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Effects of propylene glycol on in vitro ruminal fermentation, methanogenesis, and microbial community structure

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ABSTRACT

We evaluated the effects of propylene glycol (PG) on in vitro ruminal fermentation, methanogenesis, and microbial community structure. A completely randomized design was conducted in the in vitro incubation, and 4 culture PG dose levels (0, 7.5, 15, and 22.5 μL/g of dry matter) were used in the trial. Based on the fermentation results, the control group (0 μL/g of dry matter, CON) and the second treatment group (15.0 μL/g of dry matter, TRT) were chosen for further analysis to explore the effects of PG on the bacterial and archaeal community structure. The concentrations of propanol, propanal, and succinate increased linearly, whereas the concentration of L-lactate decreased linearly as PG doses increased. The molar proportion of propionate demonstrated a linear increase with increasing PG doses. In contrast with propionate, the molar proportion of acetate and butyrate, and acetate-to-propionate ratio decreased linearly with increasing PG doses. The addition of PG markedly decreased methane production without negative effects on nutrient degradability. In the archaeal level, the relative abundance of *Methanobrevibacter* tended to decrease, but that of *Methanomassiliicoccus* significantly increased in TRT group. At the bacterial level, the relative abundance of *Bacteroidetes* and *Prevotella* in TRT group was numerically higher than that in CON group. The analysis of the *Negativicutes* class showed that the relative abundance of *Succiniclasticum* tended to increase, whereas that of *Selenomonas* tended to decrease in TRT group. These results demonstrated that PG might be used as an inhibitor to mitigate methane emission. However, the small decrease in methane production will limit the application of PG as a methane inhibitor in production practices. Further research is needed to determine whether use together with other inhibitors may improve the effects of PG on the utilization of reducing equivalents ([H]) and methane production.

Key words: propylene glycol, propionate formation, methanogenesis, bacterial community, archaeal community

INTRODUCTION

Propylene glycol (**PG**) has served as a glucogenic precursor in the early lactation of dairy cows to diminish the negative energy balance and treat ketosis since 1954 (Johnson, 1954). It efficiently treats ketosis by decreasing blood concentrations of free fatty acids and BHB, and PG was more effective to produce propionate and increase plasma glucose concentration than other precursors, such as glycerol (Piantoni and Allen, 2015; Ferraro et al., 2016). There are 2 metabolic fates for PG in the rumen: it is absorbed intact or fermented into volatile components and absorbed by rumen wall. Emery et al. (1967) suggested that PG was mainly absorbed intact and rarely fermented into propionate in the rumen, based on a study with a single cow. However, in vitro incubation studies indicated that apart from propionate, propanal, and propanol were also important products or intermediates during PG fermentation (Czerkawski and Breckenridge, 1973; Czerkawski et al., 1984). These results were also observed in similar studies that were conducted in other ecosystems (Veltman et al., 1998; Driehuis et al., 1999). Kristensen et al. (2002) reported that PG has a low rate of metabolism in cows from a study under washed rumen conditions. Another similar study conducted by Kristensen and Raun (2007) confirmed that the hepatic extraction of PG was relatively low, and PG was extensively fermented into volatile components, such as propanal and propanol, but not propionate. These observations indicated that ruminal fermentation was of considerable importance in cattle, and rumen microbiota were responsible for the majority of PG metabolism in cattle.

Methanogenesis in the rumen results in a significant influence on greenhouse gas emission and represents a direct energy loss of 2 to 12% for animals with different

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feedstuffs (Johnson and Johnson, 1995). Redirecting reducing equivalents ([H]) from methane (CH_4) toward other electron sinks that are nutritional to animals is an excellent CH_4 mitigation strategy, which not only decreases the loss of digestible energy but also avoids fermentation inhibition (Lan and Yang, 2019). Propanal is the intermediate during PG fermentation, and it will serve either as an electron donor oxidized to propionate or as an electron acceptor reduced to propanol (Veltman et al., 1998). Kristensen and Raun (2007) suggested that a larger proportion of propanal might be metabolized to propanol and thereby used as an electron sink in the rumen, based on an in vivo study. Propanol has been confirmed to be an important product during PG fermentation (Czerkawski et al., 1984; Kristensen and Raun, 2007), and propanol taken up by the rumen wall is oxidized to propionate in the liver; this endogenously produced propionate is further available for gluconeogenesis (Kristensen and Raun, 2007). Although numerous PG studies have been conducted in dairy cows, only a few studies have reported the effects of PG on methanogenesis, and no studies have measured changes in the structure of microbial community in response to the addition of PG in the diet of dairy cows. Czerkawski and Breckenridge (1972) reported that PG competed with the methanogenesis pathway for [H] and resulted in small but consistent inhibition of CH_4 production. However, Costa et al. (2020) reported the contradictory results that PG has no beneficial effects in mitigating sheep CH emission. Therefore, the objectives of this study were to explore the effects of PG on in vitro ruminal fermentation, methanogenesis, and to focus on changes in the bacterial and archaeal community structure by high-throughput sequencing.

