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Effect of a garlic and citrus extract supplement on performance, rumen fermentation, methane production, and rumen microbiome of dairy cows

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ABSTRACT

The aim of this trial was to determine the effect of a garlic and citrus extract supplement (GCE) on the performance, rumen fermentation, methane emissions, and rumen microbiome of dairy cows. Fourteen multiparous Nordic Red cows in mid-lactation from the research herd of Luke (Jokioinen, Finland) were allocated to 7 blocks in a complete randomized block design based on body weight, days in milk, dry matter intake (DMI), and milk yield. Animals within each block were randomly allocated to a diet with or without GCE. The experimental period for each block of cows (one for each of the control and GCE groups) consisted of 14 d of adaptation followed by 4 d of methane measurements inside the open circuit respiration chambers, with the first day being considered as acclimatization. Data were analyzed using the GLM procedure of SAS (SAS Institute Inc.). Methane production (g/d) and methane intensity (g/kg of energy-corrected milk) were lower by 10.3 and 11.7%, respectively, and methane yield (g/kg of DMI) tended to be lower by 9.7% in cows fed GCE compared with the control. Dry matter intake, milk production, and milk composition were similar between treatments. Rumen pH and total volatile fatty acid concentrations in rumen fluid were similar, whereas GCE tended to increase molar propionate concentration and decrease the molar ratio of acetate to propionate. Supplementation with GCE resulted in greater abundance of *Succinivibrionaceae*, which was associated with reduced methane. The relative abundance of the strict anaerobic *Methanobrevibacter* genus was reduced by GCE. The change in microbial community and rumen propionate proportion may explain the decrease in enteric methane emissions. In conclusion, feeding GCE to dairy cows for 18 d modified rumen fermentation and microbiota, leading to reduced methane production and intensity without compromising DMI or milk production in dairy cows. This could be an effective strategy for enteric methane mitigation of dairy cows.
Key words: garlic and citrus extract, methane production, methane intensity, dairy cow, rumen microbiome

INTRODUCTION

Methane (CH_4) is one of the main contributors to global greenhouse gas emissions and therefore to climate change. Compared with carbon dioxide (CO_2), CH_4 has a shorter lifetime in the atmosphere (12 vs. 1,000 yr), but 28 times higher 100-year global warming potential (EPA, 2020). The environmental impact of ruminant livestock has attracted increasing attention because of the direct emission of CH_4 from the fermentation of feed in the rumen. In the anaerobic environment of the rumen, complex feed compounds are fermented by microorganisms to VFA. During this process, CO_2 and hydrogen (H_2) are produced, which are then used by methanogens to produce CH_4 (Morgavi et al., 2010; Ungerfeld, 2020). Enteric CH_4 not only affects the environment but also leads to a loss of, on average, 6% of the total feed energy consumed by high-producing dairy cows (Niu et al., 2018), which makes it an economic issue for farmers.

Research suggests that enteric CH_4 mitigation is possible either by inhibiting methanogenic archaea directly by reducing hydrogen (H^+) production or by alternative pathways to utilize H^+ (McAllister and Newbold, 2008; Martin et al., 2010). Different strategies to achieve CH_4 mitigation were tested in the recent past to use one of the above-mentioned mechanisms, with dietary manipulation and supplementation with feed additives being extensively researched (Haque, 2018; Sun et al., 2021). Dietary manipulations such as utilizing high-quality forages (e.g., corn silage) in the diet (Hassanat et al., 2013) or replacing fiber with starch by including concentrates high in fermentable carbohydrates (Jiao et al., 2014) may result in significant CH_4 reduction. This is a common practice in the diets of high-yielding dairy cows to reduce their CH_4 production potential.

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Supplementation of these diets with feed additives that either directly inhibit methanogens or modify rumen fermentation leads to less CH_4 production, which may result in a further reduction in CH_4 emission. According to a meta-analysis of enteric CH_4 mitigation strategies conducted by Arndt et al. (2022), 3 product-based strategies—increasing feeding level, decreasing grass maturity, and decreasing dietary forage-to-concentrate ratio—decreased CH_4 per unit of meat or milk by, on average, 12% and increased animal productivity by a median of 17%. Five absolute strategies—CH_4 inhibitors, tanniferous forages, electron sinks, oils and fats, and oilseeds—decreased daily CH_4 by, on average, 21%. Novel feed supplements such as the chemical 3-nitrooxypropanol (3-NOP; Melgar et al., 2020) as well as natural compounds like essential oil blends (Belanche et al., 2020) or seaweed (Roque et al., 2019a) have shown promising results in reducing CH_4 emissions from ruminants.

Other plant secondary compounds from garlic and citrus fruits have been researched to reduce CH_4 production by modifying the rumen microbiome. Garlic oil and its active compounds showed, in vitro, a strong reduction in CH_4 production and the relative abundance of methanogens (McAllister and Newbold, 2008; Patra and Yu, 2015) and a reduced acetate-to-propionate ratio with similar VFA production (Busquet et al., 2005; Kamra et al., 2012) and OM digestibility (Soliva et al., 2011). These suggest a direct inhibition of rumen methanogens without affecting microbial fermentation. In the rumen of sheep, it was observed that feeding garlic oil increased the diversity of rumen methanogens (Ohene-Adjei et al., 2008) and feeding allicin extracted from garlic reduced CH_4 production and methanogens and tended to reduce protozoa (Ma et al., 2016). Citrus fruit extract and its active compounds showed lower CH_4 production, acetate-to-propionate ratio, and relative abundance of methanogens in vitro. In heifers (Balcells et al., 2012) and steers (Seradj et al., 2018) fed citrus extract, the rumen microbiome, especially the relative abundance of lactate-consuming bacteria, was modified, which was associated with an increase in rumen propionate and a decrease in lactate concentrations. In an in vitro experiment with a batch culture (Ahmed et al., 2021a) and rumen simulation technique (**RUSITEC**; Eger et al., 2018), supplementation with garlic granules (*Allium sativum*) and citrus extract (*Citrus aurantium*) (**GCE**) led to a decrease in the abundance of methanogenic archaea, a 54% or 95% reduction in CH_4 production, respectively, and an increase in VFA production. Dairy cows fed GCE showed significantly reduced CH_4 production and higher milk yield (Vrancken et al., 2019). The supplementation of GCE has also demonstrated a reduction in CH_4 production of crossbred beef steers (Roque et al., 2019b) and Holstein veal calves (Brand et al., 2021). Therefore, supplementation with GCE could be one solution to reduce CH_4 emissions from ruminants.

