



Effects of oregano essential oil on in vitro ruminal fermentation, methane production, and ruminal microbial community

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ABSTRACT

Different inclusion rates of oregano essential oil (OEO) were investigated for their effects on ruminal in vitro fermentation parameters, total gas, methane production, and bacterial communities. Treatments were (1) control, 0 mg/L of OEO (CON); 13 mg/L (OEO1); 52 mg/L (OEO2); 91 mg/L (OEO3); and 130 mg/L (OEO4), each incubated with 150 mL of buffered rumen fluid and 1,200 mg of substrate for 24 h using the Ankom in vitro gas production system (Ankom Technology Corp., Fairport, NY). Treatment responses were statistically analyzed using polynomial contrasts. Digestibility of DM, NDF, and ADF increased quadratically with increasing OEO inclusion rates. Digestibility of DM and NDF were highest for OEO2, whereas ADF digestibility was highest for OEO3, compared with CON, with the remaining treatments being intermediate and similar. Ammonia nitrogen concentrations decreased from CON at a quadratic rate with increasing OEO inclusion rates, and OEO2 had the lowest concentration compared with the other groups. Total VFA, acetate, propionate, butyrate, valerate, and isovalerate concentrations linearly decreased with increasing OEO inclusion rates. Total gas production levels by CON and OEO4 were greater than those of OEO1, OEO2, and OEO3 in a quadratic response, and methane production linearly decreased from CON, compared with OEO4, at a decreasing rate with OEO inclusion rates. As determined by 16S rRNA sequencing, the α biodiversity of ruminal bacteria was similar among OEO inclusion rates. Increasing OEO inclusion rates linearly increased the relative abundance of *Prevotella* and *Dialister* bacteria. Several bacteria demonstrated dif-

ferent polynomial responses, whereas several bacteria were similar among increasing OEO inclusion rates. These results suggested that OEO supplementation can modify ruminal fermentation to alter VFA concentrations and reduce methane emissions by extensively altering the ruminal bacterial community, suggesting an optimal feeding rate for future animal studies of approximately 52 mg/L for mature ruminants.

Key words: bacterial composition, methane production, oregano essential oil, rumen fermentation, 16S rRNA

INTRODUCTION

Ruminant livestock production has been identified as a significant contributor to greenhouse gas emissions (Gerber et al., 2013). Seventy-one percent of the methane produced by enteric fermentation originates from ruminal fermentation (Gerber et al., 2013). In recent years, numerous strategies to mitigate methane emissions through the modulation of ruminal fermentation have been reported. Feeding natural feed additives has shown promising results (Hristov et al., 2013; Knapp et al., 2014). Among these natural additives, most are sourced from several plant types as secondary metabolites that have been reported to be useful in animal feeding (Wallace, 2004; Kamra et al., 2006), such as saponins, tannins, and essential oils (EO), which have demonstrated antimicrobial activities (Bakkali et al., 2008). Among these secondary metabolites, EO get the most attention and are being investigated as alternatives to antibiotics in animal nutrition (Greathead, 2003; Froehlich et al., 2017). In addition, EO and their bioactive compounds have been confirmed to modify ruminal fermentation by enhancing the efficiency of energy utilization while decreasing methane emissions (Tekippe et al., 2010; Joch et al., 2016). Essential oils have also been investigated as potential modifiers of ruminal biohydrogenation of dietary lipids, with the goal

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of enhancing healthful characteristics of milk and meat (Durmic et al., 2008; Lourenço et al., 2008).

Oregano (*Origanum vulgare* L.) is an herb with higher antioxidant capacity than other medicinal herbs (Dragland et al., 2003; Matsuura et al., 2003). The primary constituents of oregano EO (OEO) are carvacrol, γ -terpinene, thymol, p-cimene, and linalool, depending on origin and type of OEO (Sivropoulou et al., 1996; Baser, 2002). Oregano EO has been reported to have the second-highest oxygen radical absorbance capacity compared with clove (the highest), followed by cinnamon, ginger, and rosemary EO (Bentayeb et al., 2009). Thymol is a monoterpene with strong antimicrobial activity against a wide range of gram-positive and gram-negative bacteria and is one of the most actively researched EO (Burt, 2004). Carvacrol is a phenolic compound similar to thymol found in oregano, which also has strong antimicrobial activity. Busquet et al. (2005b) reported that carvacrol (2.2 mg/L) decreased large-peptide concentrations and increased ammonia nitrogen concentrations 2 h after feeding, using an in vitro long-term ruminal continuous culture system. Wang et al. (2009) evaluated a commercial OEO preparation (active chemical compounds: carvacrol, thymol) that demonstrated approximately a 12% decrease in methane production in sheep, similar in magnitude to methane production levels with flavomycin and *Yucca schidigera* (i.e., saponins). Oregano leaf material as an anti-methanogenic plant product, with no adverse effects on ruminal fermentation or NDF degradability, has been reported in vitro (Tekippe et al., 2010). Oregano EO was tested using a single dose and reduced ruminal methane production by positively improving total-tract apparent digestibility of dietary nutrients (Liu et al., 2017).

The study objectives were to verify our previous results and to evaluate inclusion rates of OEO to aid in determining the optimal feeding rate. Therefore, the specific study objective was to evaluate the effects of increasing OEO supplementation rates on ruminal in vitro fermentation, methane production, and ruminal bacterial communities. The hypothesis was that OEO would reduce fermenter methane production while improving feed digestibility and fermentation characteristics.

MATERIALS AND METHODS

Feeding and Management

All animal-handling protocols were approved by the Gansu Agricultural University Animal Care and Use Committee Guidelines (approved ID: 2012-2-159), fol-

Table 1. Ingredient and nutrient composition of the experimental ration (DM basis)

Ingredient	%	Nutrient level	%
Corn silage	65.50	DE ² (MJ/kg)	14.2
Corn	25.74	CP	15.1
Soybean meal	2.34	NDF	51.2
Rapeseed meal	2.34	ADF	38.0
Cottonseed meal	2.34	Ca	0.41
Limestone	0.58	P	0.27
NaCl	0.58		
1% Premix ¹	0.58		
Total	100		

¹The premix provided the following per kg of diet: vitamin A 220,000 IU, vitamin D₃ 72,000 IU, vitamin E 2,000 IU, D-biotin 40.0 mg, nicotinic acid amide 2,000 mg, Mn (as manganese sulfate) 710 mg, Zn (as zinc sulfate) 2,005 mg, Fe (as ferrous sulfate) 830.0 mg, Cu (as copper sulfate) 680.0 mg, Co (as cobalt sulfate) 12 mg.