MATERIALS AND METHODS

Treatment and Incubation

The experimental procedures were approved by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China). Rumen fluid was sampled 2 h before feeding from 3 cannulated lactating Holstein cows fed a TMR. The chemical composition of TMR was shown in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.13487151.v1). Ruminal fluid was filtered through 4 layers of cheesecloth under continuous flushing with CO_2 and was brought to the laboratory within 30 min. Equal volumes of ruminal fluid from different donor cows were mixed as the inoculum and then diluted with buffer solution (1:2 vol/vol), which was formulated as described by Menke (1988). A completely randomized design was conducted in the in vitro incubation, and 4 culture PG dose levels (0, 7.5, 15, and 22.5 μL/g of DM) were used in the trial. In the present study, 120-mL serum bottles were used as incubation vessels. Each incubation vessel contained 0.5 g of fermentation substrate with 0, 3.75, 7.50, or 11.25 μL of PG and 75 mL of inoculum-buffer mixed fluid. All incubation vessels were swirled gently by hand to mix the substrate and liquid. Then vessels were flushed with CO_2 and sealed with butyl rubber stoppers connected to vacuumed airbags to collect gases during incubation. The concentrations of PG in the in vitro cultures at the beginning of the experiment were 0, 0.68, 1.36, and 2.04 mmol/L, respectively. All cultures were incubated at 39°C for 24 h with horizontal shaking at 60 rpm. The fermentation substrate was the same as the TMR fed to donor cows, which was dried at 55°C for 48 h and ground through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA). The PG (>99.5% wt/wt with density of 1.036 g/mL) was purchased from Sigma-Aldrich (St. Louis, MO). The in vitro incubation was carried out in 3 runs, and 3 replicates per treatment were used in each run. In addition, an extra 3 blank replicates (inoculum-buffer mixed fluid only) without substrate were used to correct the analytes and gases. Each run was carried out one after another.

Sample Collection and Analysis

The incubation of cultures was stopped at 24 h, and all vessels were placed in an ice bath to terminate the incubation. Calibrated glass syringes (100 mL, Häberle Labortechnik, Lonsee-Ettlenschieß, Germany) were used to measure the total gas production of each airbag. The pH of all vessels was measured via the Seven Go portable pH meter (Mettler Toledo, Switzerland). The preweighed nylon bags (8 × 12 cm, 42 μm) were used to filter the whole biomass material of each bottle. The filtrate samples were collected to determine fermentation products and microbial analysis. The samples for determining fermentation products were frozen at −20°C, and the samples for microbial analysis were frozen immediately in liquid nitrogen and then stored at −80°C. Cold running water was used to wash the nylon bags until the effluent was clear. Afterward, the nylon bags were dried at 55°C for 48 h before analysis of the apparent disappearance of DM, NDF, and ADF. The contents of NDF and ADF were measured using the fiber analyzer (A200, Ankom Technology, Macedon, NY) according to the method described by Van Soest et al. (1991). Sodium sulfite and α-amylase were used for the analysis of NDF. The concentrations of CH_4 and H_2 in each airbag were determined using the Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a packed column (1 m

× 2 mm × 3.175 mm; Porapak Q, Agilent Technologies) and a thermal conductivity detector. The VFA concentrations were measured by the Agilent 7890B gas chromatograph (Agilent Technologies) equipped with a capillary column (30 m × 0.250 mm × 0.25 μm; BD-FFAP, Agilent Technologies) and a flame ionization detector. The detailed methods of determining gases and VFA were conducted as described by Wang et al. (2018). The concentrations of propanal and propanol were measured using the method described by Kristensen et al. (2007). The L-lactate was determined using a kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and based on the method of lactate dehydrogenase. Succinate was measured using a succinate colorimetric assay kit purchased from Sigma-Aldrich.

DNA Extraction and Bacterial and Archaeal 16S rRNA Genes Sequencing and Analysis

Total DNA was extracted using an EZNA Mag-Bind Soil DNA kit (Omega, Norcross, GA), and the quality and concentration of microbial DNA were evaluated by using 1% agarose gel electrophoresis and a Qubit 3.0 spectrometer (Invitrogen, Carlsbad, CA), respectively. Henderson et al. (2013) suggested that kit-based methods were usually poor for extracting representative DNA from rumen fluid samples. The average concentration of extracted DNA in the present study was 48.2 ng/μL, which was sufficient for further analysis following the technician's suggestion. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were selected for bacterial community analysis (Caporaso et al., 2011). The abundance of the archaeal community was much lower than that of the bacterial community, so the archaeal community was analyzed using nested PCR. The process of nested PCR was conducted using the primers Arch340F (5′-CCCTAYGGGGYGCASCAG-3′)/ Arch1000R (5′-GAGARGWRGTGCATGGCC-3′) and Arch349F (5′-GYGCASCAGKCGMGAAW-3′)/ Arch806R (50-GGACTACVSGGGTATCTAAT-3′), as previously described by Wang et al. (2018). Finally, the purified amplicons were pooled in equimolar ratios and subjected to pair-end sequencing using the Illumina MiSeq platform (2 × 300 bp). In the present study, only 2 dose levels (0 and 15.0 μL/g of DM) were chosen for microbial analysis.

