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Treatment of dual-flow continuous culture fermenters with an organic essential oil product minimally influenced prokaryotic microbiome

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Abstract

Previous research reported an essential oil (EO) product decreasing methane (CH_4) production by dual-flow continuous culture (DFCC); this product could assist organic dairy producers in decreasing emissions. Our objective was to assess the effect of this EO product on the microbial populations within DFCC. Here, we hypothesized that the EO either decreased protozoal population or induced shifts in the bacterial relative abundance to decrease CH_4 production. Metagenomic DNA was extracted from previous effluent samples taken from a DFCC system (n = 2) across four experimental periods, after which samples were sequenced the 16S rRNA gene and microbial taxonomy was assigned using the SILVA v138 database. The treatments included a control (CON) diet (60:40 concentrate:orchardgrass pellet mix, 17.1% crude protein, 33.0% neutral detergent fiber, 20.1% acid detergent fiber, and 27.1% starch) fed twice daily for a total of 80 g/d dry matter, or the same CON diet with the addition of EO at 3 mg/d. Protozoa were also quantified in both fermenter contents and unpooled daily effluent samples. The statistical model included fixed effects of treatment and fermenter, and random effect of period, using either MaAsLin2 or the adonis2 function in the vegan package of R for microbial features, or SAS mixed model for protozoal counts. The results were deemed significant at $Q < 0.05$ and $p < 0.05$ for the MaAsLin2 and adonis2/SAS analyses, respectively. For the protozoal populations, the treatments had no significant effect ($p > 0.10$) on the total counts, differentiated groups, or cell outflow. The addition of EO increased the relative abundance of *Methanobrevibacter* and decreased that of uncultured Methanomethylophilaceae ($Q < 0.05$). In contrast, EO addition had no significant effect on archaeal α- or β-diversity ($p > 0.05$). Despite not having a significant effect on the β-diversity of archaeal and bacterial communities, EO decreased ($p < 0.05$) α-diversity indices in prokaryotic communities. Moreover, EO decreased ($Q < 0.01$) the relative abundance of Clostridia UCG-014, Rikenellaceae RC9 gut group, and Christenellaceae R7 group, and increased ($Q < 0.01$) others including *Treponema*, Succinivibrionaceae UCG-002, and *Ruminococcus*. Offsetting shifts in the relative abundance of fiber-degrading bacteria and detailed methanogen communities deserves further investigation including predicted metabolic pathways impacted by population shifts

assistance in the lab.

Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Wenner BA.
Data curation: Park T, Wenner BA.
Formal analysis: Park T, Wenner BA.
Methodology: Praisler G, Wenner BA.
Software: Park T.
Validation: Park T, Wenner B.
Investigation: Praisler G.
Writing - original draft: Park T.
Writing - review & editing: Park T, Praisler G, Wenner BA.

Ethics approval and consent to participate
All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee protocol #2013A00000073.

induced by this EO combination.

Keywords: Dual-flow continuous culture, Essential oils, Methane, Rumen microbiome

INTRODUCTION

Methane (CH_4) emissions from dairy cattle pose a significant environmental challenge, notably contributing to the global greenhouse gas footprint of agricultural activities. Therefore, effectively mitigating CH_4 in dairy production is crucial to meet global environmental targets, such as those outlined in the Global Methane Pledge [1]. In this context, dietary interventions have recently garnered increasing attention as a promising approach to reduce enteric CH_4 emissions from ruminants.

Among these interventions, the use of essential oil-based additives, such as Agolin® Ruminant (AR, Agolin SA), has gained attention for its potential to modify rumen fermentation and decrease CH_4 output. Agolin® Ruminant is an essential oil (EO) product containing a blend of coriander seed oil, eugenol, geranyl acetate, and geraniol [2] geared towards reducing CH_4 emissions in cattle and some agencies have issued carbon credits to producers utilizing the product. This product is also available in an organic certified carrier for use in organic farms. The EOs in this product presumably disrupt the phospholipid membrane of archaea, leading to a decrease in CH_4 production and an increase in the cow's nutrient utilization efficiency [3]. Previous studies have employed both *in vitro* and *in vivo* experiments, revealing the additive's capacity to alter rumen fermentation patterns and reduce CH_4 production [2,4]. For example, studies have highlighted the efficacy of EO in lowering CH_4 emissions in dairy cattle [5], coinciding with improvements in milk production and components in response to EO supplementation [6]. However, the effects of EO on the rumen microbiome and their association with CH_4 mitigation remain largely unexplored.

One of the main concerns regarding the administration of AR (as well as other EO-based products) is whether it also disrupts the rumen fibrolytics when fed to cattle, as very few studies have characterized the changes in the microbiome in response to Agolin® Ruminant [7]. In a recent study, AR fed in dual-flow continuous culture (DFCC) decreased CH_4 production by 10% in less than two weeks of adaptation [8] but did not decrease fiber digestibility nor did it significantly alter volatile fatty acid production, prompting researchers to investigate which rumen microbes might be impacted by AR to both explain the mechanisms underlying decreased CH_4 emission and gauge potential drawbacks.