²DE = digestive energy.

lowing the Chinese Standards for the Use and Care of Research Animals (He et al., 2016). Three German Merino sheep \times local sheep crossbreed rams (initial live BW = 56.68 ± 2.14 kg) fitted with permanent rumen cannulas were used as ruminal inoculum donors. Sheep were selected as the inoculum source due to being readily available, and Yáñez-Ruiz et al. (2016) indicated no differences between cattle and sheep when conducting in vitro experiments. Cows were offered a 65.5:34.5 forage-to-concentrate ratio (DM basis, %) based on corn silage, ground corn, protein sources (soybean, rapeseed, and cottonseed meals), minerals, and vitamins (Table 1). The nutrient composition of the diet (% DM basis) was approximately 15.1% CP and 51.2% NDF (Table 1), to meet or exceed the nutrient requirements guidelines for sheep (NRC, 2007). Sheep were fed twice daily at 0800 and 1800 h, before and throughout the duration of the experiment, and were given ad libitum access to water.

Oregano Essential Oil Treatments

The OEO was supplied as a dry granular powder (Rum-A-Fresh, Ralco Inc., Marshall, MN) containing approximately 1.3% OEO, along with lactic acid, cobalt carbonate, and clinoptilolite as a carrier. Five inclusion rates of OEO were evaluated: 0 (CON), 13 mg/L (OEO1), 52 mg/L (OEO2), 91 mg/L (OEO3), and 130 mg/L (OEO4), adjusted for the amount of OEO in the dry granular product added to the in vitro fermentation medium.

In Vitro Fermentations

Whole ruminal contents were obtained approximately 2 h after the morning feeding from 3 sheep in

equal proportions, transported to the laboratory in a prewarmed and sealed flask, and immediately mixed. The ruminal fluid for incubations was obtained after filtering through 4 layers of 100 mm × 100 mm medical gauze (Winner Inc. Ltd., Shenzhen, China) under constant CO₂ flushing.

The in vitro experiment was carried out using the Ankom RFS gas production system (Ankom Technology Corp., Fairport, NY). The system comprised 24 fermenters having a 250-mL volume, containing 1,200 mg of feed substrate (same ration fed to donor sheep, ground to pass through a 1-mm sieve) and the corresponding OEO inclusion rate. Each treatment had 4 bottles and 4 blank bottles (rumen fluid only) with no feed substrate, to correct for background gas production. Each fermenter was filled with 150 mL of ruminal inoculum, prepared as a 1:2 ratio of rumen fluid and artificial saliva. The artificial saliva was prepared anaerobically as described by Menke and Steingass (1988). All fermenters were incubated at 39°C for 24 h in a mild shaking water bath (SPH-110X24, Shiping Ltd., Shanghai, China).

Sampling

Headspace gas (5 mL) was collected from each fermenter bottle at 24 h, using a sealed gas injection needle, for determination of methane concentrations. At the end of the 24-h incubation, fermenters were opened and pH was immediately measured using a pH meter and glass electrode (Type CG 842, BlueLine 14 pH, Schott Instruments, Weilheim, Germany). A liquid sub-sample was collected from each fermenter and stored in microcentrifuge tubes for later determination of VFA, ammonia nitrogen (NH₃-N), microbial crude protein (MCP), and bacterial community determinations. Samples were stored at −20°C for VFA and ammonia nitrogen and at −80°C for MCP and bacterial community analyses, respectively, until analyzed. The residues of each fermenter were collected after centrifugation at 4,000 × *g* for 10 min at room temperature to determine the DM, NDF, and ADF digestibility (TGL-16, Cence Ltd., Changsha, China).

Chemical Analysis

The VFA concentrations were analyzed using a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) following the procedures described by Hu et al. (2005). The NH₃-N concentration was analyzed spectrophotometrically (UV-120-01, Shimadzu) using the procedures described by Chaney and Marbach (1962). The

MCP was determined following the procedures of Lin et al. (2013a). Feed substrate and residues DM were determined following AOAC International (2016) procedures. The NDF and ADF in the feed substrate and residues were determined according to the methods described by Van Soest et al. (1991). Methane production was analyzed via gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionization detector (FID-2010, Shimadzu; Hu et al., 2005). The column (HP-INNOWAX, 19091N-133, Agilent Technologies, Santa Clara, CA) of the GC was 30 m × 0.25 mm × 0.25 μm in size, and the temperature was 80°C. The gas flow rates for nitrogen, hydrogen, and air were 30, 25, and 400 mL/min, respectively. The front inject port, column oven, and detector temperatures were 50, 375, and 200°C, respectively. The gas pressure was automatically recorded during incubation (Ankom Technology Corp.). The total gas production at 24 h was calculated based on the ideal gas equation:

$$V_x = P_{kPa} \times 1.3815886 \text{ (Ankom operations manual),}$$

where V_x (mL) = gas volume at 39°C, and P_{kPa} = gas pressure.

DNA Extraction, PCR Amplification, and 16S rRNA Sequencing

Total genomic DNA from ruminal samples stored at −80°C were extracted using the EZNA Stool DNA Kit (D4015, Omega Bio-tek Inc., Norcross, GA), following the manufacturer's instructions. The V3 to V4 region of the prokaryotic (bacterial and archaeal) small-subunit (16S) rRNA genes were amplified using slightly modified universal primer versions 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'; Fadrosch et al., 2014), where the barcode is an 8-base sequence unique to each sample on the 5' primer ends and sequencing universal primers. Amplification via PCR was performed in a total volume of 25 μL of reaction mixture containing 25 ng of template DNA, 12.5 μL of PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. The PCR conditions to amplify the prokaryotic 16S fragments consisted of an initial denaturation at 98°C for 30 s; 35 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s; and then final extension at 72°C for 10 min. The PCR products were confirmed using 2% agarose gel electrophoresis. The PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA) and quantified by Qubit (Invitrogen, Carls-

bad, CA). Amplicon sequencing was conducted on an Illumina MiSeq 2 × 3 platform to generate overlapping paired-end 2 × 300 bp (Bionew Ltd., Hohhot, China).

