation pathway are far more complex than the previous deduced from the cultured bacteria (Polan et al., 1964; Kemp et al.,

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1975; Wallace et al., 2006). This complexity is compounded by the fact that the majority of microorganisms have not yet been cultured (estimates range from 85% to 95%) (Krause et al., 2003).

Nowadays, 16S rRNA molecular technique has become the most commonly used measure in microbial ecology (Mackie and Cann, 2005). It is reasonable to expect a culture-independent technique (e.g. 16S rRNA gene PCR) could recover more of the *Butyrivibrio* group fraction from the ecosystem of rumen than culture-dependent techniques. Among the molecular techniques, DGGE, one of genetic fingerprinting techniques, has its own limitation, which is relatively small fragments with length up to 500 bp (Muyzer and Smalla, 1998). This easily leads to biases in the estimation of diversity. Complete sequences would provide more reliable phylogenetic comparison with the available database as compared with the short sequences (Stackebrandt and Goebel, 1994).

It is known that garlic (*Allium sativum*) oil (GO) has a wide spectrum of antibacterial activity against gram-negative and gram-positive bacteria (Reuter et al., 1996). Several gram-positive bacteria are involved in biohydrogenation of unsaturated FA in the rumen (Harfoot and Hazlewood, 1997). This attracted our interest in whether GO could affect the diversity of the *Butyrivibrio* group bacteria in the rumen. Therefore, the present study, using sequence analysis of nearly full-length 16S rRNA gene clones, aimed to investigate the diversity of the *Butyrivibrio* group bacteria and its response to GO supplementation in the rumen of goats.

MATERIALS AND METHODS

Animals, experimental design, and diets

Six local crossbred wethers fitted with ruminal fistulas (19.3±1.2 kg body weight [BW], mean±standard deviation) were assigned to two groups for a cross-over design with 28-d experimental period and 14-d interval. Goats were fed a basal diet without (control) or with GO infusion via ruminal fistula. The dose 0.04 g/kg BW was used in the present study according to the dose of GO in lamb research (0.01 g/kg BW) (Chaves et al., 2008) and our previous study in dairy goats (0.01, 0.02, and 0.03 g/kg BW) (Zhu et al., 2013). Accordingly, the dose was 0.8 g/d. The basal diet was formulated according to Feeding Standard of Meat-producing Sheep and Goats (NY/Y 816-2004; Ministry of Agriculture, China, 2004) in order to fulfill the maintenance requirements of goats. Diets consisted of grass hay (*Leymus chinensis*, 0.38 kg/d dry matter (DM)) and concentrate (corn meal-soybean meal-wheat bran, 0.22 kg/d DM), and were fed separately. The chemical analysis of diets has been published in our previous report (Zhu et al., 2012). Garlic

oil product was infused into rumen using a 1-mL syringe via ruminal fistulas immediately after the delivery of concentrate to animal. For each goat, the infusing lasted a few seconds. Hay was offered after the concentrate was ingested by the goats. Both diet and GO infusion were offered in equal portions at 08:00 and 18:00 h. No refusals of concentrate and no statistical difference of hay intake (0.33 kg/d for control and 0.32 kg/d for GO group) were observed in two groups. The main components of GO (stored at 4°C; Henan Yuanhua Biotechnology Co., Ltd., China) were sulfur compounds, such as the predominant diallyl trisulfide (29.3%) and diallyl disulfide (31.3%). The remaining 40% components of GO were other sulfur compounds. All goats had free access to fresh water throughout the experiment. Goats were fed individually in pens and managed in accordance with guidelines regarding animal care provided by College of Animal Science and Technology, Nanjing Agricultural University.

Sampling, DNA extraction, and PCR amplification

Ruminal contents from multiple sites within the rumen were collected via ruminal fistula before morning feeding on d 28 of each period. Sample collection was performed with a suction device which could ensure the same ratio of liquid to solid in ruminal contents across animals. Samples were subsequently stored at -25°C for latter DNA extraction. Prior to DNA extraction, samples were thawed and then homogenized using the Ultra Turrax T25 homogenizer (IKA Werke Labortechnik, Staufen, Germany). 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 µL Tris-HCl/EDTA buffer. Thereafter, DNA was stored at -25°C for latter PCR amplification.