To address this gap, our study employed samples from the previous DFCC study coupled with advanced microbiome analysis to investigate the effects of EO on rumen microbial populations and CH_4 emissions in dairy cattle. This approach allows for a controlled simulation of the rumen environment, providing detailed insights into the microbial dynamics within the rumen and their relation to CH_4 production [9].

This study aimed to determine the microbial factors associated with CH_4 inhibition by EO using a DFCC system. We hypothesized that EO either decreased the protozoal population or induced shifts in the archaeal or bacterial abundance to decrease CH_4 production. This study contributes to the evolving field of enteric CH_4 mitigation in dairy cattle, offering valuable insights for the development of sustainable and effective dietary strategies in the dairy industry.

MATERIALS AND METHODS

DFCC treatments

The DFCC system utilized for this study implements updated approaches previously characterized

[9]. Effluent samples used for DNA extraction and protozoal enumeration came from a previous study [6] evaluating the efficacy of several organic-certified CH_4 inhibitors. Briefly, DFCC (n = 4) were inoculated from two Jersey cows housed at the Waterman Dairy Farm (The Ohio State University, Columbus, OH) under care according to the Institutional Animal Care and Use Committee protocol #2013A00000073. These cows were fed a lactating diet common to the herd at the time [10] including 39% corn silage, 9.9% wet brewer's grains, 7.7% soybean meal, 4.6% bypass soy, 0.9% bypass fat, 0.9% molasses, and 3.4% vitamin mineral premix (without monensin or other feed additives). A Latin square treatment arrangement was applied to evaluate four treatments which are detailed previously. Of these, only two were considered for the current study due to a lack of efficacy previously reported for the other two treatments. The control (CON) diet was fed twice daily a total of 80 g/d dry matter (DM) diet (60:40 concentrate:orchardgrass pellet mix, 17.1% crude protein [CP], 33.0% neutral detergent fiber [NDF], 0.1% acid detergent fiber [ADF], and 27.1% starch; Table 1) and one fed CON diet with 3 mg/d dose of supplemental organically-certified EO (Agolin® Naturu, Agolin SA). Fermenter effluent samples (d8-11) were subsampled and stored at −80°C for metagenomic DNA extraction. Additionally, fermenter samples (d5) and effluent samples (d8-11) were fixed in formalin and stored for enumeration based on the procedure outlined in Dehority, 1984 [11]. The outflow of cells in effluent was contrasted to the fermenter contents populations to estimate generation time for protozoa, and protozoa were divided into the following types: *Charonina* (based on previous reports of high enrichment in DFCC [12]), Isotrichidae, *Diplodinium*, and other entodinia – either small (< 100 μm in length) or large (> 100 μm in length).

Metagenomic DNA extraction and metataxonomic analysis of the rumen bacteriome and archaeome

Metagenomic DNA from the effluent samples was extracted using the repeated bead beating plus column method [13] and purified with Qiagen mini-stool kits from Thermo Fisher Scientific. The researchers conducting the extractions were blinded to treatment during the DNA extraction and subsequent taxonomy classification. The quality and quantity of DNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, NanoDrop Technologies) and further evaluated through 1% agarose gel electrophoresis. Amplicon libraries, targeting the V4 hypervariable region of the 16S rRNA gene were generated using the 515F and 806R universal primer pair [14] and each library was uniquely barcoded for multiplexing at The Ohio State University's Molecular and Cellular Imaging Center (Wooster, OH). The libraries were then pooled and sequenced using an Illumina MiSeq sequencer (2 × 300 bp paired-end sequencing). Quality

Table 1. Control diet (CON) fed to dual-flow continuous cultures at 80 g/day, twice daily, (60:40 concentrate:orchardgrass pellet mix), with or without 3 mg/d EO[1)]

Nutrient	Diet composition
Dry matter (g/d)	80.0
Crude protein	17.1%
Starch	27.1%
Water soluble carbohydrates	8.4%
Neutral detergent fiber	33.0%
Acid detergent fiber	20.1%
Fat	2.2%
Ash	9.2%

[1)]CON, 80 g/d of control diet; EO, CON diet + 3 mg/d blended essential oil product.

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control measures, including denoising, merging, and chimera removal, were performed using QIIME2 version 2022.2 [15], following an approach similar to that described previously [16].