Bioinformatics and Analyses

All reads were processed and analyzed using the following procedures. First, paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were then merged using FLASH 1.2.7 (Magoč and Salzberg, 2011). Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to the fqtrim (version 0.94, National Center for Biotechnology Information, Bethesda, MD). Chimeric sequences were filtered using USEARCH software based on the UCHIME algorithm (Edgar et al., 2011). Bacterial operational taxonomic units (OTU) were selected using USEARCH (version 2.3.4; Edgar, 2010), applying 97% sequence similarity thresholds. Representative sequences for each OTU were chosen using Quantitative Insights Into Microbial Ecology (QIIME; Wemheuer et al., 2014) programs (version 1.9.1), which were assigned from the Ribosomal Database Project for taxonomic classification using the BLAST approach (Mittra et al., 2011). Alpha diversity analysis, including abundance-based coverage estimators (ACE), Good's coverage, Chao1, Shannon, Simpson, and observed OTU indices, were calculated with QIIME 1.9.0 according to Wemheuer et al. (2014).

Statistical Analyses

All data were checked for normality and outliers using the UNIVARIATE procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) before any statistical analyses were conducted. We performed ANOVA using the R package (version 3.4.1, R Core Team, 2017) or SAS. The Mann-Whitney U test was applied to determine the significance of α diversity (Zeng et al., 2015). The data of ruminal fermentation parameters and nutrient digestibility were statistically analyzed using the PROC MIXED procedure of SAS. The statistical model used was as follows: $Y_{ij} = \mu + R_i + T_j + e_{ij}$, where Y_{ij} = dependent variable, μ = overall mean, R_i = replication, T_j = treatment, and e_{ij} = random error. Treatment effects were considered fixed, and replication was considered to be random effect. Polynomial contrasts were used to test the linear, quadratic, cubic, and quartic effects of treatments (i.e., OEO inclusion rate). Differences among treatments were separated using the PDIF statement of SAS. Statistical significance was set to P

< 0.05 , and a tendency of difference was declared at $P < 0.10$.

RESULTS

Effects on Rumen Fermentation Characteristics

The digestibility of DM, NDF, and ADF increased quadratically ($P < 0.03$) with increasing OEO inclusion rates (Table 2). In slight contrast, DM and NDF digestibility was highest for OEO2, whereas ADF digestibility was highest for OEO3 compared with CON, with the remaining treatments being intermediate and similar ($P > 0.05$). It thus appears that OEO3 would be the optimal feeding rate for future in vivo studies.

Fermenter pH demonstrated a linear ($P < 0.05$) increase with increasing OEO inclusion rate, with the highest pH observed for the highest inclusion rate of OEO (OEO4). In contrast with fermenter pH, $\text{NH}_3\text{-N}$ concentrations demonstrated a quartic response ($P < 0.01$) with increasing OEO inclusion rates, such that OEO2 had the lowest ($P < 0.05$) $\text{NH}_3\text{-N}$ concentrations compared with the CON and OEO1 fermenters. Inclusion rate of OEO demonstrated mixed effects on fermenter VFA concentrations (Table 2). Microbial CP concentrations demonstrated a quadratic response ($P < 0.10$), such that the OEO2 fermenter had the highest MCP concentration compared with fermenters CON, OEO3, and OEO4. The MCP concentrations were inverse of the fermenter ammonia concentrations, indicating that ammonia was being incorporated into the synthesis of MCP.

Total concentrations of VFA, propionate, butyrate, isovalerate, and acetate-to-propionate ratio demonstrated trends ($P < 0.10$) or significant ($P < 0.05$) quartic responses to increasing OEO inclusion rate, decreasing to lower concentrations for OEO4 compared with CON fermenters. Acetate demonstrated a quadratic response ($P < 0.10$), with OEO4 decreasing to a lower concentration compared with fermenters CON or OEO1. Isobutyrate concentrations linearly decreased ($P < 0.01$) with increasing OEO inclusion rate. As the first OEO inclusion rate was added to the fermenters, most VFA remained similar ($P > 0.10$) to CON, but further increases in OEO inclusion rate decreased VFA concentrations ($P < 0.05$), meaning that very high OEO inclusion rates can become detrimental or inhibitory to VFA production.

Effects on Total Gas and Methane Production

Total 24-h gas production demonstrated a quartic ($P < 0.10$) response, decreasing to lowest for OEO2

Table 2. Fermenter nutrient digestibility, pH, ammonia N (NH₃-N), microbial crude protein (MCP), VFA, and gas production for fermenters fed a control (CON) ration supplemented with increasing levels of oregano essential oil (OEO)