Bacterial 16S rRNA gene sequence of each sample was amplified on an Eppendorf Mastercycler Gradient (Eppendorf, Germany) with universal primers, forward 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse 1510r (5'-GGC TAC CTT GTT ACG A-3') (Ding and Yokota, 2004). A reaction mixture (50 µL) consisted of 1 µL DNA extract, 1 µL of each primer (10 µmol/L), 1 µL deoxynucleotide triphosphate mixture (10 mmol/L each), 5 µL 10× PCR buffer, 6 µL MgCl₂ (25 mmol/L), 0.25 µL Taq polymerase (5 U/µL) and lastly 34.75 µL PCR-grade H₂O. The PCR was performed under the following conditions: one cycle of 94°C for 3 min for denaturation, followed by 30 cycles of 94°C for 30 s for denaturation, 57°C for 30 s for annealing, and 68°C for 1.5 min for extension, and final extension at 68°C for 7 min. 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) to check its size.

Construction of clone library

According to the method from Pei et al. (2010), equal quantities of PCR products of six samples in each experimental group were mixed together for clone library construction. The mixture was purified by kit (Invitrogen, China) and then cloned into *Escherichia coli* strain TOP10 (Tiangen Biotech, China) using pGEM-T Easy vector (Promega, USA). Transformants from each library were randomly picked up. Recombinant plasmids were extracted by lysis (95°C for 15 min). All plasmids were checked by amplification with pGEM-T-specific primers Sp6 (forward, 5'-ATT TAG GTG ACA CTA TAG-3') and T7 (reverse, 5'-AAT ACG ACT CAC TAT AGG-3'). PCR reaction mixtures of primers Sp6 and T7 were the same to that of primers 8f and 1510r. PCR amplification was carried out with an initial DNA denaturation step at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 1.5 min, and final extension at 68°C for 7 min. The plasmids containing an insert approx. 1.5 kbp based on the DNA marker ladder run on the same gel were used to construct a total bacterial clone library.

Butyrivibrio group bacterial library was constructed with the plasmids selected from the total bacterial library by PCR amplification using *Butyrivibrio*-specific forward primer B395f (5'-GYG AAG AAG TAT TTC GGT AT-3') and reverse primer B812r (5'-CCA ACA CCT AGT ATT CAT C-3') (Boeckert et al., 2008). PCR reaction mixtures of primers B395f and B812r were the same to that of primers 8f and 1510r. The amplification conditions were as follows: 94°C for 5 min, 34 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s, and a final extension of 72°C for 7 min. The amplicons were checked on a 1.5% agarose gel (w/v). Then the plasmids amplified by the specific primers were sequenced in both directions commercially (Beijing Genomics Institute, China).

Sequence analysis, diversity estimation, and phylogenetic analysis

All chimeric sequences were discarded before submitting to GenBank database. Classification analysis of sequence was conducted by comparison with Ribosomal Database Project (RDP) database. Similarity searches were performed with BLAST tool in GenBank database. The proportion of the *Butyrivibrio* group bacteria in total bacteria was expressed by the ratio of the number of clones selected by *Butyrivibrio*-primers to the number of clones in the total bacterial library. The proportion of each kind of

clone (according to the nearest known relative and the similarity) in the *Butyrivibrio* group bacterial library was calculated as follows: the number of that clone type divided by the total clone numbers of this library.

The Shannon-Weaver diversity index (H_{shannon}) was calculated for each library with the DOTUR program (Schloss and Handelsman, 2005) to characterize the diversity of the *Butyrivibrio* group bacteria.

Sequences were aligned using Clustalx1.83, and a phylogenetic tree was constructed by Neighbor-Joining of Bootstrap Test in MEGA4 software. Percentage bootstrap values were calculated from 1,000 replicates and were shown at nodes if $\geq 50\%$.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of clones reported in this paper have been deposited in GenBank database under accession numbers HQ326588 to HQ326624 (control) and JN008400 to JN008437 (GO).