In this analysis, the final number of quality amplicon sequencing variants (ASVs) was 3,662,173 (3,653,369 bacterial ASVs and 8,804 archaeal ASVs) and they were classified taxonomically based on a 99% similarity . This classification was conducted using the weighted Silva 16S pre-trained classifier (NR 138 version; [17–19]) to enhance classification accuracy. Only phyla, families, and genera with a relative ASV abundance ≥ –0.5% in at least one treatment were included in the analysis. ASV BIOM tables for archaea and bacteria were separated prior to downstream analysis. Alpha diversity indices such as richness, Chao1, Shannon's index, Pielou's evenness, Good's coverage, and Faith's phylogenetic diversity were derived from the average rarefied ASV table (repeated 100 times, referenced in [20]). The microbial metabolic functions were predicted using PICRUSt2 [21] utilizing 16S ASVs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were reconstructed from these predictions, representing microbial metabolic functions based on KEGG ortholog profiles derived from PICRUSt2.

Statistical analysis

Statistical assessment of the microbial relative abundance data was carried out using MaAsLin2 [22] to examine the impact of EO. This analysis involved centered log-ratio normalization and a linear model without data transformation. The statistical model encompassed the fixed effects of both the treatment and fermenter, along with the random effect of the period. The mixed model was implemented using either MaAsLin2 or SAS 9.4 (SAS Institute), particularly for analyzing effluent protozoal counts. Alpha diversity metrics and effluent protozoal counts were examined using the SAS mixed model. A significance threshold of $Q < 0.05$ (Benjamini-Hochberg FDR-corrected *P*-values) was set for MaAsLin2 analyses, and a $p < 0.05$ was used for SAS analyses. Bray-Curtis and Jaccard distance matrices were compared to evaluate the overall microbial community differences resulting from EO. This was done using the adonis2 function within the 'vegan' package (version 2.5–7) of R [23]. The same statistical models were applied to conduct these comparisons. Additionally, PCA results were graphically represented through plots created using the 'ggfortify' package in R (version 3.5.3) [24].

RESULTS

Protozoal populations were primarily entodiniomorphids (74%, Table 2) – mostly shorter in length than 100 μm – while another 16% of the protozoal population was *Charonina* spp. Both protozoal populations within fermenters and daily flow of protozoa in effluent were unchanged by treatment, as was generation time ($p > 0.10$). In the control treatment, no members of Isotrichidae were detected in effluent samples despite being identified in fermenter populations.

Fig. 1 provides a comprehensive overview of the primary bacterial communities identified in the effluent samples at the phylum level. Our analyses revealed five major phyla – each representing more than 0.5% of the average relative abundance in either the control or treatment groups – accounting for 98.4% of the relative abundance of the detected ASVs. These phyla include Bacteroidota (45.6%), Firmicutes (27.6%), Proteobacteria (19.7%), Spirochaetota (3.5%), and Patescibacteria (1.9%) (Fig. 1A). At the genus level, 34 major bacterial genera accounted for 90.3% of the total bacterial population, as shown in Fig. 1B.

In terms of archaeal communities, all ASVs were classified into two families: Methanobacteriaceae (97.2%) and Methanomethylophilaceae (2.8%), with only three genera detected (*Methanobrevibacter*, *Methanosphaera*, and uncultured genus within the Methanomethylophilaceae