On the basis of these findings, we hypothesize that GCE might be able to reduce enteric CH_4 emissions of lactating dairy cows by altering the ruminal microbial community without affecting animal performance. The objective of this study was to investigate the effect of GCE on CH_4 emissions, performance, rumen fermentation, and rumen microbiome of lactating dairy cows.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted at the Natural Resources Institute Finland (Luke) under Regional State Administrative Agency permission ESAVI/7012/2019 in accordance with the guidelines established by the European Community Council Directive 2010/63/EU for animal experiments complying with the ARRIVE guidelines (Kilkenny et al., 2010). Fourteen healthy multiparous Nordic Red cows in mid-lactation from the research herd of Luke (Jokioinen, Finland) were used in this experiment. The sample size of 7 replicates per treatment was calculated before the study by assuming the power of the study $(1 - \beta)$ as 0.85, the level of significance (α) in a one-sided test to be 0.05, and an assumption to detect at least a 10% reduction in daily CH_4 production. To minimize the differences between the groups, the animals were allocated to 7 blocks in a complete randomized block design on the basis of BW (650 ± 49 kg), DIM (110 ± 37 d), DMI (28.2 ± 1.6 kg/d), and milk yield (39.8 ± 3.9 kg/d). The 2 animals within each block were randomly allocated to a diet without (control; **CTRL**) or with GCE applied at 44 g/d (Table 1). The GCE consisted of garlic and citrus extract (50%) and limestone and vegetable oil (50%); it contained 960 g/kg DM as fed, and 377 g/kg ash, 79 g/kg CP, 53 g/kg ether extract, and 151 g/kg crude fiber on a DM basis. Half of the daily GCE (22 g/d) was mixed with 3 kg of TMR offered in the morning (0800 h) and the other half during the evening (1630 h) feeding, and animals were allowed to consume the mixtures for 2 h. The proportion of consumed TMR was recorded, and the leftovers were top-dressed on the main TMR for the rest of the feeding period. The same procedure was followed for the CTRL treatment without adding GCE. The main portions of TMR were delivered to cows by an automatic feeding wagon (TR Feeding Robot, Pellon Group Ltd.) at 1000, 1300, 1830, and 2000 h. The TMR had a forage-to-concentrate ratio of 50:50 on a DM basis and comprised grass silage and concen-

trate pellets. The formulation of TMR and the nutrient composition of grass silage and concentrate pellets are presented in Table 1. The grass was ensiled from swards of mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) with a formic acid–based ensiling additive (AIV 2 Plus, Eastman Ltd.) applied at a rate of 5.0 L/t of fresh grass. The grass was lightly wilted before ensiling to achieve a DM content of 25%. All cows received the same TMR. The cows were housed in a freestall barn (140 freestalls) with controlled access to their feed using individual feed bins controlled by neck collars and automated opening gates (RIC, Insentec), with free access to water and salt blocks during the adaptation period. The cows were milked twice daily at 0700 and 1645 h in a 2 × 6 tandem milking parlor. The animals were monitored daily for health problems, and any abnormalities and infections were recorded and treated according to the general barn guidelines. All cows completed the whole experimental period (18 d for each cow and 60 d in total) without major symptoms that could affect the results. The experimental period for each block of 2 cows (one for each of the CTRL and GCE groups) consisted of 14 d of adaptation followed by 4 d of CH_4 measurements inside the open circuit respiration chambers, with the first day being used for acclimation. During the chamber measurements, the cows were milked in situ. Each block of 2 cows entered the experiment 7 d after the previous block to ensure the availability of respiration chambers for CH_4 measurements. The cows were restrained using a neck yoke in a dedicated platform (180 × 126 cm) covered with a rubber mat and had continuous access to experimental feeds, fresh water, and salt blocks. The cows from each block were randomly allocated to different chambers.

Measurements and Chemical Analyses

Feed refusals were measured on a daily basis, and daily feed intake was determined as the amount of TMR offered minus refusals for every cow. Cows were weighed on a daily basis except for 4 d during CH_4 measurements in respiration chambers by a walk-through static scale (Pellon Group) every time they left the milking parlor.

Grass silage and concentrates were collected over sampling days, kept at 4°C, composited for each block, and stored at −20°C until chemical analysis using routine procedures as described by Ahvenjärvi et al. (2018). Silage DM content and consequently DMI were corrected for the loss of volatiles according to Huida et al. (1986). Milk yields were recorded gravimetrically (Pellon SAC) throughout the trial. Milk samples, taken from 4 consecutive milkings starting in the afternoon of d 16 for each block while the cows were in respiration chambers, were preserved with Bronopol tablets (Valio Ltd.) and stored at 4°C until analysis of fat, protein, lactose, total solids, urea, and SCC (MilkoScan FT6000, Foss Electric). The cows entered the respiration chambers in the morning (0900 h) of d 15 for 4 d to provide 3 full days of measurements. Over 3 d, gas exchanges were recorded by measuring concentrations of CH_4, CO_2, and H_2 in the inlet and outlet air using dedicated analyzers (Columbus Instruments) located in the Minkiö dairy barn (Jokioinen, Finland). Respiration chambers and their calibration are described in detail by Bayat et al. (2022). Briefly, gas analysis was configured to allow automatic measurements at 3.5-min intervals from each chamber and the reference air. The zero and span calibrations of analyzers were conducted at the beginning of every 3-d measurement for each block of cows using standard gases (AGA Ltd.). The concentration of measured gases and airflow (corrected based on standard temperature and pressure) were recorded and monitored (Oxymax v. 4.86, Columbus Instruments), and the data captured were used for further calculations. Environmental control of temperature across a range from 15 to 22°C and relative humidity of 50 to 70% was maintained through an adjustable air conditioning system.