Sequencing reads were assigned to different samples based on their unique barcode. Paired-reads from the original DNA fragments were merged by FLASH (Magoč and Salzberg, 2011), and then quality control of these merged reads was conducted by using PRINSEQ (Schmieder and Edwards, 2011). The barcode and primers sequences were removed, and PCR chimeras were filtered using UCHIME algorithm (Edgar et al., 2011). After the removal of singletons, operational taxonomic units (**OTU**) were clustered at 97% sequence identity by UPARSE (Edgar et al., 2011). The taxonomic classification of the sequences was carried out using the RDP classifier at the bootstrap cutoff of 80%. Bacterial sequences were aligned with the SILVA (version 138) database and archaeal sequences were aligned with RDP database (version 11.5) at the confidence threshold of 70%. The α diversity indices were calculated by using the QIIME 2 software package. The bioinformatics software STAMP (https://beikolab.cs.dal.ca/software/STAMP) was used to visualize the relative abundance difference of bacteria and archaea by extended error bar plot (Parks et al., 2014). All the raw sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/), under accession number SRP198193.

Statistical Analysis

All data were checked to ensure normal distribution, and some data were transformed to $\log_{10}(n+1)$ if necessary. The in vitro fermentation variables, the apparent disappearance of nutrient, and α diversity index of bacteria and archaea were analyzed using PROC MIXED of SAS 9.4 (SAS Institute, Inc., Cary, NC) as shown in the following model:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean, A_i is the effect of treatment (considered fixed), B_j is the effect of run (j = 1, 2, 3, considered fixed), AB_{ij} is the interaction between A_i and B_j (considered fixed), and e_{ijk} is the residual. Polynomial contrasts were used to test the linear and quadratic effects of treatments. The fixed effect of run and the interaction of treatment and run that were not significant were dropped from the model, and the reduced model run again. Differences were declared significant at $P < 0.05$, and a tendency of difference was declared at $0.05 \leq P < 0.10$.

RESULTS

Data on pH, nutrient degradability, products of fermentation, the proportion of VFA, and CH_4 and H_2 production are presented in Table 1. Based on the previous study that PG was metabolized after 24 h in

ruminal cultures in vitro, we terminated the incubation process at 24 h (Trabue et al., 2007). The concentrations of propanol, propanal, and succinate increased linearly ($P < 0.01$), whereas the concentration of L-lactate decreased linearly as PG doses increased ($P < 0.01$). The molar proportion of propionate demonstrated a linear ($P < 0.01$) increase with increasing PG doses. In contrast with propionate, the molar proportion of acetate and butyrate and the acetate-to-propionate ratio demonstrated a linear ($P < 0.01$, $P = 0.019$, and $P < 0.01$) decrease with increasing PG doses. Methane production was linearly decreased ($P = 0.045$), whereas H_2 detected in the gas phase was unaffected when the fermentation substrate was supplemented with increasing dose levels of PG. The apparent disappearance of DM, NDF, ADF, and pH were not significantly affected in response to the addition of PG.

Changes of Microbial Community Structure in Response to PG

Based on the results of ruminal fermentation characteristics and gas production (Table 1), the molar proportion of acetate, propionate, acetate-to-propionate ratio, and CH_4 production in the second treatment group (15.0 μL/g of DM) was significantly ($P < 0.05$) different from that in the control group (0 μL/g of DM), but was similar to that in the third treatment group (22.5 μL/g of DM). In the present study, the PG dose level in the second treatment group (15.0 μL/g of DM) was the best dose to affect ruminal fermentation in vitro. Therefore, the control group (0 μL/g of DM, **CON**) and the second treatment group (15.0 μL/g of DM, **TRT**) were chosen for further analysis to explore the effects of PG on the bacterial and archaeal community structure by high-throughput sequencing.

Bacteria

At the bacterial phylum level, *Bacteroidetes* and *Firmicutes* were the dominant phyla, representing 51.52 and 27.23% of the total sequences, respectively. *Proteobacteria*, *Actinobacteria*, *Patescibacteria*, and *Verrucomicrobia* represented average percentages of 13.26, 3.37, 1.56, and 1.29%, respectively (Supplemental Figure S1, https://doi.org/10.6084/m9.figshare.13487151.v1). At the bacterial genus level, the 10 predominant genera were *Prevotella* (23.02%), *Rikenellaceae_RC9_gut_group* (11.66%), *Ruminobacter* (11.14%), *Christensenellaceae_R-7_group* (3.3%), *Olsenella* (3.17%), *Pseudobutyrivibrio* (2.51%), *Prevotellaceae_UCG-003* (2.48%), *NK4A214_group* (2.36%), *Succiniclasticum* (1.74%), and *Succinivibrionaceae_UCG-002* (1.27%; Supplemental Figure S2, https://doi

Table 1. The in vitro fermentation pH, nutrient digestibility, products of fermentation, the proportion of VFA, and gas production for fermenters used a control fermentation substrate with increasing dose levels of propylene glycol (PG; n = 9)