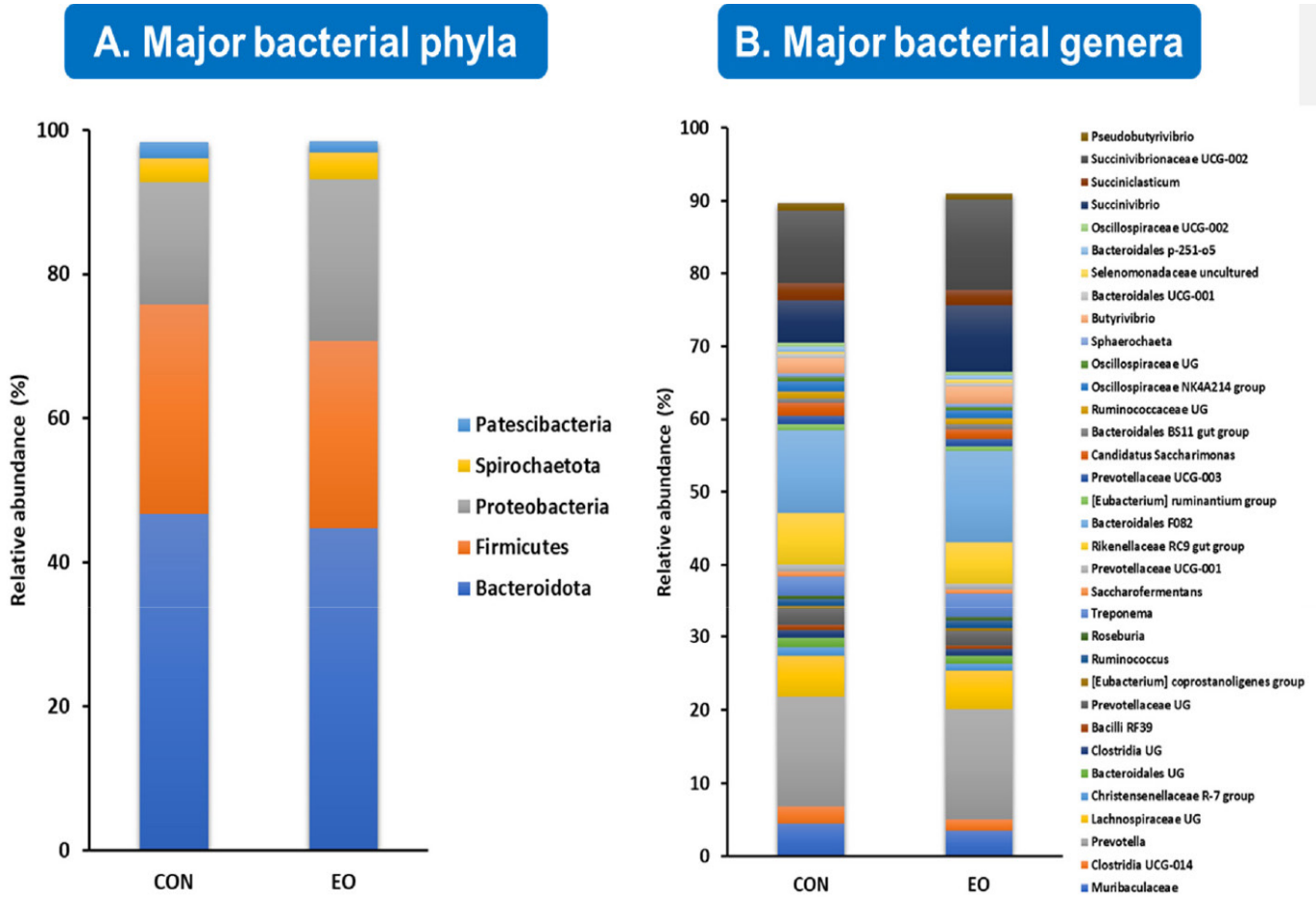
Table 2. Counts of protozoal populations, calculated daily effluent flow, and generation time for dual-flow continuous culture fermenters fed either a control diet or a control diet with 3 mg/d supplemental EO

	CON[1)]	EO	SEM	*p*-value[2)]
Fermenter populations [cells (× 10^3)/mL]	24.5	21.1	5.12	0.17
Small entodinia	18.2	16.0	3.84	0.18
Large entodinia	0.200	0.200	0.108	0.80
Diplodinium	1.40	1.60	0.568	0.76
Isotrichid	0.800	0.700	0.367	0.58
Charonina	3.90	2.60	1.40	0.13
Daily flow [cells (× 10^7)/d]	36	33.9	0.360	0.55
Small entodinia	30.4	31.0	2.78	0.83
Large entodinia	0.00736	0.00723	0.00806	0.99
Diplodinium	0.244	0.331	0.0994	0.33
Isotrichid	-	0.00737	0.00534	0.27
Charonina	0.098	0.148	0.0419	0.41
Generation time[3)] (h)	42.9	30.8	14.6	0.51

[1)]CON, 80 g/d of control diet; EO, CON diet + 3 mg/d blended essential oil product.

[2)]*p*-values reported for the main effect of EO versus CON.

[3)]Generation time = total pool size of cells (i.e., fermenter counts × fermenter volume / effluent flow of cells/d × 24 h.



Fi. 1. Relative abundance of the major bacterial phyla (A) and genera (B) (only phyla and genera with a relative abundance of ≥ 0.5% in at least one treatment are shown).

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family). Notably, all major bacterial and archaeal taxa were identified in both the control and EO treated groups.

This study also investigated the impact of EO on the diversity of the prokaryotic microbiome, revealing a significant reduction in all alpha-diversity indices – including species richness, evenness, phylogenetic diversity, and comprehensive indices (Shannon's index and Simpson's index) – across both the bacteriota and archaeota (Table 3). Despite these changes, the overall composition of the prokaryotic microbiome, as assessed by Bray-Curtis and Jaccard distance matrices, remained unaffected by EO treatment ($p > 0.1$; Fig. 2).

Comparative analysis between the control and EO-treated groups at the phylum level revealed differential abundances in Proteobacteria, Spirochaetota, and Patescibacteria (Table 4). At the genus level, eight bacterial genera (Clostridia UCG-014, Christensenellaceae R-7 group, unclassified genus within Bacteroidales, Bacilli RF39, Prevotellaceae UCG-001, Rikenellaceae RC9 gut group, and Candidatus *Saccharimonas*) exhibited a positive association with the control group, whereas four genera (*Ruminococcus*, *Treponema*, *Butyrivibrio*, and Succinivibrionaceae UCG-002) were more closely associated with the EO treatment (Table 4). Moreover, among the archaeal genera, *Methanobrevibacter* and an uncultured Methanomethylophilaceae genus exhibited positive and negative associations with EO treatment, respectively (Fig. 3).