Rumen Fermentation and Microbial Analysis

On the last day of the sampling period for each block, a 500-mL rumen fluid sample was collected from each cow at about 1000 h using stomach tubing (Ruminator,

Table 1. Formulation and chemical composition (% of DM) of grass silage, concentrate, and TMR

Item[1]	Grass silage	Concentrate	TMR
Feed ingredient			
Grass silage[1]	—	—	50.0
Barley	—	22.0	11.0
Wheat	—	11.0	5.50
Oat	—	17.0	8.50
Molassed sugar beet pulp	—	12.5	6.25
Rapeseed meal	—	35.0	17.5
Mineral and vitamin premix[2]	—	2.50	1.25
Chemical composition			
DM	34.0	87.9	49.0
OM	90.4	92.6	91.3
CP	13.8	21.2	17.8
NDF	47.8	24.3	37.0

[1]Mean fermentation characteristics of experimental silage: pH, 4.35 ± 0.07; in DM (%) lactic acid, 3.58 ± 1.02; acetic acid, 1.32 ± 0.18; propionic acid, 0.064 ± 0.049; butyric acid, 0.012 ± 0.008; soluble N (% total N), 40.3 ± 4.0; ammonium N (% total N), 3.09 ± 0.29.

[2]Proprietary mineral and vitamin supplement (Lypsykivennäinen Tiineys+) containing (g/kg) calcium (210), magnesium (90), sodium (95), phosphorus (15), (mg/kg) inorganic selenium (20); organic selenium (10); α-tocopheryl acetate (2,000); and D-biotin (30).

Geishauser). Rumen fluid collected from the ventral sac of the rumen was immediately measured for pH using a portable pH meter (VWR International). A subsample of 5.0 mL of rumen fluid was immediately preserved with 0.5 mL of saturated mercury (II) chloride and 2.0 mL of 1 *M* sodium hydroxide and stored at −20°C for VFA determination. Another subsample of 15.0 mL of rumen fluid for the determination of ammonia was immediately preserved with 0.3 mL of sulfuric acid (50%, vol/vol) and stored at −20°C. Rumen VFA and ammonia N concentrations were determined according to Huhtanen et al. (1998) and McCullough (1967), respectively.

For microbial analyses, samples were aliquoted into 2-mL tubes, snap-frozen in dry ice, and stored at −80°C until DNA extraction. Total DNA was extracted from 0.5 mL of rumen liquid, as described by Rius et al. (2012). For microbial amplicon sequencing, universal primers 515F and 806R (Caporaso et al., 2011) targeting the bacterial 16S rRNA gene V4 region, and 316F and 539R primers (Sylvester et al., 2004) targeting the ciliate protozoa 18S rRNA gene were used. Libraries were prepared and sequenced on the Illumina MiSeq platform using 2 × 250 bp chemistry in the Finnish Functional Genomics Centre (Turku, Finland). Demultiplexing of sequences, adapter removal and sorting sequences by barcode was performed by the sequencing data provider. The sequence reads are available in the National Center for Biotechnology Information Sequence Read Archive under BioProject PRJNA872093.

Bacterial sequencing data were processed using Qiime 2 (Bolyen et al., 2019). Briefly, quality control, filtering of chimeric reads, and clustering of bacterial sequences into amplicon sequence variants (**ASV**) was performed using DADA2 (Callahan et al., 2016). Bacterial ASV taxonomy was assigned using the Silva 138 database (Quast et al., 2013), where sequences were trimmed to only include 250 bases from the V4 region, bound by the 515F/806R primer pair. Archaeal taxonomy was assigned using the RIM-DB database (Seedorf et al., 2014). Ciliate protozoa sequencing data were processed using Qiime v. 1.9.1 (Caporaso et al., 2010). Briefly, the alignment of paired-end reads was performed using SeqPrep in Qiime. Quality-filtered (>q20) bacterial sequences were clustered into operational taxonomic units (**OTU**) at 97% similarity using UCLUST (Edgar, 2010). Chimeric reads were filtered out using Usearch61 (Edgar, 2010). The ciliate protozoa OTU taxonomy was assigned using the ciliate protozoa database (Kittelmann et al., 2015). After quality control, bacterial sequencing data presented 21,646 to 35,090 sequencing reads, archaea 668 to 1,848 sequencing reads, and ciliate protozoa 38,930 to 51,541 sequencing reads per sample.

Calculations

Energy-corrected milk was calculated using the equation proposed by Sjaunja et al. (1990) based on milk fat, protein, and lactose yields. Methane, CO_2, and H_2 production were calculated by multiplying air flow and differences in gas concentrations in the inlet and outlet chambers. Methane yield was calculated as grams of CH_4 emitted per kilogram of DMI by individual animals per day, and CH_4 intensity was calculated as grams of CH_4 emitted per kilogram of ECM from individual animals per day. Data on SCC were $\log_{10}$-transformed before statistical analysis to ensure normal distribution.

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (version 9.4, SAS Institute Inc.), with block and treatment included as fixed effects in the model. The natural variation between experimental animals was corrected by blocking the animals on average DMI and milk yield, collected 2 wk before the start of the trial when all the cows received the CTRL diet. Least squares means and standard error of means are reported, and differences between treatments were considered significant when $P \leq 0.05$ and a tendency was recorded when $0.05 < P \leq 0.10$.