Item[1]	PG dose[2] (μL/g of DM)				SEM	P-value[3]	
	0	7.5	15.0	22.5		L	Q
pH	6.72	6.71	6.71	6.72	0.004	0.780	0.446
DMD,[3] %	65.1	67.6	67.1	67.6	0.01	0.325	0.521
NDFD,[4] %	59.5	56.5	56.2	56.0	0.01	0.201	0.574
ADFD,[5] %	56.3	52.8	52.8	52.3	0.01	0.271	0.613
Propanol, mM	0.13[d]	0.53[c]	0.97[b]	1.59[a]	0.092	<0.01	<0.01
Propanal, mM	0.00[d]	0.13[c]	0.16[b]	0.30[a]	0.018	<0.01	0.317
L-Lactate, mM	0.35[a]	0.30[a]	0.26[b]	0.22[c]	0.009	<0.01	0.343
Succinate, mM	0.16[c]	0.19[b]	0.22[b]	0.27[a]	0.008	<0.01	0.128
Total VFA, mM	70.1	70.1	71.3	71.3	0.44	0.230	0.987
Individual, mol/100 mol							
Acetate	64.5[a]	63.7[b]	63.1[b]	62.2[c]	0.15	<0.01	0.878
Propionate	22.8[c]	23.8[b]	24.6[ab]	25.7[a]	0.20	<0.01	0.974
Isobutyrate	0.6	0.6	0.6	0.6	0.01	0.823	0.798
Butyrate	10.1[a]	9.9[ab]	9.7[b]	9.5[b]	0.08	0.019	0.980
Isovalerate	1.1	1.1	1.0	1.0	0.01	0.879	0.635
Valerate	0.9	0.9	0.9	0.9	0.01	0.635	0.553
Acetate/propionate	2.85[a]	2.71[b]	2.56[bc]	2.43[c]	0.031	<0.01	0.962
Total gas production (mL)	78.9	87.6	75.0	82.0	2.91	0.897	0.891
gH_2 (mL)	0.09	0.09	0.09	0.10	0.005	0.414	0.293
CH_4 (mL)	7.23[a]	6.51[ab]	6.28[b]	6.34[b]	0.049	0.045	0.438

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]DMD = apparent disappearance of DM; NDFD = apparent disappearance of NDF; ADFD = apparent disappearance of ADF; gH_2 = hydrogen detected in the gas phase.

[2]Data were analyzed using PG dose levels of 0, 7.5, 15, and 22.5 μL/g DM.

[3]L = linear; Q = quadratic.

.org/10.6084/m9.figshare.13487151.v1). The relative abundance of most phyla and genera was not significantly affected when the fermentation substrate was supplemented with PG. The relative abundance of *Bacteroidetes* in TRT group was numerically higher than that in CON group (52.45 vs. 50.58%, $P = 0.471$; Figure 1). The relative abundance of *Prevotella* in TRT group was numerically higher than that in CON group (25.17 vs. 21.06%, $P = 0.269$; Figure 2). To further study changes in the bacterial community structure, differences in the relative abundance of individual OTU belonging to *Prevotella* were tested using the bioinformatics software (STAMP). The relative abundance of 2 dominant OTU belonging to *Prevotella* in TRT group was numerically higher than that in CON group. The relative abundance of OTU1314 belonging to *Prevotella* in TRT group significantly increased ($P = 0.034$) in response to the addition of PG (Figure 3a). The *Negativicutes* class of *Firmicutes* was found to be correlated with propionate formation from carbohydrates (Reichardt et al., 2014). Differences in the relative abundance of genera belonging to *Negativicutes* class were tested using the bioinformatics software (STAMP). In *Negativicutes* class, the relative abundance of *Succiniclasticum* tended to increase ($P = 0.052$), whereas that of *Selenomonas* tended to decrease ($P = 0.085$) after PG supplementation (Figure 3b). Alpha diversity indices of the bacterial community were presented in Table 2. No significant differences were observed between treatments based on the α diversity indices of coverage, Chao1, ACE, Shannon, and Simpson, showing that the bacterial community richness and diversity were not affected by the addition of PG.

Archaea

At the archaeal phylum level, Euryarchaeota was the dominant phylum, representing nearly100% of the total sequences. At the archaeal genus level, *Methanomassiliicoccus* (67.15%), *Methanobrevibacter* (29.90%), *norank_f__Methanobacteriaceae* (1.35%), *Methanomicrobium* (0.91%), and *Methanosphaera* (0.53%; Supplemental Figure S3, https://doi.org/10.6084/m9.figshare.13487151.v1) were the 5 predominant genera. The relative abundance of *Methanomassiliicoccus* significantly increased ($P = 0.044$), whereas that of *Methanobrevibacter* tended to decrease ($P = 0.072$), and that of *Methanosphaera* significantly decreased ($P = 0.011$) after PG supplementation (Figure 4). Alpha diversity indices of the archaeal community were presented in Table 2. The α diversity indices of Chao1 ($P = 0.018$) and ACE ($P = 0.007$) were significantly decreased in response to the addition of PG, showing that PG changed the archaeal community richness. The α diversity index of Simpson was significantly increased ($P = 0.023$), and Shannon index tended to decrease ($P = 0.067$) in TRT, indicating the PG affected the diversity of archaeal community.