Measurement	OEO dose ¹ (mg/L)					SEM	Contrast ² ($P < $)			
	CON	OEO1	OEO2	OEO3	OEO4		L	Q	C	Qu
Fermenter digestibility (%)										
DMD ³	38.9 ^b	41.2 ^{ab}	44.9 ^a	44.8 ^a	41.4 ^{ab}	1.60	0.13	<0.01	0.90	0.97
NDFD ⁴	26.3 ^b	28.2 ^{ab}	32.0 ^a	29.5 ^{ab}	27.8 ^b	1.35	0.42	0.01	0.25	0.54
ADFD ⁵	25.6 ^b	27.0 ^{ab}	28.1 ^{ab}	29.5 ^a	25.4 ^b	1.53	0.77	0.03	0.47	0.50
pH	5.54 ^b	5.59 ^b	5.60 ^b	5.67 ^b	5.72 ^a	0.05	<0.01	0.89	0.47	0.40
NH ₃ -N (mg/dL)	15.8 ^a	15.6 ^a	11.3 ^b	12.7 ^b	12.2 ^b	0.65	<0.01	<0.01	0.11	<0.01
MCP (mg/mL)	0.31 ^c	0.40 ^{ab}	0.49 ^a	0.36 ^b	0.35 ^{bc}	0.03	0.84	<0.01	0.10	0.87
VFA (mmol/L)										
Total	162.5 ^a	161.4 ^a	155.3 ^b	147.8 ^c	145.6 ^d	1.71	<0.01	0.29	0.13	0.07
Acetate	106.9 ^a	104.8 ^a	98.5 ^b	97.0 ^b	95.5 ^b	0.86	<0.01	<0.01	0.26	0.32
Propionate	44.9 ^b	46.2 ^{ab}	48.3 ^a	43.6 ^b	43.3 ^b	1.13	0.03	0.02	<0.01	0.06
Butyrate	6.41 ^a	5.81 ^b	5.30 ^{bc}	4.12 ^c	4.01 ^{cd}	0.70	<0.01	0.09	0.49	0.03
Valerate	1.34 ^{ab}	1.45 ^a	1.01 ^b	0.94 ^b	0.80 ^b	0.08	<0.01	0.21	0.65	0.36
Isobutyrate	0.77 ^a	0.72 ^a	0.50 ^{ab}	0.46 ^{ab}	0.37 ^b	0.08	<0.01	0.46	0.66	0.48
Isovalerate	2.29 ^a	2.37 ^a	1.78 ^b	1.73 ^b	1.56 ^b	0.06	<0.01	0.02	0.98	<0.01
Acetate/propionate	2.38 ^b	2.27 ^{ab}	2.73 ^a	2.23 ^{ab}	2.21 ^{ab}	0.04	0.10	<0.01	<0.01	0.07
Total gas (mL)	137.6 ^a	124.4 ^b	124.2 ^b	125.0 ^b	128.9 ^a	5.00	0.40	0.06	0.20	0.08
Methane (mL)	43.0 ^a	41.7 ^{ab}	38.8 ^{ab}	36.6 ^{bc}	38.2 ^b	1.81	0.01	0.09	0.60	0.86
Methane/total gas (%)	31.3 ^{ab}	33.5 ^a	31.3 ^{ab}	29.4 ^{ab}	29.7 ^b	1.55	0.04	0.94	0.21	0.44

^{a-d}Means within a row with unlike superscripts differ ($P < 0.05$).

¹Data were analyzed using OEO dose levels of 0 (CON), 13 mg/L (OEO1), 52 mg/L (OEO2), 91 mg/L (OEO3), or 130 mg/L (OEO4).

²Probability of contrast being less than the value in the column; L = linear; Q = quadratic; C = cubic; Qu = quartic.

³DMD = dry matter digestibility.

⁴NDFD = neutral detergent fiber digestibility.

⁵ADFD = acid detergent fiber digestibility.

then increasing with increasing OEO inclusion rate (Table 2), whereas 24-h total methane production and methane/total gas were linearly decreased ($P < 0.05$) with increasing OEO inclusion rate, such that OEO3 had the lowest methane production and methane/total gas compared with the CON and OEO1 fermenters. However, as total gas and methane decreased, methane as a percentage of total gas production declined faster as the OEO inclusion rate increased. Methane decreasing at a greater rate than total gas production should direct more energy into VFA production.

Effects on Rumen Bacterial Community

A total of 379,768 raw bacterial 16S rRNA gene sequences were obtained. After quality control and filtering, 25,317 valid sequences were analyzed for each sample. A total of 4,410 OTU based on 97% sequence similarity were generated. The α diversity of 16S rRNA gene OTU was not altered by increasing OEO inclusion rate ($P > 0.10$; Table 3), except for Chao1, which demonstrated a linear ($P < 0.05$) reduction such that OEO3 fermenters were lowest ($P < 0.05$) compared with remaining fermenters.

Based on the obtained OTU, a total of 24 genera were observed to have a relative abundance greater

than 1% (Figure 1). The most abundant microbial genus detected via 16S rRNA gene sequence data was *Prevotella*, which demonstrated a linear ($P < 0.05$) response, with increasing OEO inclusion rate being greatest ($P < 0.05$) for OEO4 fermenters compared with CON fermenters and the remaining treatments being intermediate (Table 4). Several microbial genera were similar ($P > 0.05$) among all OEO inclusion rates. *Firmicutes* unclassified, *Eubacterium*, *Lachnospiraceae* unclassified, *Bifidobacterium*, and *Oxobacter* demonstrated quartic ($P < 0.05$) responses with increasing OEO inclusion rates that were not consistent across microbial genera. *Firmicutes* unclassified, *Eubacterium*, *Bifidobacterium*, and *Oxobacter* were lower for OEO1 fermenters compared with CON fermenters, whereas *Lachnospiraceae* unclassified was lower for CON, OEO2, and OEO3 fermenters compared with OEO1 fermenters, with the remaining treatments being intermediate. The microbial genera with more than 2% relative abundance demonstrate greater relative abundance of *Prevotella* and *Dialister*, while reducing ($P < 0.010$) *Veillonellaceae* unclassified, *Porphyromonadaceae* unclassified, and *Firmicutes* unclassified via linear, quadratic, cubic, or quartic responses with increasing OEO inclusion rate compared with CON.

Table 3. Changes in richness and diversity of the ruminal bacterial community in fermenters fed a control (CON) or ration supplemented with increasing inclusion rates of oregano essential oil (OEO)

Measurement index	OEO dose ¹ (mg/L)					SEM	Contrast ² ($P <$)			
	CON	OEO1	OEO2	OEO3	OEO4		L	Q	C	Qu
Observed OTU ³	1,297.0	1,310.3	1,314.0	1,317.3	1,323.3	20.17	0.32	0.081	0.071	0.76
Shannon	8.01	7.98	8.06	7.98	8.12	0.09	0.23	0.49	0.50	0.41
Simpson	0.99	0.99	0.99	0.99	0.99	0.00	—	—	—	—
Chao1	1,979.1 ^a	1,955.9 ^a	1,962.5 ^a	1,879.6 ^b	1,928 ^a	44.44	0.05	0.26	0.16	0.36
ACE ⁴	2,038.93	2,026.15	2,058.70	2,014.73	2,011.79	40.89	0.30	0.34	0.59	0.46
Good's coverage	96.33	97.33	96.33	96.33	97.00	0.09	0.95	0.48	0.44	0.29

^{a,b}Means within a row with unlike superscripts differ ($P < 0.05$).