Rumen microbial community α-diversity was calculated using the Shannon index, Pielou evenness estimate, and observed number of ASVs. For α-diversity, all samples were subsampled to the same depth, equivalent to the lowest number of reads per sample. To evaluate whether treatment was significantly associated with rumen microbial community composition, between-sample diversity was calculated as Bray–Curtis dissimilarities following Hellinger transformation and visualized using principal coordinate analysis (**PCoA**) as implemented in the *MicrobiotaProcess* R package (Xu and Yu, 2021). The significance of groups was evaluated by distance-based permutational multivariate ANOVA (adonis) and defined at $P < 0.05$ level after 999 permutations.

The treatment effect on individual microbial taxa was evaluated by ANOVA as described above. Before the test, low-abundance taxa with <0.01% relative abundance across all samples were filtered out. The number of reads was log base transformed $[\log_2(x + 1)]$ and standardized by data centering. For easier interpretation of results, the number of reads of microbial taxa identified as significantly affected by treatment in ANOVA analysis was converted to compositional data

Table 2. Effect of garlic and citrus extract supplement on intake of diet components and nutrients by dairy cows

	Treatment[1]			
Intake (kg/d)	CTRL	GCE	SEM	*P*-value
Silage DM	13.2	13.0	0.24	0.60
Concentrate DM	13.8	13.6	0.24	0.64
GCE-TMR mix[2]	5.81	5.13	0.31	0.18
DM	27.0	26.7	0.42	0.66
OM	24.7	24.4	0.43	0.65
CP	4.82	4.76	0.08	0.64
NDF	9.91	9.78	0.18	0.62

[1]CTRL = control; GCE = garlic and citrus extract supplement.

[2]Intake of GCE-TMR mix with or without garlic and citrus extract supplement (0 or 44 g/d).

and presented as relative percent abundances within each microbial category.

RESULTS

Dry matter intake was similar ($P \geq 0.66$) between treatments (Table 2). The intake of GCE and CTRL mash concentrates was not different ($P = 0.18$). The BW of cows tended ($P = 0.08$) to be lower for GCE than for CTRL. Milk yield ($P = 0.80$) and ECM yield ($P = 0.57$) were not different between treatments (Table 3). Similarly, protein, fat, and lactose concentrations and yields and SCC were unaffected ($P \geq 0.31$) by treatment. Milk production efficiency calculated as milk yield or ECM divided by DMI was also not affected ($P \geq 0.34$) by GCE.

Methane production (g/d) was 10.3% lower ($P = 0.018$) for GCE than for CTRL (Table 4). The GCE also indicated a tendency ($P = 0.06$) toward lower CH_4 yield (g/kg of DMI) of 9.7%. Methane intensity (g/kg of ECM) was 11.7% lower ($P = 0.039$) for GCE compared with CTRL. Daily H_2 and CO_2 emissions did not differ ($P \geq 0.11$) between the treatments.

Table 4. Effect of garlic and citrus extract supplement on methane, carbon dioxide, and hydrogen emissions of dairy cows

	Treatment[1]			
Item	CTRL	GCE	SEM	*P*-value
Methane				
(g/d)	575[a]	516[b]	12.8	0.018
(g/kg of DMI)	21.8	19.7	0.63	0.059
(g/kg of ECM)	13.7[a]	12.1[b]	0.42	0.039
Carbon dioxide				
(g/d)	16,342	15,697	323	0.21
Hydrogen				
(g/d)	1.68	1.19	0.18	0.11

[a,b]Mean values in the same row with different superscripts differ ($P < 0.05$).

[1]CTRL = control; GCE = garlic and citrus extract supplement.

Table 3. Effect of garlic and citrus extract supplement on milk yield, milk composition, and feed efficiency of dairy cows

	Treatment[1]			
Item	CTRL	GCE	SEM	*P*-value
Yield (kg/d)				
Milk	39.3	38.9	1.13	0.80
ECM[2]	42.1	42.8	0.83	0.57
Fat	1.74	1.80	0.04	0.30
Protein	1.42	1.45	0.03	0.60
Lactose	1.82	1.78	0.05	0.55
TS	5.40	5.44	0.11	0.83
Concentration (%)				
Fat	4.46	4.64	1.27	0.35
Protein	3.65	3.73	1.09	0.60
Lactose	4.65	4.58	0.45	0.31
TS	13.8	14.1	2.4	0.60
Urea (mg/100 mL)	32.1	32.5	1.41	0.85
SCC[3] ($\times 10^3$/mL)	40.7	26.3	0.23	0.60
Feed efficiency				
Milk/DMI	1.47	1.46	0.03	0.92
ECM/DMI	1.57	1.61	0.02	0.34

[1]CTRL = control; GCE = garlic and citrus extract supplement.

[2]ECM calculated according to Sjaunja et al. (1990).

[3]Data transformed using $\log_{10}$ for statistical analysis and back-transformed for interpretation.

Rumen pH and total VFA concentration were not affected ($P \geq 0.32$) by GCE (Table 5). The molar concentration of propionate tended ($P = 0.089$) to be higher and that of caproate lower ($P = 0.029$) for GCE. Tendencies toward a lower molar ratio of acetate-to-propionate and acetate plus butyrate-to-propionate ($P \leq 0.09$) were observed for animals fed GCE.