An examination of the major KEGG pathways predicted from the bacterial communities revealed differential abundances in 26 pathways between the control and treatment groups as shown in Table 5. Similarly, for the archaeal microbiome, differential abundances were observed in seven and eight major KEGG pathways between the control and EO treatment groups (Table 6).

DISCUSSION

Prior research indicated that EO did not significantly alter fermentation characteristics, such as VFA profiles, various nutrient digestibility estimates, and ammonia concentration in effluent samples from this DFCC experiment but there was a 10% decrease in CH_4 for this study [25], aligning with the findings of a meta-analysis of the effects of AR treatment [2]. Our findings also demonstrated that EO had no impact on protozoal populations, including the specific genera identified in the present study. This is particularly noteworthy, as protozoal inhibition has been suggested as a potential mechanism for EO-induced CH_4 mitigation by targeting hydrogen producers and the protozoa-associated methanogen community [26–30]. However, previous

Table 3. Changes in the alpha-diversity indices of bacteriota and archaeota in response to EO treatment[1)]

Group	Observed ASVs	Chao1 estimates	Pielou's evenness	Faith's phylogenetic diversity	Shannon's index	Simpson's index
Archaea						
CON[2)]	3.75	3.75	0.788	0.437	1.505	0.572
EO	3.25	3.25	0.619	0.374	1.226	0.471
SEM	0.979	0.979	0.217	0.083	0.504	0.184
p-value	0.0008	0.0008	< 0.0001	0.0005	0.0006	< 0.0001
Bacteria						
CON	1,109	1,157	0.773	52.005	7.815	0.972
EO	1,066	1,125	0.728	51.179	7.317	0.953
SEM	104.479	120.563	0.033	2.801	0.408	0.016
p-value	0.0013	0.0023	< 0.0001	< 0.0001	0.0003	< 0.0001

[1)]Good's coverage was at least over 99.4% for all samples.

[2)]CON, control diet; EO, essential oil fed at 3 mg/d.

ASV, amplicon sequencing variant.

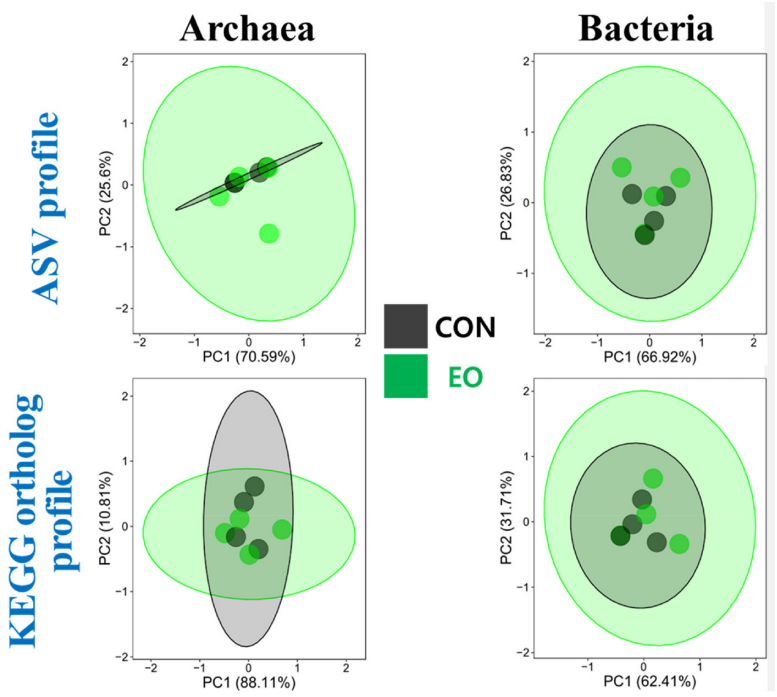

Fig. 2. Principle component analysis plots of the archaeal and bacterial microbiome. The grey dots and ellipse represent the CON group fed 80 g/d dry matter of a control diet and the green dots and ellipse represent the EO group fed the CON diet supplemented with 3 mg/d essential oil. The overall archaeal and bacterial microbiome was not affected by EO ($p > 0.10$). ASV, amplicon sequencing variant; EO, essential oil; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 4. Maaslin2[1] analysis of the bacterial phyla, families, and genera associated with EO treatment