¹Data were analyzed using OEO dose levels of 0 (CON), 13 mg/L (OEO1), 52 mg/L (OEO2), 91 mg/L (OEO3), or 130 mg/L (OEO4).

²Probability of a contrast being less than value in column; L = linear; Q = quadratic; C = cubic; Qu = quartic. Dashes indicate no statistical test because all values = 0.99.

³OTU = operational taxonomic unit.

⁴ACE = abundance-based coverage estimator.

DISCUSSION

Effects on Rumen Fermentation Characteristics

For a ruminant animal, most of the DM and fiber of a feed or ration is ruminally digested. Therefore, ruminal DM and fiber digestibility are appropriate indices for evaluating OEO effects (Lin et al., 2009). In the literature, the EO effects on nutrient digestibility by the rumen microbiome have been mixed. Some researchers have suggested that EO have no effects on fiber degradation but can reduce the degradation of readily degradable substrates, such as protein and starch, ow-

ing to OEO inhibition of amylolytic and proteolytic bacteria (Wallace et al., 2002; Hart et al., 2008). On the contrary, other authors have reported positive effects (Benchaar et al., 2006; Yang et al., 2007) or no effects (Meyer et al., 2009; Sallam et al., 2009) of EO on ruminal digestibility. In the current study, supplementation of 52 mg/L and 91 mg/L OEO increased in vitro fermenter DM, ADF, and NDF digestibility (Table 2) compared with CON, with digestibility increasing with increasing OEO inclusion rate to a point of being maximal at 52 or 91 mg/L. These results corresponded to a linear decrease in VFA production, to the extent that a high OEO inclusion rate can be detrimental by

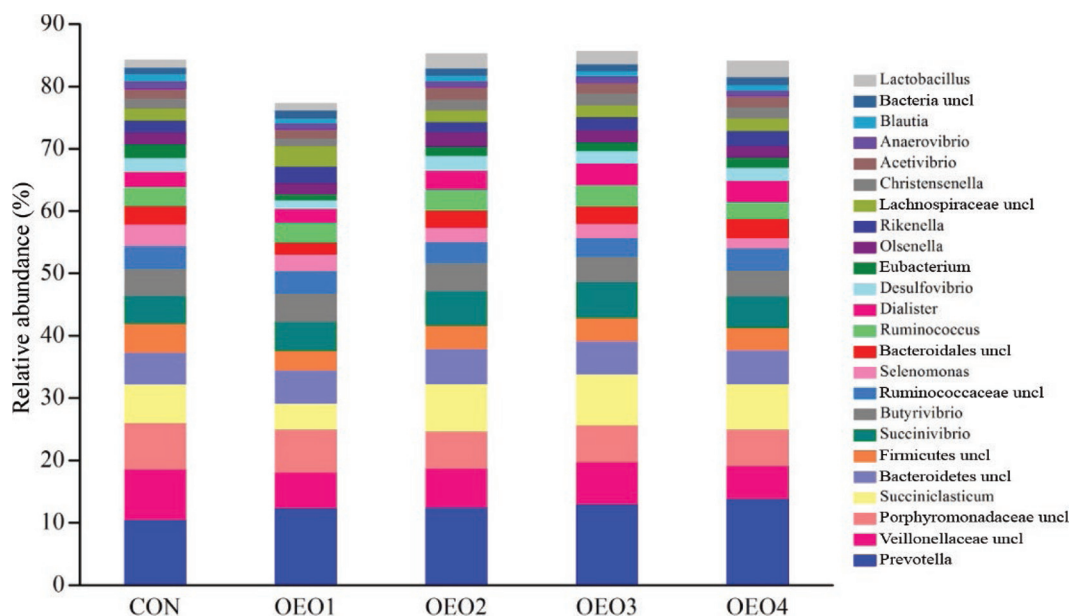
**Figure 1.** Ruminal bacterial community composition of genera with a relative abundance greater than 1%. Data were analyzed using inclusion rates of oregano essential oil (OEO) of 0 (CON), 13 mg/L (OEO1), 52 mg/L (OEO2), 91 mg/L (OEO3), and 130 mg/L (OEO4). Uncl = unclassified.

Table 4. Relative abundances of microbial genera (% of total sequences) of the microbial community in fermenters fed a control (CON) ration with increasing inclusion rates of oregano essential oil (OEO)