Bacterial, archaeal, and ciliate protozoan community composition was explored at the phylum, family, and species levels, respectively. The α-diversity of rumen bacteria, archaea, and ciliate protozoa, estimated as

Table 5. Effect of garlic and citrus extract supplement on rumen fermentation characteristics of dairy cows

	Treatment[1]			
Item	CTRL	GCE	SEM	*P*-value
Rumen pH	6.72	6.78	0.04	0.36
Ammonia N (m*M*)	2.48	2.35	0.09	0.35
Total VFA (m*M*)	101	96.5	3.40	0.32
VFA profile (mol/100 mol)				
Acetate (A)	67.5	66.0	6.00	0.13
Propionate (P)	16.5	19.5	9.97	0.089
Butyrate (B)	13.1	11.6	5.73	0.14
Isobutyrate	0.64	0.61	0.25	0.50
Valerate	1.13	1.24	0.54	0.22
Isovalerate	0.66	0.66	0.63	1.00
Caproate	0.45[a]	0.35[b]	0.23	0.029
A:P	4.10	3.50	0.19	0.085
A + B:P	4.89	4.13	0.25	0.082

[a,b]Mean values in the same row with different superscripts differ ($P < 0.05$).

[1]CTRL = control; GCE = garlic and citrus extract supplement.

Table 6. Effect of garlic and citrus extract supplement on bacterial, archaeal, and protozoal ciliates' α-diversity indices

Item	Treatment[1]		SEM	*P*-value
	CTRL	GCE		
Bacteria				
Pielou evenness	0.94	0.92	0.006	0.14
Observed no. of ASV[2]	798	723	31.3	0.15
Shannon entropy	9.03	8.74	0.10	0.11
Archaea				
Pielou evenness	0.52	0.55	0.02	0.41
Observed no. of ASV	10.2	9.5	0.43	0.33
Shannon entropy	1.20	1.24	0.05	0.59
Protozoal ciliates				
Pielou evenness	0.65	0.63	0.02	0.61
Observed no. of ASV	19.7	19.3	0.91	0.80
Shannon entropy	1.92	1.87	0.04	0.44

[1]CTRL = control; GCE = garlic and citrus extract supplement.
[2]ASV = amplicon sequence variants.

Shannon index, Pielou evenness, and observed number of ASVs, was not affected ($P \geq 0.11$) by treatment (Table 6). Rumen bacterial and archaeal community structure, assessed by PCoA, did not indicate differences between CTRL and GCE treatments (adonis test $P > 0.1$; Figure 1A and 1B). However, the ciliate protozoa community structure was influenced by the dietary additive ($P = 0.017$; Figure 1C).

The bacterial community at the phylum level (Figure 2), irrespective of diet, was dominated by *Firmicutes* (39–49%), *Bacteroidota* (29–45%), and *Proteobacteria* (3–16%). *Patescibacteria*, *Spirochaetota*, and *Verrucomicrobiota* were detected at low abundance (1–4%), and the remaining phyla were rare (<2%).

Family and genus *WCHB1–41* affiliated with phylum *Verrucomicrobiota* were lower ($P = 0.02$), whereas *Succinivibrionaceae* were more abundant ($P = 0.04$) in the GCE compared with the CTRL diet (Table 7). At the species level, both *WCHB1–41* sp. ($P = 0.02$) and *Prevotella* sp. ($P = 0.05$) were less abundant in the GCE compared with the CTRL group.

Archaeal community (Figure 3), irrespective of diet, was dominated by *Methanobrevibacter gottschalkii* clade (33–72%), *Methanobrevibacter ruminantium* clade (11–51%), and *Methanosphaera* sp. *ISO3-F5* (10–16%). Among *Methanomassiliicoccaceae*, *Group10* (2–5%), *Group12* sp. *ISO4-H5* (1–6%), and *Group8* sp. *WGK1* (1–3%) were the most abundant. At the genus level, *Methanobrevibacter* was detected at lower abundance in GCE than in CTRL ($P < 0.01$). At the species level, however, no difference was detected between groups (Table 7).

The ciliate protozoa community (Figure 4) was dominated by *Epidinium caudatum* (26–48%), *Isotricha* sp. (7–31%), *Isotricha prostoma* (4–19%), *Entodinium furca monolobum* (2–17%), *Dasytricha rumiantium* (4–17%), and *Isotricha intestinalis* (1–16%). Compared with CTRL, the GCE diet increased abundances of *Isotricha* sp. ($P = 0.04$) and *Entodinium furca monolobum* ($P = 0.05$), and tended to decrease *Entodinium longinucleatum* ($P = 0.05$; Table 7).

DISCUSSION

Enteric Methane Emissions

In this study, GCE decreased daily CH_4 emissions significantly compared with CTRL. A similar CH_4-reducing effect of GCE was observed in various in vitro studies (Eger et al., 2018; Ahmed et al., 2021a; Brede