	Associated categorical feature	Coefficient	Relative abundance (%)		SEM	*p*-value	Q-value
			CON[2]	EO			
Bacterial phylum							
Patescibacteria	CON	0.200	2.197	1.554	0.388	< 0.0001	< 0.0001
Proteobacteria	EO	0.241	17.027	22.415	2.891	< 0.0001	< 0.0001
Spirochaetota	EO	0.085	3.324	3.770	0.435	< 0.0001	< 0.0001
Bacterial genus							
Clostridia UCG-014	CON	0.225	2.347	1.525	0.249	< 0.0001	< 0.0001
Christensenellaceae R-7 group	CON	0.106	1.240	0.921	0.125	< 0.0001	< 0.0001
Bacteroidales UG	CON	0.033	1.266	1.115	0.094	< 0.0001	< 0.0001
RF39	CON	0.089	0.668	0.470	0.059	< 0.0001	< 0.0001
Prevotellaceae UCG-001	CON	0.022	0.970	0.847	0.080	< 0.0001	0.001
Rikenellaceae RC9 gut group	CON	0.155	7.098	5.683	0.689	< 0.0001	< 0.0001
Candidatus *Saccharimonas*	CON	0.144	1.823	1.316	0.310	< 0.0001	< 0.0001
Pseudobutyrivibrio	CON	0.071	1.023	0.812	0.119	< 0.0001	< 0.0001
Treponema	EO	0.191	2.602	3.263	0.416	< 0.0001	< 0.0001
Ruminococcaceae UG	EO	0.032	0.902	0.884	0.082	< 0.0001	< 0.0001
Butyrivibrio	EO	0.097	2.140	2.326	0.128	< 0.0001	< 0.0001
Succinivibrionaceae UCG-002	EO	0.155	10.019	12.383	2.405	< 0.0001	< 0.0001

[1]Only the major bacterial phyla and genera, representing at least ≥ 0.5% of the average relative abundance of at least one of the treatment groups that were significantly different ($Q \leq 0.05$) are shown.

[2]CON, control diet; EO, essential oil fed at 3 mg/d.

UCG, uncultured genus-level group; UG, unclassified genus (highest classified taxon level was presented).

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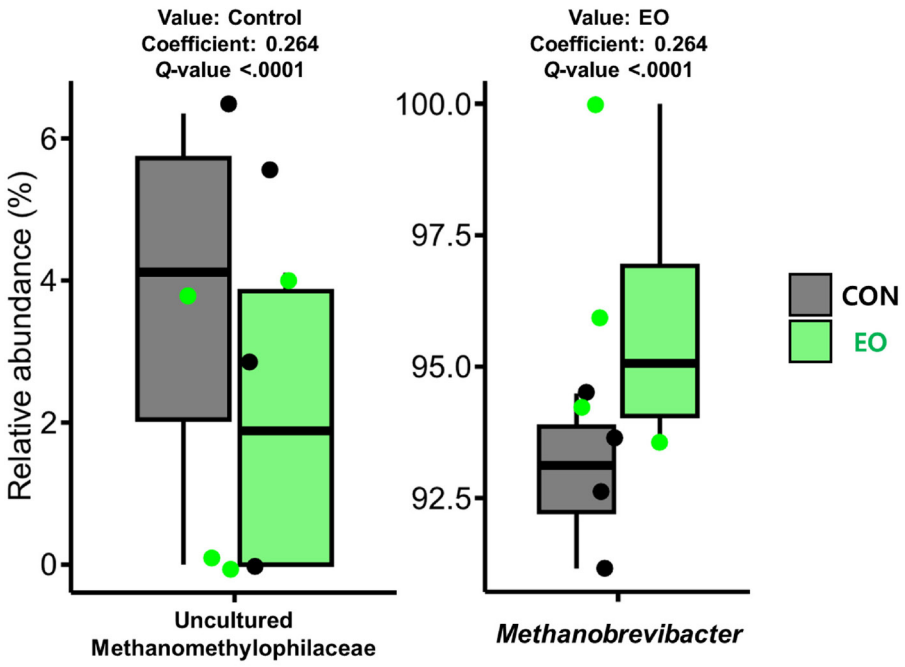

Fig. 3. Differentially abundance of archaeal genera in response to EO treatment. EO, essential oil.

studies on various EOs have not consistently demonstrated this effect [31], highlighting the need for further research to elucidate the potential mechanisms through which EOs influence CH_4 production under different ruminal conditions. Further, DFCC systems modified for protozoal retention tend to harbor fewer protozoa – a drawback which has been documented in previous work [12] and the specific effect of EO on protozoa is recommended for further investigation in other *in vitro* models.

In the current study, analyses using Bray-Curtis and Jaccard matrices revealed that the overall archaeal and bacterial microbiome remained unaffected by EO treatment, aligning with results from previous studies on EO treatments both *in vitro* and *in vivo* [32–34]. Despite minor microbial shifts, the overall microbiome exhibited relative stability.

EO treatment decreased the alpha diversity of the archaeota and bacteriota, which was consistent with findings from an *in vivo* study on lactating dairy cows [7]. This reduction in prokaryotic diversity could be linked to more efficient feed utilization and decreased CH_4 emissions [35]. Previous EO treatments have been reported to inhibit methanogens and decrease CH_4 production [31,36–38], suggesting that the observed lower methanogenic diversity and abundance of methylotrophic methanogen might result from the direct inhibitory effects of EOs. However, further research, particularly involving AR treatment, is needed to validate these suggestions.