Genus	OEO dose ¹ (mg/L)					SEM	Contrast ² ($P < $)			
	CON	OEO1	OEO2	OEO3	OEO4		L	Q	C	Qu
<i>Prevotella</i>	10.54 ^b	12.43 ^{ab}	12.58 ^{ab}	13.03 ^a	13.88 ^a	1.03	0.05	0.64	0.35	0.42
<i>Veillonellaceae</i> uncl ³	8.12 ^a	5.76 ^b	6.21 ^{ab}	6.82 ^{ab}	5.36 ^b	0.64	0.09	0.86	0.02	0.15
<i>Porphyromonadaceae</i> uncl	7.45 ^a	6.87 ^{ab}	5.98 ^{ab}	5.92 ^b	5.83 ^b	0.60	0.02	0.25	0.59	0.96
<i>Succiniclasticum</i>	6.17 ^b	4.14 ^c	7.56 ^{ab}	8.14 ^a	7.24 ^{ab}	0.61	<0.01	0.05	0.04	<0.01
<i>Bacteroidetes</i> uncl	5.15	5.37	5.68	5.34	5.47	0.69	0.74	0.70	0.63	0.88
<i>Firmicutes</i> uncl	4.58 ^a	3.10 ^c	3.71 ^{bc}	3.71 ^{ab}	3.59 ^b	0.44	0.29	0.30	0.10	<0.01
<i>Succinivibrio</i>	4.46	4.72	5.50	5.74	5.08	0.64	0.26	0.18	0.76	0.96
<i>Butyrivibrio</i>	4.41	4.48	40.61	4.16	4.09	0.53	0.51	0.71	0.67	0.81
<i>Ruminococcaceae</i> uncl	3.64	3.53	3.29	2.99	3.64	0.41	0.78	0.28	0.58	0.62
<i>Selenomonas</i>	3.49 ^a	2.73 ^a	2.31 ^{bc}	2.23 ^{bc}	1.66 ^c	0.43	<0.01	0.55	0.29	0.66
<i>Bacteroidales</i> uncl	2.99	1.99	2.78	2.86	3.09	0.51	0.27	0.59	0.28	0.17
<i>Ruminococcus</i>	2.95	3.14	3.28	3.30	2.59	0.57	0.49	0.22	0.79	0.85
<i>Dialister</i>	2.43 ^{ab}	2.23 ^b	3.12 ^a	3.53 ^a	3.48 ^a	0.44	0.03	0.35	0.47	0.47
<i>Desulfovibrio</i>	2.26	1.37	2.34	1.95	2.07	0.45	0.55	0.85	0.61	0.07
<i>Eubacterium</i>	2.18 ^a	0.95 ^b	1.48 ^a	1.40 ^{ab}	1.63 ^a	0.32	0.75	0.13	0.12	<0.01
<i>Olsenella</i>	1.96	1.80	2.37	1.97	1.82	0.43	0.87	0.27	0.84	0.18
<i>Rikenella</i>	1.91	2.64	1.65	2.17	2.49	0.42	0.57	0.23	0.69	0.09
<i>Lachnospiraceae</i> uncl	1.91 ^b	3.27 ^a	1.87 ^b	1.84 ^b	2.03 ^{ab}	0.57	0.13	0.29	0.50	0.05
<i>Christensenella</i>	1.61	1.21	1.71	1.91	1.78	0.41	0.33	0.74	0.45	0.34
<i>Acetivibrio</i>	1.51	1.54	2.00	1.70	1.75	0.34	0.53	0.36	0.55	0.54
<i>Anaerovibrio</i>	1.25	0.84	0.92	0.98	0.83	0.24	0.39	0.73	0.27	0.38
<i>Blautia</i>	1.14	0.89	0.93	0.85	0.96	0.44	0.71	0.58	0.86	0.56
<i>Bacteria</i> uncl	1.00	1.31	1.14	1.15	1.23	0.12	0.46	0.92	0.15	0.12
<i>Lactobacillus</i>	1.06 ^b	0.94 ^b	2.17 ^a	1.93 ^a	2.44 ^a	0.33	<0.01	0.43	0.48	0.12
<i>Anaerovorax</i>	0.87	0.88	0.83	0.75	0.91	0.10	0.89	0.43	0.52	0.88
<i>Clostridium</i>	0.83	0.74	0.82	0.80	0.80	0.11	0.96	0.99	0.77	0.48
<i>Ethanoligenens</i>	0.69	0.56	0.76	0.65	0.73	0.14	0.58	0.94	0.92	0.31
<i>Bulleidia</i>	0.64	0.54	0.62	0.49	0.50	0.11	0.17	0.97	0.84	0.28
<i>Mitsuokella</i>	0.59 ^a	0.43 ^b	0.28 ^{bc}	0.23 ^c	0.15 ^c	0.07	<0.01	0.06	0.20	0.69
<i>Erysipelothrix</i>	0.58	0.40	0.44	0.45	0.44	0.10	0.55	0.45	0.33	0.33
<i>Coriobacteriaceae</i> uncl	0.51 ^a	0.34 ^{bc}	0.42 ^a	0.29 ^c	0.27 ^c	0.06	0.02	0.81	0.58	0.05
<i>Saccharibacteria</i>	0.49 ^{bc}	0.35 ^c	0.60 ^{ab}	0.62 ^a	0.52 ^{bc}	0.08	0.08	0.07	0.12	0.13
<i>Paraprevotella</i>	0.44 ^b	0.41 ^b	0.51 ^b	0.64 ^{ab}	0.80 ^a	0.11	<0.01	0.41	0.77	0.74
<i>Saccharofermentans</i>	0.39 ^{ab}	0.29 ^b	0.37 ^{ab}	0.40 ^{ab}	0.54 ^a	0.11	0.07	0.32	0.89	0.27
<i>Schwartzia</i>	0.36	0.20	0.31	0.31	0.25	0.09	0.71	0.76	0.23	0.18
<i>Oribacterium</i>	0.36	0.35	0.38	0.43	0.36	0.06	0.45	0.38	0.39	0.96
<i>Alloprevotella</i>	0.34	0.27	0.36	0.33	0.41	0.07	0.27	0.71	0.96	0.28
<i>Syntrophococcus</i>	0.31	0.24	0.27	0.25	0.29	0.05	0.91	0.27	0.56	0.13
<i>Prevotellaceae</i> uncl	0.29	0.15	0.25	0.28	0.27	0.04	0.34	0.83	0.14	0.07
<i>Moryella</i>	0.28	0.26	0.20	0.25	0.26	0.05	0.84	0.29	0.55	0.71
<i>Erysipelotrichaceae</i> uncl	0.26 ^a	0.12 ^b	0.33 ^a	0.27 ^a	0.35 ^a	0.07	0.07	0.95	0.91	<0.01
<i>Lachnospirillum</i>	0.25	0.45	0.18	0.22	0.23	0.07	0.25	0.41	0.37	0.10
<i>Pyramidobacter</i>	0.20	0.15	0.15	0.17	0.12	0.05	0.38	0.98	0.21	0.77
<i>Turicibacter</i>	0.19	0.18	0.20	0.17	0.18	0.06	0.82	0.93	0.75	0.65
<i>Intestinimonas</i>	0.18	0.09	0.12	0.09	0.12	0.04	0.40	0.31	0.57	0.18
<i>Desulfovulbus</i>	0.16	0.015	0.22	0.31	0.25	0.20	0.44	0.71	0.74	0.99
<i>Oscillibacter</i>	0.15	0.61	0.17	0.21	0.18	0.48	0.48	0.96	0.43	0.37
<i>Atopobium</i>	0.15	0.10	0.13	0.13	0.13	0.05	0.97	0.76	0.67	0.53
<i>Roseburia</i>	0.14	0.46	0.19	0.21	0.22	0.37	0.74	0.99	0.52	0.46
<i>Streptococcus</i>	0.14	0.09	0.31	0.23	0.26	0.25	0.47	0.72	0.91	0.48
<i>Mogibacterium</i>	0.14	0.12	0.16	0.16	0.12	0.05	0.96	0.48	0.67	0.66
<i>Oscillospira</i>	0.13	0.24	0.13	0.11	0.13	0.12	0.39	0.70	0.30	0.44
<i>Fretibacterium</i>	0.13	0.08	0.21	0.18	0.24	0.07	0.09	0.74	0.78	0.09
<i>Pseudomonas</i>	0.11	0.08	0.05	0.07	0.05	0.07	0.40	0.68	0.60	0.95
<i>Bifidobacterium</i>	0.57 ^{bc}	0.46 ^c	0.97 ^a	0.81 ^{ab}	0.76 ^{ab}	0.14	0.10	0.05	0.87	0.04
<i>Oxobacter</i>	0.34 ^{ab}	0.15 ^b	0.25 ^a	0.22 ^{ab}	0.26 ^{ab}	0.05	0.87	0.39	0.31	0.04