DISCUSSION

Studies from sheep or cows indicated that the main products of fermentation of PG were propanol and propionate, and to a lesser extent, propanal (Czerkawski and Breckenridge, 1973; Kristensen and Raun, 2007). Propylene glycol has a significant effect on rumen fermentation pattern, causing an increase in the molar

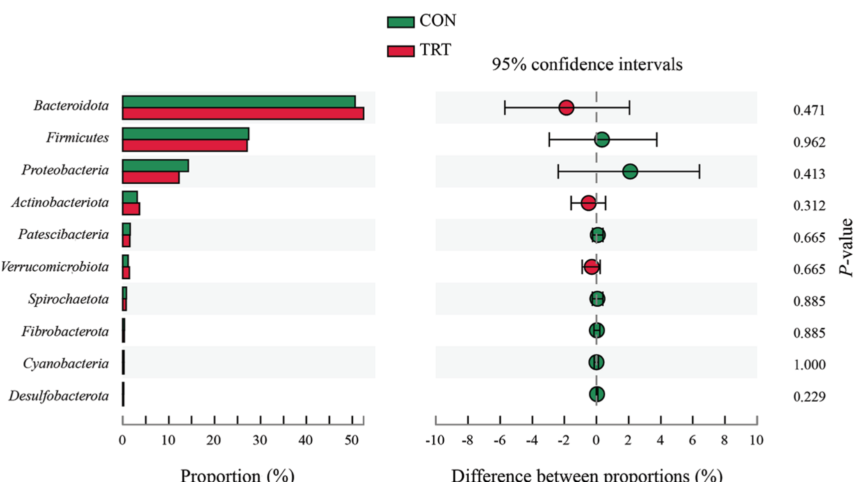

Figure 1. Differences in the relative abundance of the predominant bacterial phyla. The propylene glycol dose for the control group (CON) was 0 μL/g of DM; the second treatment group (TRT) was 15.0 μL/g of DM.

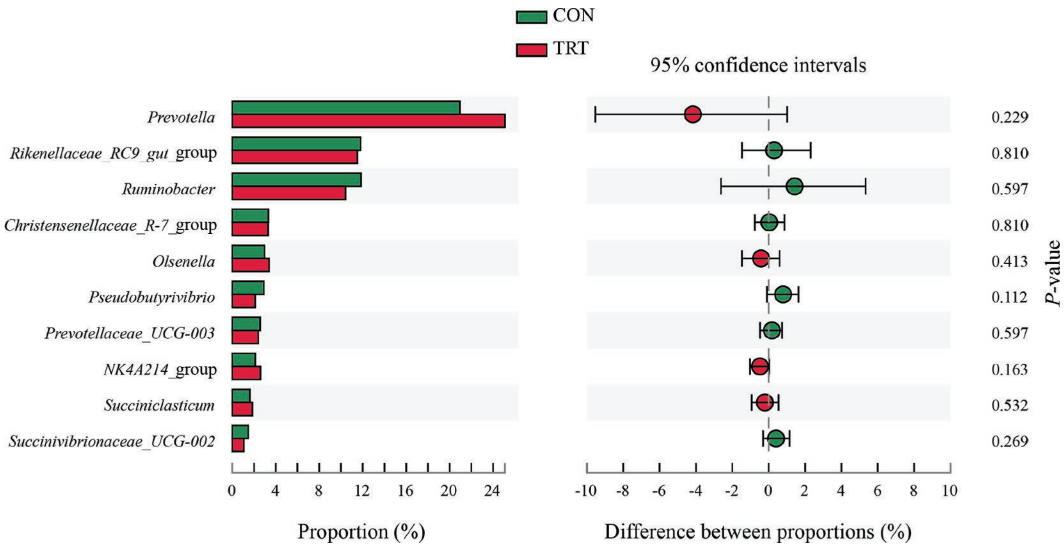

Figure 2. Differences in the relative abundance of the predominant bacterial genera. The propylene glycol dose for the control group (CON) was 0 μL/g of DM; the second treatment group (TRT) was 15.0 μL/g of DM.

proportion of propionate and a decrease in the acetate/propionate molar ratio. Numerous studies have shown significant higher proportion of propionate in the rumen in response to the use of PG (Nielsen and Ingvartsen, 2004). Effects of PG on butyrate were not consistent; Trabue et al. (2007) and Ferraro et al. (2016) have reported that the addition of PG increased the proportion of propionate and decreased butyrate in ruminal cultures in vitro, which was similar to our results. Christensen et al. (1997) and Shingfield et al. (2002) have found no change in the proportion of butyrate in the rumen response to the use of PG. Different dietary components used in different studies may be responsible for the different VFA results. The observed similarity in pH among treatments was consistent with the observed similarity in total VFA concentrations among treatments in the present study. The formation of propionate is one of the important [H] disposal pathways in the rumen, and the increase in ruminal propionate proportion is stoichiometrically associated with a reduction in methanogenesis. The formation of acetate and butyrate results in more [H] production, whereas propionate formation is an alternative pathway for [H] use in the rumen, accompanied by a decline in CH_4 production (Moss et al., 2000). A negative correlation between propionate formation and CH_4 production was reported by Janssen (2010). Although propionate formation is an alternative pathway to dispose excess [H] and the thermodynamically favored pathway under high H_2 pressures (Janssen, 2010), the relation between propionate formation and methanogenesis in the rumen is not clear. As reported by Melgar et al. (2020), inhibition of rumen methanogenesis resulted from 3-nitrooxypropanol increased the hydrogen emission and did not change the molar proportion of propionate, suggesting a possible redirection of [H] to alternative metabolic pathways. Ungerfeld (2015) reported that inhibition of methanogenesis led to the redirection of [H] toward propionate and H_2 rather than butyrate, and a substantial proportion of the reducing equivalents were still unaccounted for in other fermentation products. There are 3 known pathways for propionate production: succinate pathway, acrylate pathway, and PG pathway. During the formation of propionate, the succinate pathway and acrylate pathway could provide alternative electron sinks to competitively inhibit methanogenesis in the rumen. Strictly speaking, the PG pathway could not provide alternative electron sinks to compete [H] with methanogenesis in the rumen. However, as an intermediate during PG fermentation, propanal can be used as an electron sink, and it will be either an electron donor oxidized to propionate or an electron acceptor reduced to propanol. Veltman et al. (1998) reported that decomposition of PG in wastewater digesters resulted in the same production of propanol and propionate. Kristensen and Raun (2007) suggested that a larger fraction of propanal might be diverted to propanol and used as an electron sink in the rumen. Similarly, the observed net sums of propanol and propanal for each treatment (0.53, 1.00, and 1.76 m*M*) in the present study were very close to the concentrations of PG added for each level of treatment (0.68, 1.36, and 2.04 m*M*), which suggested that most PG was metabolized to these 2 compounds, leaving just a little PG for con-