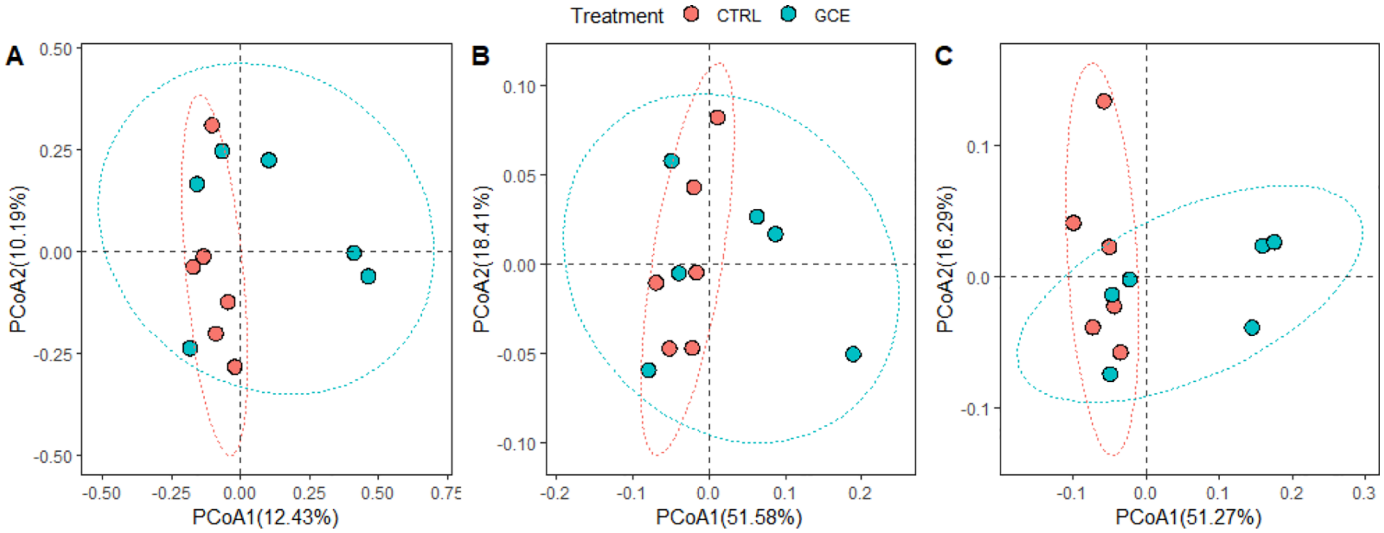

Figure 1. Beta-diversity visualized as principal coordinate analysis (PCoA) plots for (A) rumen bacteria (adonis test: $P = 0.185$), (B) archaea ($P = 0.545$), and (C) ciliate protozoa ($P = 0.017$) from cows receiving control (CTRL) or garlic and citrus extract (GCE) supplement.

Table 7. Effect of garlic and citrus extract supplement on significant changes in rumen microbiota

	Treatment[1]			
Taxonomy	CTRL	GCE	SEM	*P*-value
Family				
Succinivibrionaceae	0.032[a]	0.082[b]	0.012	0.037
Verrucomicrobiota WCHB1–41	0.022[a]	0.012[b]	0.002	0.024
Genus				
Methanobrevibacter	0.0145[a]	0.0081[b]	0.002	<0.01
Succiniclasticum	0.0157[a]	0.0160[b]	0.0008	0.029
Verrucomicrobiota WCHB1–41	0.022[a]	0.012[b]	0.002	0.015
Species				
Prevotella sp.	0.057[a]	0.047[b]	0.003	0.048
Verrucomicrobiota WCHB1–41 sp.	0.019[a]	0.011[b]	0.001	0.016
Ciliate protozoa species				
Entodinium furca monolobum	0.057	0.108	0.019	0.053
Entodinium longinucleatum	0.025	0.007	0.007	0.055
Isotricha sp.	0.098[a]	0.191[b]	0.028	0.045

[a,b]Mean values in the same row with different superscripts differ ($P < 0.05$).
[1]CTRL = control; GCE = garlic and citrus extract supplement.

et al., 2021) and in vivo trials with large ruminants (Roque et al., 2019b; Vrancken et al., 2019; Brand et al., 2021; Bitsie et al., 2022).

The CH_4 reduction in vitro was almost 95% by GCE added to a dairy cow diet with rumen fluid in a RUSITEC system (Eger et al., 2018). This was achieved by alteration of the archaeal community without negatively affecting rumen fermentation. In the present trial, the lower abundance of *Methanobrevibacter* at the genus level in the GCE group establishes the inhibitory effect of GCE on methanogens, leading to reduced enteric CH_4 emissions. The organosulfur compounds in garlic lead