RESULTS

Sequence analysis

Total bacterial library consisted of 285 and 347 clones in control and GO groups, in which 37 and 38 clones were selected by *Butyrivibrio*-specific primers and constructed as the *Butyrivibrio* group bacterial library, respectively. Accordingly, the *Butyrivibrio* group represented 12.98% (control) and 10.95% (GO) of total bacteria in the rumen. In both libraries, clones were classified to the genus *Pseudobutyrvibrio*, *Butyrivibrio* and others within the family Lachnospiraceae based on comparisons with RDP database. Some clones only observed in GO group were classified to the genus *Ruminococcus* and others within the family Ruminococcaceae.

Using the criterion that the similarity was 97% or greater with database sequences, 29.73% and 18.42% of clones were identified as known isolates (*B. proteoclasticus* and *Ps. ruminis*) in control and GO groups, respectively (Table 1). The majority of clones resembled *Ps. ruminis* with similarity ranging from 98% to 99%, except for Lachnospiraceae bacterium (similarities from 87% to 92%) in the two libraries. In addition to *B. proteoclasticus* and *Ps. ruminis* in both libraries, clones identified as *B. fibrisolvens* (5.41%) and *R. flavefaciens* (7.89%) were specifically observed in control and GO groups, respectively. Accordingly, *B. fibrisolvens* and *R. flavefaciens* accounted for 0.70% and 0.86% of total bacteria, respectively.

The Shannon index (a diversity index that accounts for both abundance and evenness of the species present in the community) of control and GO group libraries were 2.47 and 2.91, respectively.

Table 1. 16S rRNA gene clones of the *Butyrivibrio* group bacteria from goat rumen

Name	Similarity (%)	Library	Size of clone (bp)	No. of clone	Proportion (%)
Nearest known relative					
<i>Butyrivibrio proteoclasticus</i>	97.2-98.6	Control	1,505-1,506	3	8.11
	98.7	Garlic oil	1,506	1	2.63
<i>Pseudobutyrvibrio ruminis</i>	98.5-99.2	Control	1,489-1,494	8	21.62
	98.0-99.3	Garlic oil	1,492-1493	6	15.79
<i>Butyrivibrio fibrisolvens</i>	99.2, 99.5	Control	1,491, 1,493	2	5.41
<i>Ruminococcus flavefaciens</i>	98.1-99.1	Garlic oil	1,474-1,475	3	7.89
Unidentified ^a					
<i>Butyrivibrio hungatei</i>	89.4	Control	1,491	1	2.70
	89.0	Garlic oil	1,493	1	2.63
<i>Butyrivibrio proteoclasticus</i>	89.4, 91.6	Control	1,495, 1,503	2	5.41
	92.1	Garlic oil	1,508	1	2.63
Clostridiaceae bacterium	85.8-90.2	Control	1,483-1,500	3	8.11
	84.7, 90.6	Garlic oil	1,483, 1,488	2	5.26
<i>Eubacterium oxidoreducens</i>	92.1, 92.3	Control	1,509, 1,511	2	5.41
	91.0, 93.6	Garlic oil	1,493, 1,509	2	5.26
<i>Eubacterium ruminantium</i>	90.3, 92.5	Control	1,491, 1,507	2	5.41
	94.2, 94.2	Garlic oil	1,504	2	5.26
Lachnospiraceae bacterium	87.3-88.8	Control	1,470-1,476	12	32.43
	88.1-92.0	Garlic oil	1,473-1,492	8	21.05
<i>Clostridium phytofermentans</i>	92.0	Control	1,482	1	2.70
<i>Clostridium saccharolyticum</i>	92.0	Control	1,478	1	2.70
<i>Clostridium</i> sp.	89.0-91.0	Garlic oil	1,493-1,506	3	7.89
Clostridiales bacterium	92.2, 94.2	Garlic oil	1,485, 1,530	2	5.26
<i>Eubacterium contortum</i>	91.0	Garlic oil	1,511	1	2.63
<i>Eubacterium coprostanoligenes</i>	90.6	Garlic oil	1,490	1	2.63
<i>Eubacterium ventriosum</i>	94.2, 94.3	Garlic oil	1,512	2	5.26
<i>Papillibacter cinnamivorans</i>	86.6, 90.0	Garlic oil	1,475, 1,497	2	5.26
<i>Ruminococcus flavefaciens</i>	93.7	Garlic oil	1,473	1	2.63

^a Similarity is less than 97%.