Carbohydrate-fermenting Clostridia such as Clostridia UCG-014 and Christensenellaceae R-7 group, which accounted for a significant portion of ruminal hydrogenase transcripts in a previous study [39], play a key role in hydrogen production. Therefore, reducing the abundance of these primary hydrogen producers could decrease CH_4 production. The differential distribution of two butyrivibrios might affect fermentation characteristics, especially butyrate production, due to their similar phenotypic characteristics but distinct phylogenetic classification [40]. Propionate-producing bacteria such as Succinivibrionaceae UCG-002, commonly found in the CH_4-inhibited conditions in the rumen of dairy cattle [41,42], were prevalent in the EO treatment, supporting previous observations of numerically increased propionate levels with EO treatment [25]. The

Table 5. Maaslin2[1)] analysis of the bacterial KEGG pathways associated with EO treatment

KEGG pathways	Associated categorical feature	Coefficient	Relative abundance (%)		SEM	*p*-value	*Q*-value	KEGG pathway description	KEGG objects
			CON[2)]	EO					
ko00030	CON	0.005	1.147	1.138	0.008	< 0.0001	< 0.0001	Pentose phosphate pathway	Carbohydrate metabolism
ko00190	CON	0.009	0.614	0.601	0.006	< 0.0001	< 0.0001	Oxidative phosphorylation	Energy metabolism
ko00260	CON	0.007	1.265	1.252	0.008	< 0.0001	< 0.0001	Glycine, serine and threonine metabolism	Amino acid metabolism
ko00550	CON	0.001	2.206	2.205	0.010	< 0.0001	0.003	Peptidoglycan biosynthesis	Glycan biosynthesis and metabolism
ko00630	CON	0.011	0.965	0.945	0.008	< 0.0001	< 0.0001	Glyoxylate and dicarboxylate metabolism	Carbohydrate metabolism
ko00650	CON	0.010	0.767	0.750	0.005	< 0.0001	< 0.0001	Butanoate metabolism	Carbohydrate metabolism
ko00720	CON	0.009	1.176	1.159	0.008	< 0.0001	< 0.0001	Carbon fixation pathways in prokaryotes	Energy metabolism
ko00730	CON	0.013	1.804	1.771	0.017	< 0.0001	< 0.0001	Thiamine metabolism	Metabolism of cofactors and vitamins
ko00770	CON	0.005	1.936	1.924	0.008	< 0.0001	< 0.0001	Pantothenate and CoA biosynthesis	Metabolism of cofactors and vitamins
ko00970	CON	0.001	2.332	2.329	0.012	< 0.0001	0.002	Aminoacyl-tRNA biosynthesis	Translation
ko01110	CON	0.001	0.618	0.617	0.002	< 0.0001	< 0.0001	Biosynthesis of secondary metabolites	
ko01200	CON	0.006	1.035	1.023	0.005	< 0.0001	< 0.0001	Carbon metabolism	
ko03420	CON	0.005	1.106	1.096	0.011	< 0.0001	< 0.0001	Nucleotide excision repair	Replication and repair
ko00061	EO	0.011	2.069	2.107	0.017	< 0.0001	< 0.0001	Fatty acid biosynthesis	Lipid metabolism
ko00130	EO	0.023	0.611	0.652	0.030	< 0.0001	< 0.0001	Ubiquinone and other terpenoid-quinone biosynthesis	Metabolism of cofactors and vitamins
ko00540	EO	0.041	1.207	1.303	0.045	< 0.0001	< 0.0001	Lipopolysaccharide biosynthesis	Glycan biosynthesis and metabolism
ko00740	EO	0.006	0.746	0.759	0.011	< 0.0001	< 0.0001	Riboflavin metabolism	Metabolism of cofactors and vitamins
ko00780	EO	0.023	2.325	2.406	0.040	< 0.0001	0.016	Biotin metabolism	Metabolism of cofactors and vitamins
ko00790	EO	0.012	0.869	0.894	0.016	< 0.0001	< 0.0001	Folate biosynthesis	Metabolism of cofactors and vitamins
ko02026	EO	0.028	0.721	0.771	0.024	< 0.0001	< 0.0001	Biofilm formation - Escherichia coli	Cellular community - prokaryotes
ko03010	EO	0.001	2.408	2.413	0.012	< 0.0001	0.014	Ribosome	Translation
ko03070	EO	0.010	1.208	1.234	0.020	< 0.0001	< 0.0001	Bacterial secretion system	Membrane transport
ko04122	EO	0.023	1.057	1.110	0.044	< 0.0001	< 0.0001	Sulfur relay system	Folding, sorting and degradation

[1)]Only the major KEGG pathways, representing at least ≥ 0.5% of the average relative abundance of at least one of the treatment groups that were significantly different ($Q \leq 0.05$) are shown.

[2)]CON, control diet; EO, essential oil fed at 3 mg/d.