version to propionate. Perhaps the fact that most of the PG ended up in propanol plus propanal explained why the total VFA concentration did not significantly differ among treatments. In the present study, CH_4 production significantly decreased in TRT group, but a small decrease of 13.14% made it an imperfect inhibitor of CH_4 production. In agreement with our results, Czerkawski and Breckenridge (1972) reported that PG can successfully compete with the methanogenesis pathway for [H] and resulted in small but consistent inhibition of CH_4 production. In the present study, the fermentation of PG produced more propanol and less propionate, which means that more of the intermediate product propanal could be used as an electron acceptor to competitively inhibit methanogenesis. This should be one reason that a small decrease in CH_4 production was observed in the present study when the fermentation substrate was supplemented with PG. In addition, the significant increase of succinate concentration and the significant decrease of L-lactate concentration were observed in the present study. These results indicated that the change of fermentation pattern resulting from PG metabolism affected the other 2 propionate production pathways. The enhanced succinate pathway could provide alternative electron sinks to competitively inhibit methanogenesis. This should be another reason that the addition of PG in fermentation substrate decreased the CH_4 production. In fact, compared with the control group, the extra propionate production for each treatment (0.70, 1.56, and 2.34 m*M*) was more than

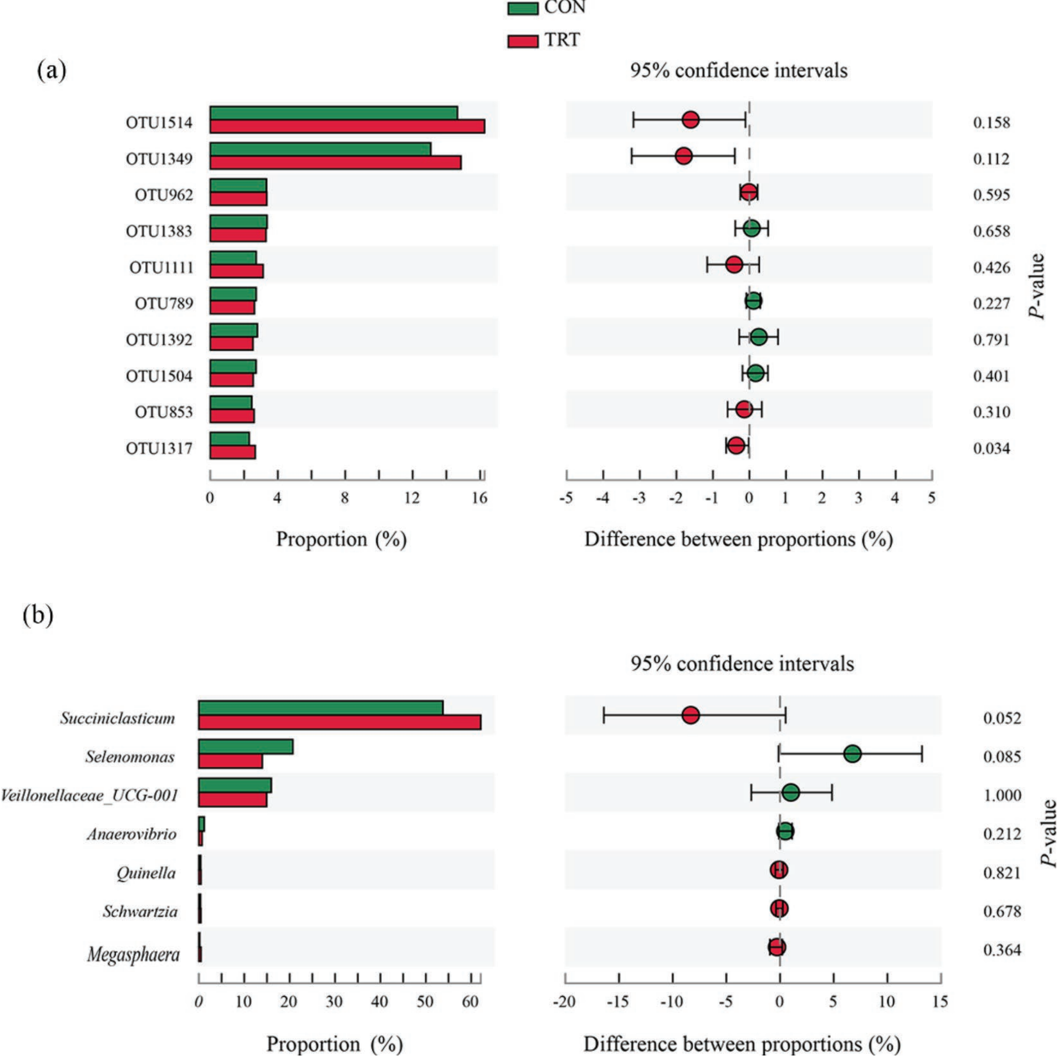

Figure 3. Changes in specific bacterial community structure. (a) The difference in the relative abundance of operational taxonomic units (OTU) in *Prevotella*. (b) The difference in the relative abundance of genera in the *Negativicutes* class. The propylene glycol dose for the control group (CON) was 0 μL/g of DM; the second treatment group (TRT) was 15.0 μL/g of DM.

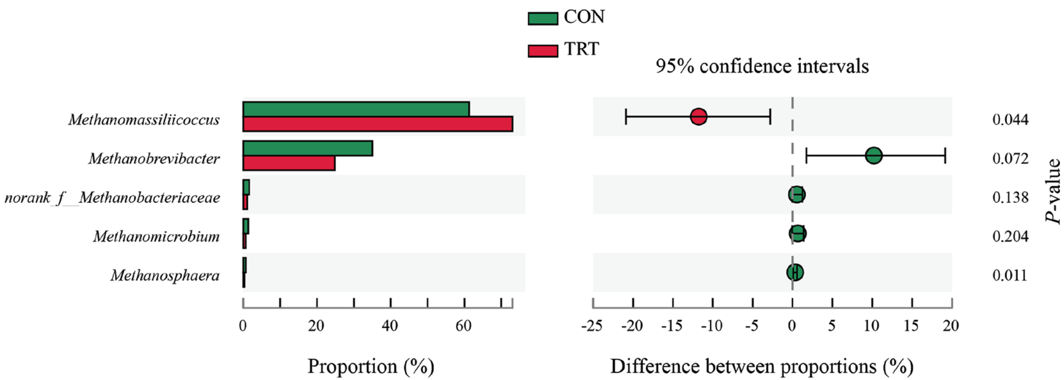

Figure 4. Differences in the relative abundance of the predominant archaeal genera. The propylene glycol dose for the control group (CON) was 0 μL/g of DM; the second treatment group (TRT) was 15.0 μL/g of DM.

that from the maximum PG conversion for each level of treatment (0.15, 0.36, and 0.28 m*M*). Consistent with our results, a decrease of L-lactate concentration was also observed in the PG study conducted by Kristensen and Raun (2007), but the succinate concentration was not reported. Similarly, Czerkawski and Breckenridge (1973) reported that the dissimilation of PG by rumen microbes resulted in an increased intake of hydrogen, and [H] appeared to be better utilized than gaseous hydrogen.