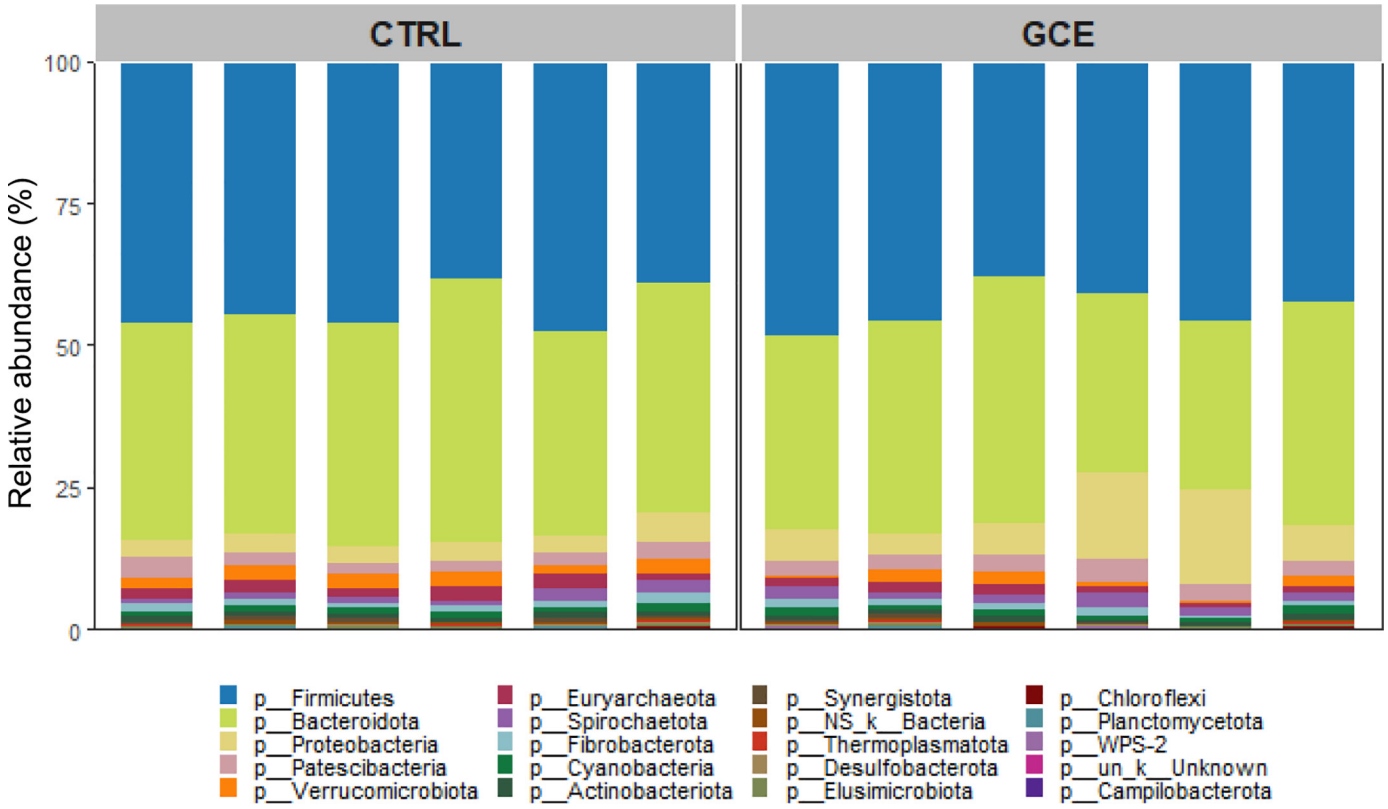

Figure 2. Bacterial community composition at the phylum (p_) level from individual cows receiving control (CTRL) or garlic and citrus extract (GCE) supplement.

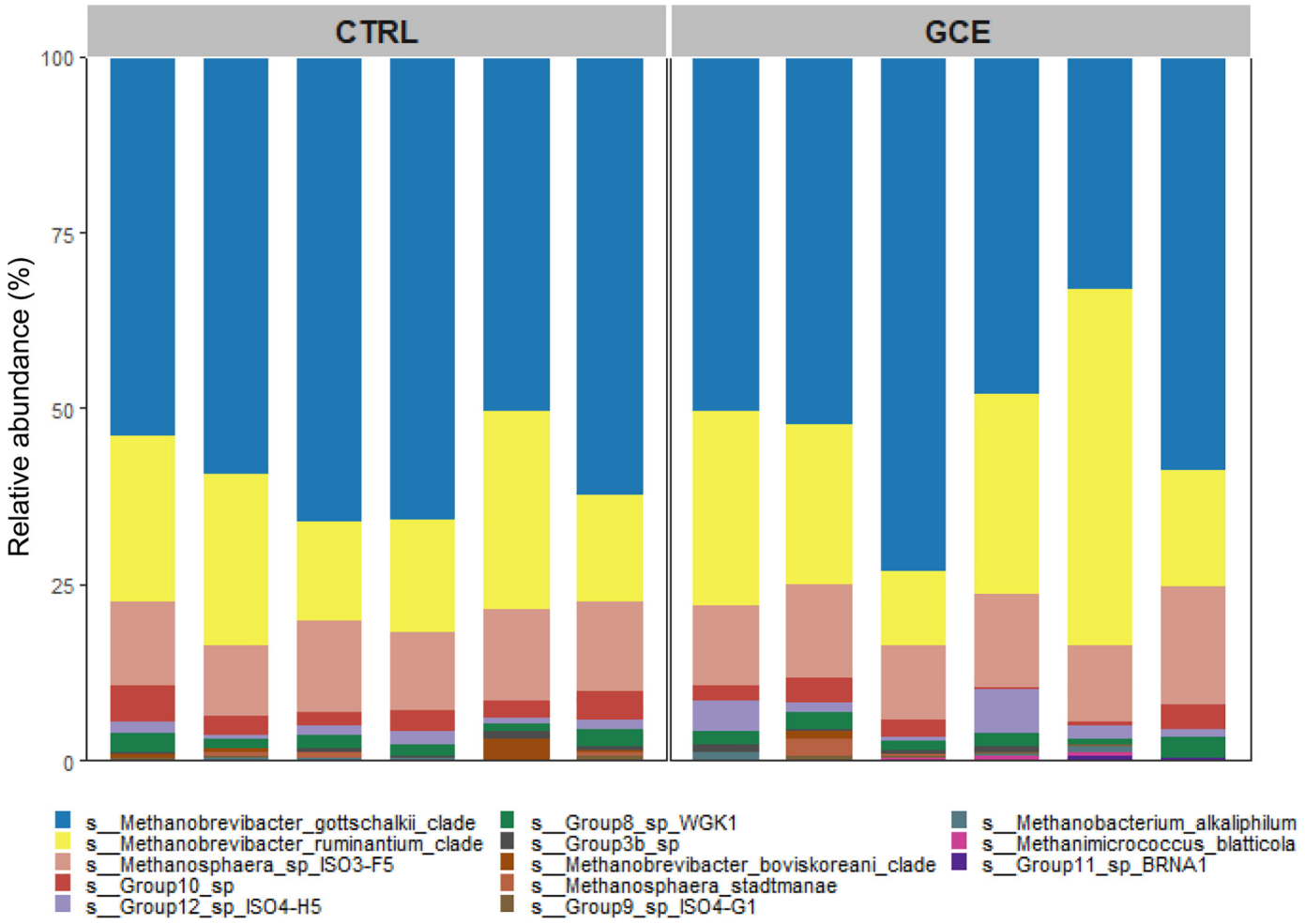

Figure 3. Archaeal community composition at the species (s_) level from cows receiving control (CTRL) or garlic and citrus extract (GCE) supplement.

to the direct inhibition of methanogenic archaea in the rumen by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase. This enzyme catalyzes the synthesis of the isoprenoid units in methanogenic archaea as they have unique membrane lipids that contain glycerol joined by ether linkages to long-chain isoprenoid alcohols (Busquet et al., 2005). In another long-term (38-d) RUSITEC experiment (Brede et al., 2021), CH_4 production was significantly reduced until d 18, which was also due to a selective effect on archaeal community composition.