^{a-c}Means in the same row with unlike superscripts differ, $P < 0.05$.¹Data were analyzed using OEO dose levels of 0 (CON), 13 mg/L (OEO1), 52 mg/L (OEO2), 91 mg/L (OEO3), or 130 mg/L (OEO4).²L = linear; Q = quadratic; C = cubic; and Qu = quartic.³Uncl = unclassified.

reducing VFA concentrations. Why digestibility could be increased with a decrease in VFA may be related to the production of microbial extracellular enzymes enhancing DM and fiber digestibility (Priest, 1977), but the hydrolysis products were not fermented to VFA. These findings are in contrast with the results reported by Tager and Krause (2011), who reported that high levels of EO negatively affected ruminal fiber digestibility, but do agree that they theoretically could reduce ruminal VFA production.

The potential uses of EO as a ruminal fermentation modifier have been reported in the literature (Molero et al., 2004; Simitzis, 2017). Numerous in vitro experiments have reported that different types and doses of EO could inhibit NH_3 concentrations (Cardozo et al., 2005; Lin et al., 2012; Lin et al., 2013b). Hristov et al. (2013) also observed a decrease of NH_3 and butyrate concentration when feeding dairy cows *Origanum vulgare* L. leaves (250, 500, and 750 g/cow). The various EO available as natural feed additives could be considered useful in ruminant nutrition by reducing ruminal ammonia N concentrations and protein deamination by inhibiting hyper-ammonia producing (HAP) bacteria (Patra, 2011). The quartic response in decreasing ruminal ammonia nitrogen concentrations with increasing OEO inclusion rate observed in the present study likely resulted from the inhibition of proteolysis, peptidolysis, amino acid deamination, or an increase in microbial protein synthesis, or some combination of these factors—a premise substantiated by the reduced concentrations of isobutyrate and isovalerate reported by Patra and Yu (2012). In the present study, the pH increased linearly with increasing OEO inclusion rate, likely due to the reduction in VFA production, which is in agreement with previous studies (Lin et al., 2012; Patra and Yu, 2015a). Several studies have observed that EO supplementation can cause a reduction in VFA concentrations (Busquet et al., 2006; Calsamiglia et al., 2007; Benchaar et al., 2008). Patra and Yu (2012) reported, using an in vitro test, that total VFA concentrations were decreased by adding oregano and clove EO (0.25, 0.50, and 1.0 g/L). Additionally, a mixed EO blend (50 or 200 mg/L eugenol, carvacrol, citral, and cinnamaldehyde oils) fed to Hu sheep was effective in reducing the acetate:propionate ratio, $\text{NH}_3\text{-N}$, and total VFA concentrations (Lin et al., 2013a). As confirmed by the present study, total VFA, acetate, and butyrate concentrations were markedly reduced with increasing OEO inclusion rates, whereas the propionate concentration and acetate:propionate ratio were increased with addition of 52 mg/L of OEO. Even though VFA concentrations were reduced with increasing OEO inclusion rate, the propionate concentration increased and then decreased in a quadratic response. Inclusion

of OEO caused a shift in ruminal fermentation for lower acetate and greater propionate concentrations. Thus, it is plausible that OEO supplementation causes an ionophore-like shift in the ruminal microbial ecology. Several studies have demonstrated that some EO have positive effects on VFA concentrations by decreasing acetate production and increasing propionate production (Mohammed et al., 2004; Busquet et al., 2005a; Poudel et al., 2019). Poudel et al. (2019) demonstrated that feeding the same OEO product used in this study resulted in an increase in ruminal propionate concentrations from Holstein calves, along with an increase in Prevotellaceae. In the present study, supplementation with increasing OEO inclusion rates decreased isobutyrate and isovalerate concentrations. These results are in agreement with the findings reported by Pinski et al. (2016), who suggest that different doses of OEO have different potencies to inhibit proteolysis and aminogenesis. Additional studies using HAP bacterial populations need to be conducted to elucidate the effects of OEO on aminogenesis.

Effects on Total Gas and Methane Production

In the present study, total gas production was linearly decreased by increasing OEO inclusion rate. These results are in agreement with the in vitro results reported by Patra and Yu (2012) and Cobellis et al. (2016b), that supplementation with cinnamon bark oil, Ceylon cinnamon bark oil, and oregano oil demonstrated a pronounced inhibition of total gas production. The novel finding in our study is an increasing reduction in methane as a percentage of total gas production with increasing OEO inclusion rate. However, Agarwal et al. (2009) found an increase in gas production with addition of 0.33 and 1.0 $\mu\text{L/mL}$ of peppermint oil. These results demonstrate that the OEO type and dose may elicit different results. Numerous studies have reported strong inhibition in methane production by EO (Wang et al., 2009; Knapp et al., 2014; Cobellis et al., 2016b). For example, Macheboeuf et al. (2008) observed a 98% reduction in methane production using 5 mM of OEO or cinnamon EO. Evaluating 5 different EO (clove, eucalyptus, garlic, oregano, and peppermint), Patra and Yu (2012) reported that methane production linearly decreased with increasing EO doses, with the greatest methane reduction (87%) using OEO at a dose of 1.0 g/L. Garlic oil can inhibit in vitro methane production (38.5%) with a dose of 167 $\mu\text{L/L}$ (Pawar et al., 2014). The finding of Pawar et al. (2014) is an increasing methane reduction as a % of total gas production with increasing OEO inclusion rate. In the present study, methane production decreased linearly with increasing OEO supplementation, suggesting that OEO inhibits