Many microbial species belonging to the *Firmicutes* are good H_2 producers, such as *Ruminococcus* and *Eubacterium*. In contrast, *Bacteroidetes* were commonly considered to be net H_2 utilizers (Lan and Yang, 2019). Belanche et al. (2016) reported that chitosan decreased the CH_4 production and increased the proportion of propionate in ruminal cultures in vitro, partly achieved by substitution of *Firmicutes* by *Bacteroidetes*. Wang et al. (2018) also reported that a modified dietary formulation strategy trended to decrease the CH_4 production and increased the proportion of propionate by substitution of *Firmicutes* by *Bacteroidetes*. *Prevotella* spp. are usually considered as the main bacterial genus represented in the rumen, with many different species observed (Kim et al., 2011). *Prevotella* spp. can degrade starch, hemicellulose and protein by producing a variety of extracellular degradative enzymes (Stevenson and Weimer, 2007). *Prevotella* species produced different fermentation end products due to the different enzymes produced by different species. *Prevotella albensis* produced acetate, but some of *Prevotella* species produced propionate as the fermentation end product, including *Prevotella brevis*, *Prevotella bryantii* and *Prevotella ruminicola* (Emerson and Weimer, 2017). *Prevotella copri* produced succinate as the main fermentation product and the accumulation of succinate has been observed in the rat gut (De Vadder et al., 2016). The function of different *Prevotella* species varies greatly, some of which are related to a high CH_4 production phenotype (Kittelmann et al., 2014), whereas others are related to a low CH_4 production phenotype (Danielsson et al., 2017). In the present study, comparison at genus level of *Prevotella* did not reveal a significant difference between CON group and TRT group. To find an explanation for the difference in CH_4 production in the present study, the bacterial composition of *Prevotella* genus and the *Negativicutes* class was examined more in detail. The analysis of *Prevotella* genus showed that the relative abundance of predominant OTU belonging to *Prevotella* was higher in TRT group, but not significantly increased. These predominant OTU belonging to *Prevotella* in the present study may be correlated with the propionate formation.