Phylogenetic analysis

In a phylogenetic tree, a tight and unique cluster (*Pseudobutyrvibrio*) was formed by clones resembling *Ps. ruminis* and *B. fibrisolvens*, in which all the sequences were classified to the genus *Pseudobutyrvibrio* (Figure 1). The majority of clones classified to the genus within the family Lachnospiraceae formed many clusters, which were genetically distinct from clusters of *Pseudobutyrvibrio* and *Butyrivibrio*. In addition, some *Butyrivibrio* bacterial clones found in the GO group clustered closely with *R. flavefaciens* and uncultured Ruminococcaceae bacterium.

DISCUSSION

In this study, the *Butyrivibrio* group bacteria represented 12.98% of total bacteria in the rumen of goats. This result was in line with the previous report with respect to the genus *Butyrivibrio* isolates which accounted for 10% to 30% of total culturable bacteria in the rumen (Kopečný et al., 2003; Jarvis and Moore, 2010). The proportion of the *Butyrivibrio* group in total bacteria tended to be reduced as

GO supplementation in the rumen. This effect may be due to the wide spectrum of antibacterial activity of GO (Reuter et al., 1996), which consequently leads to changes in the number of bacteria.

This study revealed that the *Butyrivibrio* group bacteria are complex with considerable unknown species in goat rumen. In addition to the genus *Pseudobutyrvibrio*, *Butyrivibrio* and others within the family Lachnospiraceae in both libraries, some clones classified to the genus *Ruminococcus* and others within the family Ruminococcaceae were specifically found in the GO group. The family Lachnospiraceae includes the genus *Butyrivibrio*, *Lachnospira* and *Roseburia* (Cotta and Forster, 2006). *Butyrivibrio* isolates have high genetic diversity (Kopečný et al., 2003), in which the roles of *B. fibrisolvens* and *B. proteoclasticus* in biohydrogenation were investigated widely (Jenkins et al., 2008). Previous study found the concomitant disappearance of unknown Lachnospiraceae strains and a decrease in ruminal C18:0 concentration, suggesting that these unknown bacteria might play a role in *in vivo* C18:0 production (Boeckaert et al., 2008). However,