methane synthesis. The decrease in methane production with the addition of OEO is similar to the findings of Tekippe et al. (2011). Feeding lactating cows (8 cows) *Origanum vulgare* L. leaves (500 g/cow per d) led to an approximately 31% decrease in ruminal methane production. The inhibition of methane production by OEO may be due to indirect or direct inhibition (or both) of methanogens via a decline in H₂ production due to reduced acetate and butyrate and more propionate production (Cieslak et al., 2013; Kumar et al., 2014). Shifting ruminal fermentation to more propionate would inhibit hydrogen-producing bacteria, such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, and protozoa (Cobellis et al., 2016b). Further studies are needed to elucidate the exact mechanism by which OEO reduces methane production.

Rumen Microbial Community

In previous studies, very few bacteria genera were analyzed (Fiorentini et al., 2013; Martínez-Fernández et al., 2014; Zhou et al., 2017), which prevented a deeper understanding of the influence of feed additives on ruminal bacterial communities. In the present study, 16S rRNA sequencing was used to comparatively examine OEO influence on the ruminal bacterial community. Ruminant animals have a very diverse bacterial community, especially at the species level, containing about 5,200 OTU (Kim et al., 2011). A similar result (4,410 OTU) was observed in our study. The phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the prevalent bacteria in the current study, which agreed with the results of previous studies (Zhou et al., 2017; Stewart et al., 2018; Yan et al., 2018). Antibacterial potency of OEO (containing phenol) may contribute to the antimicrobial activities of OEO (Ultee et al., 2002). Generally, gram-positive bacteria are thought to be more susceptible to EO than are gram-negative bacteria, due to the lack of a protective outer membrane surrounding the cell wall (Patra and Yu, 2012). In the present study, although *Firmicutes* was not affected by OEO. Members of *Firmicutes*, *Selenomonas*, and *Mitsuokella*, gram-positive genera, were linearly decreased by OEO inclusion rates (Table 4). *Dialister* and *Lactobacillus* were linearly increased by OEO inclusion rates, suggesting that these bacteria may play a key role in ruminal biohydrogenation (Huws et al., 2011; Patra and Yu., 2015b). In the present study, supplementation with 13 mg/L of OEO increased the relative abundance of *Lachnospiraceae* unclassified, and the relative abundances of *Lactobacillus* were increased by the 3 highest OEO inclusion rates, suggesting that these bacteria may play a key role in ruminal biohydrogenation (Huws et al., 2011; Patra and Yu., 2015b).

Therefore, changes of microbial compositions via OEO inclusion may be associated with changes in the ruminal biohydrogenation process (Ramos-Morales et al., 2013). Thoetkiattikul et al. (2013) reported that *Bacteroidetes* was a major non-cellulosic plant constituent degrader in the rumen. Most *Bacteroidetes* bacterial strains are hemicellulolytic, proteolytic, or amylolytic (Evans et al., 2011). The relative abundance of the top 2 most abundant bacteria of the *Bacteroidetes* family (*Prevotella* and *Dialister*) were increased by OEO, possibly due to reduced competition from other bacteria that are inhibited by OEO (Patra and Yu, 2015b). *Prevotella*, a gram-negative genus of *Bacteroidetes*, linearly increased with OEO inclusion rates, maybe due to reduced competition from other bacteria, which are inhibited by OEO (Patra and Yu, 2015b). In addition, we observed that *Prevotella* was the dominant genus among all treatments, in accordance with some previous in vivo studies (Jami et al., 2013; Thoetkiattikul et al., 2013; Paz et al., 2016). It has been suggested that EO could reduce ruminal protein degradation and ammonia concentrations (McIntosh et al., 2003; Patra, 2011). We also report that OEO supplementation significantly lowered ammonia nitrogen, which is in agreement with previous studies that reported that EO decreased ruminal ammonia concentrations (Patra and Yu, 2012; Patra and Yu, 2014). The methanogens *Euryarchaeota* are a phylum belonging to the archaea community; in the current study, *Euryarchaeota* showed very low relative abundance and were decreased at all levels of OEO inclusion, consistent with the decrease of CH₄ production in OEO treatments. These results indicate that ruminal methane production may be much more influenced by the relative abundance of archaea, rather than by the microbial population structure (Wallace, 2004; Duarte et al., 2017). We noted that *Fibrobacteres* accounted for less than 1.0% of the sequences among all treatments. This result is similar to a study by Patra and Yu (2015b), reporting 1 OTU *Fibrobacteres* with the addition of 0.5 g/L OEO. In addition, the cellulolytic *Fibrobacteres* bacteria decreased for the 52 and 91 mg/L OEO treatments, which may be positively correlated with acetate concentration (Varzaneh et al., 2018). In the present study, OEO supplementation also reduced a few other bacteria, such as *Verrucomicrobia*, *Synergistetes*, *Spirochaetes*, and *Planctomycetes*. However, rumen metabolism of these genera are not well understood, and further studies are needed to elicit their functions.

CONCLUSIONS

Previous studies using 16S rRNA analysis demonstrate that OEO supplementation can influence rumi-

nal bacterial communities to increase digestibility of dry matter and fiber, while shifting fermentation to reduce molar concentrations of acetate and butyrate to increasing propionate. The modulation of fermentation by OEO inclusion rate altered many ruminal bacterial genera that are associated with feed digestibility and ruminal fermentation characteristics. However, a large number of ruminal bacteria remain to be cultured, cautioning that the results obtained in the present study might have limitations. Future *in vivo* studies are needed to optimize OEO inclusion rate so that effective methane mitigation can be achieved without altering ruminal feed digestion and fermentation. However, an optimal OEO feeding rate for future animal studies appears to be 52 mg/L for mature ruminants.

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