Species in the *Negativicutes* class have been proved to participate in the propionate formation by the acrylate pathway or the succinate pathway (Reichardt et al., 2014). The analysis of *Negativicutes* class showed that

Table 2. Alpha diversity indices of bacteria and archaea among treatments in vitro (n = 9)

Item[1]	PG dose[2] (μL/g of DM) 0	15	SEM	*P*-value
Bacteria				
Coverage	0.99	0.99	0.001	0.316
Chao1	1,431	1,413	10.8	0.428
ACE	1,421	1,405	8.0	0.336
Shannon	5.68	5.59	0.038	0.269
Simpson	0.01	0.01	0.001	0.572
Archaea				
Coverage	1.00	1.00	0.001	0.189
Chao1	769	623	32.2	0.018
ACE	1,369	1,054	62.5	0.007
Shannon	1.98	1.79	0.053	0.067
Simpson	0.26	0.33	0.018	0.023

[1]ACE = abundance-based coverage estimator.

[2]PG = propylene glycol; data were analyzed using PG dose levels of 0 and 15 μL/g of DM.

the relative abundance of *Succiniclasticum* tended to increase, whereas that of *Selenomonas* tended to decrease in TRT group. The same changes of the relative abundance of *Succiniclasticum* and *Selenomonas* with our study were observed in a similar study that explored the effect of calcium propionate on rumen microbiota (Cao et al., 2020). *Succiniclasticum* could ferment succinate to propionate (Van Gylswyk, 1995), so its high relative abundance in the rumen generally indicated the large production of succinate. And the high relative abundance of *Succiniclasticum* was commonly observed in dairy cows fed high levels of concentrate or corn (Petri et al., 2013; Bi et al., 2018). *Selenomonas* could utilize lactate to produce propionate (Paynter and Elsden, 1970), so its relative abundance in the rumen was generally related to the production of lactate. A previous study reported that *Selenomonas ruminantium* alone fermented lactate to propionate, acetate, and CO_2, but coculture with methanogen caused a significant decrease in the production of propionate and an increase in acetate formed from lactate (Chen and Wolin, 1977). The changes of the relative abundance of *Succiniclasticum* and *Selenomonas* in the present study further confirmed that the change of fermentation pattern resulting from PG metabolism affected the other 2 propionate production pathways, and the enhanced succinate pathway could provide alternative electron sinks to competitively inhibit methanogenesis.

Methanogenic archaea most commonly use H_2 and CO_2 as substrates for methanogenesis, but some species can also metabolize formate, methanol, or acetate to produce CH_4. Hydrogenotrophic pathway and methylotrophic pathway are commonly considered as the 2 major pathways of methanogenesis in the rumen. The genus *Methanobrevibacter* is considered to be the most common hydrogenotrophic archaea, which produces 1 mole of CH_4 for each mole of CO_2 by hydrogenotrophic pathway (Hook et al., 2010). The genus *Methanomassiliicoccus* utilizes methylamine substrates to generate CH_4 through H_2-dependent methylotrophic pathway (Moissl-Eichinger et al., 2018). Some methanogens present inside and on the surface of protozoa utilize hydrogen produced by protozoa to improve the efficiency of methanogenesis (Embley et al., 2003). *Methanobrevibacter* is commonly the predominant protozoa-associated methanogens, which can encode a kind of sugar protein to attach on the surface of protozoa (Belanche et al., 2014). A positive correlation between the relative abundance of *Methanobrevibacter* and CH_4 formation has been found by Zhou et al. (2011) and Danielsson et al. (2012). The relationship between *Methanomassiliicoccus* and protozoa has not been reported in published literatures. Comparisons of the pathways for CH_4 formation by *Methanobrevibacter* and *Methanomassiliicoccus* indicated that *Methanobrevibacter* might be more effective than *Methanomassiliicoccus* in methanogenesis. In our previous study, we also found that nonforage fiber sources would mitigate CH_4 emission by decreasing the relative abundance of *Methanobrevibacter* and increasing that of *Methanomassiliicoccus* at the archaea level (Wang et al., 2018). In the present study, the addition of PG in fermentation substrate may reduce CH_4 production by changing the structure of archaeal community, increasing the relative abundance of *Methanomassiliicoccus* and decreasing that of *Methanobrevibacter*.

CONCLUSIONS

In the present study, we showed that the fermentation of PG produced more propanol and less propionate, which meant that more of the intermediate product propanal could be used as an electron acceptor to competitively inhibit methanogenesis. In addition, the change of fermentation pattern resulting from PG metabolism affected the other 2 propionate production pathways, and the enhanced succinate pathway could provide alternative electron sinks to competitively inhibit methanogenesis. A small decrease (13.14%) in CH_4 production was observed in the present study when the fermentation substrate was supplemented with PG. Changes in the bacterial and archaeal community structure may be responsible for these results. Propylene glycol may not be a perfect inhibitor of CH_4 production. However, if the methanogenesis pathway is inhibited by some other inhibitors, the use of PG might be beneficial by improving the utilization of [H] associated with inhibition.

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