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Table 6. Essential oil (EO) treatment associated archaeal KEGG pathways analyzed by Maaslin2[1)]

KEGG pathways	Associated categorical feature	Coefficient	Relative abundance (%)		SEM[3]	*p*-value	*Q*-value	KEGG pathway description	KEGG objects
			CON[2)]	EO					
ko00190	CON	0.012	0.962	0.939	0.016	< 0.0001	< 0.0001	Oxidative phosphorylation	Energy metabolism
ko00330	CON	0.009	0.965	0.949	0.011	< 0.0001	< 0.0001	Arginine and proline metabolism	Amino acid metabolism
ko00900	CON	0.002	3.557	3.550	0.027	< 0.0001	< 0.0001	Terpenoid backbone biosynthesis	Metabolism of terpenoids and polyketides
ko03030	CON	0.001	4.885	4.882	0.037	< 0.0001	0.004	DNA replication	Replication and repair
ko03050	CON	0.002	1.211	1.209	0.009	< 0.0001	< 0.0001	Proteasome	Folding, sorting and degradation
ko04112	CON	0.013	1.319	1.291	0.015	< 0.0001	< 0.0001	Cell cycle - Caulobacter	Cell growth and death
ko00121	EO	0.013	2.408	2.455	0.022	< 0.0001	< 0.0001	Secondary bile acid biosynthesis	Lipid metabolism
ko00450	EO	0.003	2.924	2.939	0.017	< 0.0001	0.001	Selenocompound metabolism	Metabolism of other amino acids
ko00550	EO	0.011	3.239	3.291	0.027	< 0.0001	< 0.0001	Peptidoglycan biosynthesis	Glycan biosynthesis and metabolism
ko00790	EO	0.002	3.728	3.743	0.032	< 0.0001	0.001	Folate biosynthesis	Metabolism of cofactors and vitamins
ko02010	EO	0.006	0.891	0.905	0.017	< 0.0001	< 0.0001	ABC transporters	Membrane transport
ko03420	EO	0.002	3.613	3.629	0.042	< 0.0001	0.004	Nucleotide excision repair	Replication and repair
ko03430	EO	0.007	3.247	3.282	0.016	< 0.0001	< 0.0001	Mismatch repair	Replication and repair
ko04122	EO	0.002	6.180	6.198	0.032	< 0.0001	0.001	Sulfur relay system	Folding, sorting and degradation

[1)]Only the major KEGG pathways, representing at least ≥ 0.5% of the average relative abundance of at least one of the treatment groups that were significantly different ($Q \leq 0.05$) are shown.

[2)]CON, control diet; EO, essential oil fed at 3 mg/d.

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abundance of Spirochaetes, specifically *Treponema* spp., was significantly higher in the EO treatment, and also exhibited greater abundance in EO-treated lactating dairy cows [7]. However, the metabolic versatility of *Treponema* spp. makes it difficult to pinpoint the exact reason for their increased abundance in response to EO treatment [43].

The inhibition of CH_4 production could stimulate anabolic processes requiring metabolic hydrogen, such as fatty acid synthesis [44]. Although we did not observe significant changes in metabolic hydrogen concentrations previously for this study [24], shifts in microbial pathways related to these fermentation end-products are anticipated following EO treatment.

Among the KEGG pathways related to CH_4 metabolism (ko00680), the control group exhibited significant differences in the relative abundance of pathways involved in the pentose phosphate pathway, glycine, serine and threonine metabolism, glyoxylate and dicarboxylate metabolism, and carbon fixation pathways in prokaryotes, whereas EO treatment stimulated pathways related to the metabolism of cofactors and vitamins including riboflavin metabolism and folate biosynthesis.

Acetogens require folate to produce acetate and ATP by reducing two molecules of carbon dioxide through the Wood-Ljungdahl pathway [45,46]. Therefore, although we did not detect significant changes in acetogen growth, our findings suggest that there was a shift in their growth patterns, especially under conditions that inhibited CH_4 production. The stimulation of riboflavin metabolism, associated with the biosynthesis of coenzyme F_{420} [47], could potentially counteract EO-mediated methanogenesis inhibition. However, this function and other vitamin B complex metabolic functions were also more prevalent in the rumen microbiome of cows exhibiting high milk yield and milk protein content [48]. Additionally, archaeal metabolic pathways enriched in response to EO administration, such as the biosynthesis of secondary bile acids and ABC transporters, were found to be negatively correlated with CH_4 emissions [49], suggesting that they play a key role in modulating the microenvironment and facilitating host-microbiome interactions [50–52].

Collectively, our findings suggest that the lack of substantial effects of EO on fermentation and digestion parameters measured parallel to the current study [24] might be due to the absence of a direct inhibitory effect of EO on methanogens. Offsetting shifts in the relative abundance of fiber-degrading bacteria and detailed methanogen communities deserve further investigation including predicted metabolic pathways impacted by population changes. It appears that EO moderates CH_4 production primarily by modulating the ruminal prokaryotic microbiome.

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