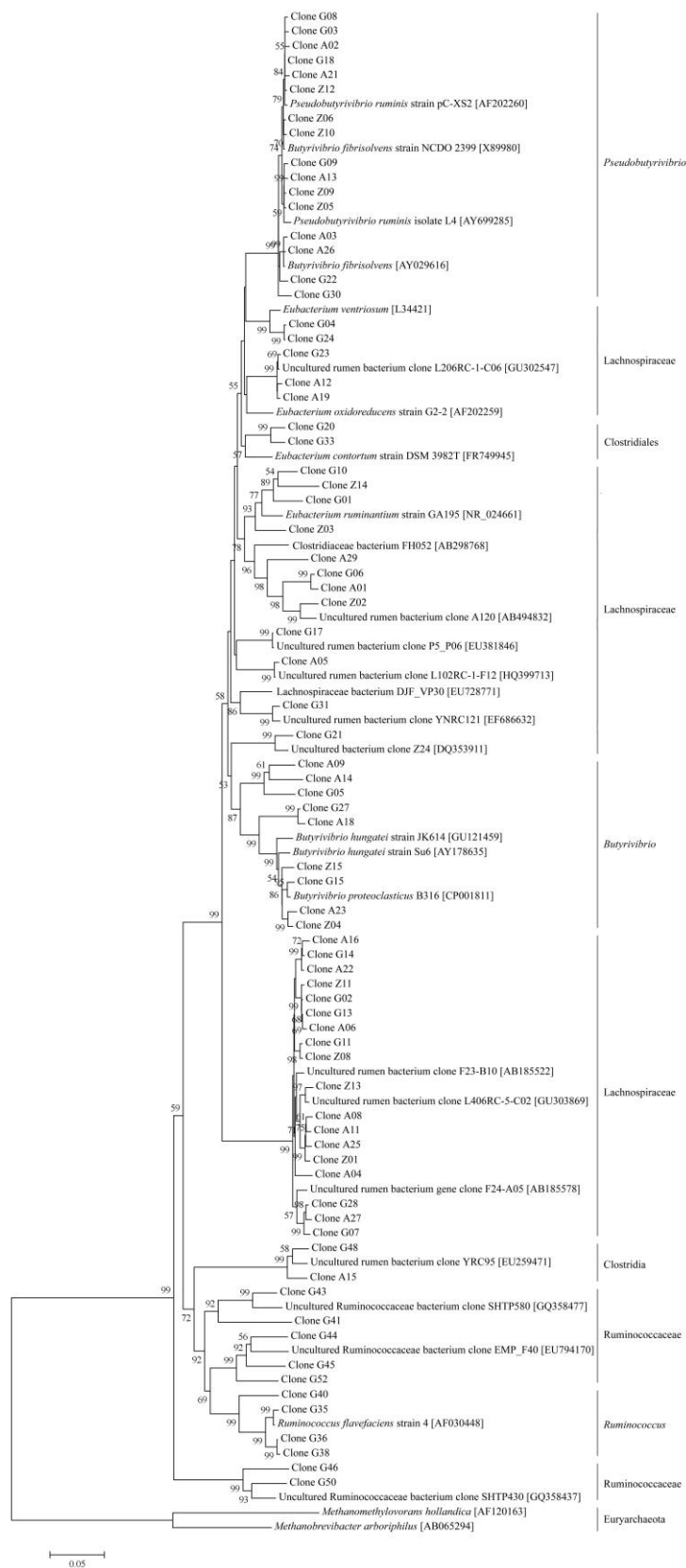


Figure 1. Phylogenetic tree of the *Butyrivibrio* group bacterial 16S rRNA gene clones from goat rumen. Clone A and clone Z are from control (HQ326588 to HQ326624), clone G (JN008400 to JN008437) are from goats treated by garlic oil infusion (0.8 g/d) via ruminal fistula. GenBank accession numbers of the known bacteria are shown in brackets. The bar represents 1 nt substitution per 100 nt.

putative taxonomic identification of terminal restriction fragments revealed that uncultured bacteria including those belonging to unclassified Lachnospiraceae were likely associated with TVA and *c9, t11*-CLA production (Huws et al., 2011). *Ruminococcus* has been implicated in biohydrogenation in the rumen as reviewed by Durmic et al. (2008). Additionally, recent research showed that the unclassified Ruminococcaceae, as shown by DGGE sequences, associated not only with the ruminal concentrations of TVA, *c9, t11*-CLA, but also with C18:0 (Huws et al., 2011). In the present phylogenetic tree, clones classified to the genus *Ruminococcus* and others within the family Ruminococcaceae and Lachnospiraceae formed genetically distinct clusters, indicating that their roles in biohydrogenation may be different from the genus *Pseudobutyrvibrio* and *Butyrvibrio*. However, to our knowledge, the actual roles of bacteria belonging to the genus *Ruminococcus* and others within the family Ruminococcaceae and Lachnospiraceae are not determined and need further investigation. Meanwhile, the vast majority of clone sequences had low similarity (<97%) with the closest known isolates in both libraries, suggesting that there were many yet unknown species within the *Butyrvibrio* group. The majority of microorganisms in the rumen have not been cultured (estimates range from 85% to 95%) (Krause et al., 2003). This partly explains the low similarity of cloned sequences with the known isolates in the present study.

In the complex community, the majority of clones resembled the known isolate *Ps. ruminis* with $\geq 97\%$ similarity in the *Butyrvibrio* group bacterial library. To our knowledge, Van Gylswyk et al. (1996) for the first time investigated the feature of *Ps. ruminis* from the rumen, and reported that *Ps. ruminis* differed sufficiently from the genus *Butyrvibrio* and was placed in a new genus. *Ps. ruminis* closely resembles *B. fibrisolvans* in many phenotypical characteristics (Van Gylswyk et al., 1996). Presently, clones closely resembling *Ps. ruminis* and *B. fibrisolvans* formed a tight and unique cluster (*Pseudobutyrvibrio*) in the phylogenetic tree, in which all the sequences were classified to the genus *Pseudobutyrvibrio*. *Ps. ruminis* may have a role similar to *B. fibrisolvans* in biohydrogenation. Nonetheless, there were differences between them. *Ps. ruminis* contains high medium chain FA (C12 to C15), whereas *B. fibrisolvans* contains high cellular compositions of long chain FA (C16 to C18) (Van Gylswyk et al., 1996; Kopečný et al., 2003). Afterwards, *Ps. ruminis* was reported to have high isomerase activity which converts the *cis*-12 bonds of linoleic acid (*c9, c12*-CLA) to *trans*-11 bonds (Koppová et al., 2006). Therefore, species resembling *Ps. ruminis* may contribute to the accumulation of *c9, t11*-CLA in the rumen. In addition, *B. fibrisolvans* is capable of hydrogenating

linoleic acid and forming *c9, t11*-CLA, and TVA as well (Polan et al., 1964; Kepler et al., 1966). Considering the higher proportion of *Ps. ruminis* in the libraries, *Ps. ruminis* may play a more important role than *B. fibrisolvans* in the biohydrogenation of linoleic acid to *c9, t11*-CLA.

The diversity of the *Butyrvibrio* group bacteria was modified by GO supplementation in the rumen as revealed by Shannon index. The reason behind this was not very clear. A previous report showed that high concentration (312 mg/L of culture fluid) of GO did not have detrimental effects on rumen microbial fermentation in a continuous culture trial (8 d), in contrast with the effects observed in a batch fermentation trial (24 h) (Busquet et al., 2005). Busquet et al. (2005) suggested that after longer adaptation, the microbial population inhibited by GO could be replaced by other resistant bacterial groups. This replacement might lead to modification of the *Butyrvibrio* group bacterial community.

Among the composition of bacteria, specific appearances of *B. fibrisolvans* and *R. flavefaciens* were observed in control and GO groups, respectively. *R. flavefaciens* is one of the most predominant fibrolytic bacteria in the rumen (Hespell et al., 1997). In the future, it is important to verify the roles of *R. flavefaciens* in the biohydrogenation in rumen. In the present study, the bacterial species (>97% similarity) resembling *B. fibrisolvans* and *R. flavefaciens* accounted for 0.70% and 0.86%, respectively, of total bacteria in goat rumen. A previous report showed that the proportion of 16S rRNA gene copies attributable to *B. fibrisolvans* and *R. flavefaciens* were <0.03% and 0.5% to 1%, respectively, of the total bacteria from the rumen of lactating cows, as investigated by relative quantification real-time PCR (Stevenson and Weimer, 2007). In addition to animal species, diets and methodology used were the possible reasons that caused this discrepancy in the proportion of *B. fibrisolvans* in total ruminal bacteria between the two experiments. The low proportion of *B. fibrisolvans* in the rumen as reported by Stevenson and Weimer (2007) may lead to its unlikely selection from plasmids in the total bacterial library. Owing to the antibacterial activity (Reuter et al., 1996), the possible inhibition of GO on *B. fibrisolvans* may also partly illustrate the disappearance of *B. fibrisolvans* in GO group library. For the lack of appearance of *R. flavefaciens* in control library, the report that bacteriocins produced by *B. fibrisolvans* inhibited ruminococci growth (Krause et al., 1999) may in part explain this phenomenon.

In conclusion, this study revealed that the *Butyrvibrio* group represented 12.98% of total bacteria the rumen of goats. Many unique unknown bacterial sequences were found in the *Butyrvibrio* group bacterial library. Nevertheless, the roles of these unknown bacteria need

further investigation.

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