Ahmed et al. (2021a), in an in vitro batch culture experiment conducted on rumen fluid from sheep, observed that GCE dosed at 20% of substrate (50% grass: 50% concentrate) led to an up to 54% reduction in CH_4 production. Although the forage-to-concentrate ratio in the present trial was similar to 50:50, the dosage of GCE was lower and might explain the lesser reduction in enteric CH_4 emission in vivo. It may not be possible to observe a similar CH_4 reduction effect in in vitro and in vivo trials as physiological processes come into play in live animals compared with batch cultures.

In beef cattle on a feedlot diet supplemented with 15 g/d GCE included in an alfalfa pellet, a reduction in CH_4 yield of 23.2% (Roque et al., 2019b) was observed, and with 16 g/d GCE in a pellet, a reduction in CH_4 yield of 24.6% (Bitsie et al., 2022) was observed. The tendency for reduced CH_4 yield in the current trial was lower than in the previous trials. The daily dosage of GCE in the beef trials was similar but higher per unit of DMI, which may explain the higher CH_4 reduction. Moreover, the feedlot diets were low in forage (fiber) and high in corn (starch), although a forage effect was observed by Bitsie et al. (2022) as the steers fed 41.5% corn silage emitted more CH_4 on a grams per kilogram of DMI basis compared with steers fed the 15% corn silage diet. The feedlot diets are high in concentrates, and greater CH_4 emissions are expected from cattle fed

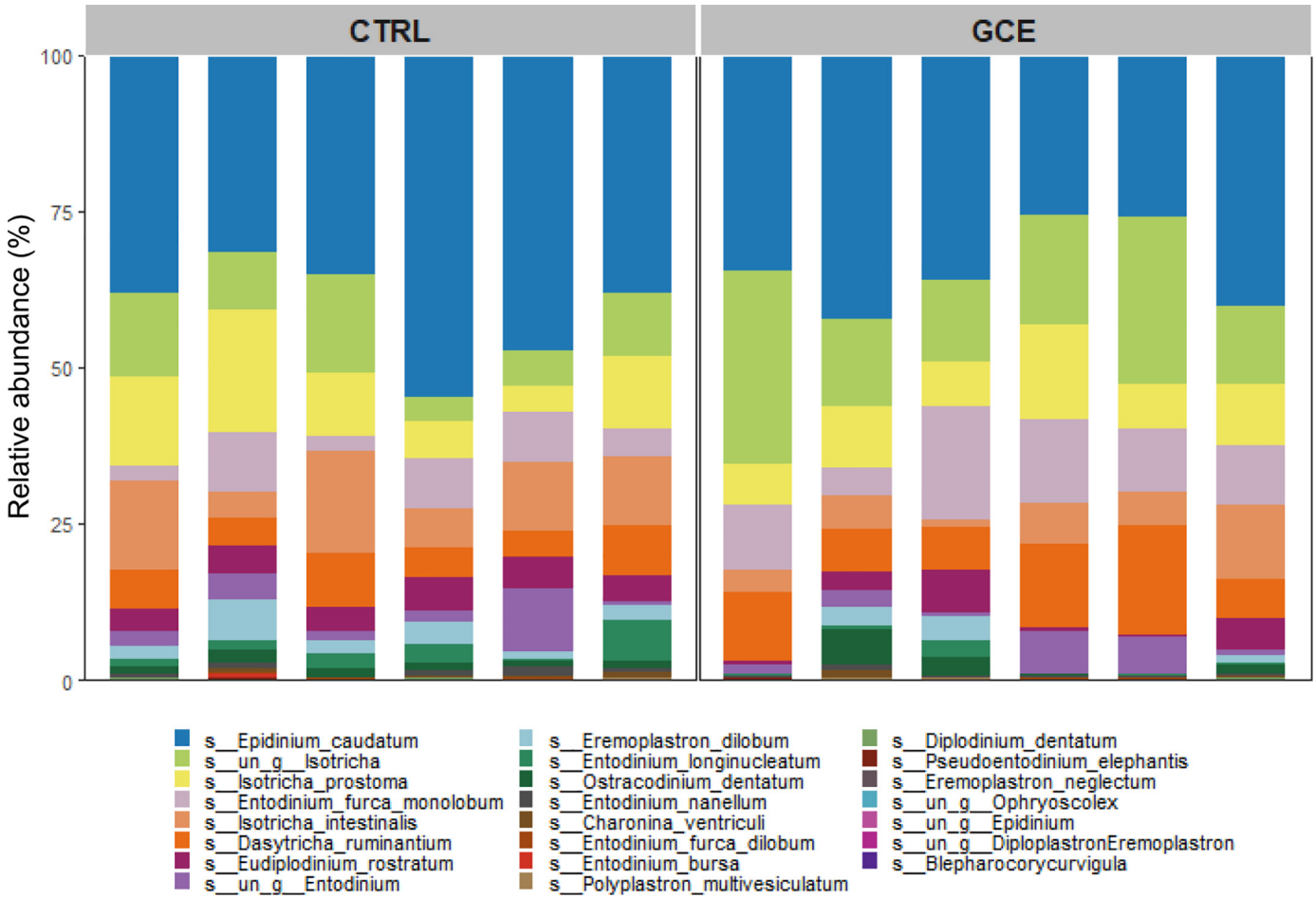

Figure 4. Ciliate protozoa community composition at the species (s_) level from cows receiving control (CTRL) or garlic and citrus extract (GCE) supplement.

50% forage from grass silage in the diets, as observed in the present trial. A 22.8% decrease in CH_4 production by preweaning Holstein-Friesian bull calves fed milk replacer and a 52.3% concentrate starter feed and supplemented with GCE at 4 g/d for 70 d could also be attributed to lower dietary fiber levels (Brand et al., 2021).

The use of grass silage–based rations in milk production is typical practice in Norway, with feed rations for dairy cows comprising 45% and 42% silage and concentrates, respectively (Åby et al., 2014). Lower CH_4 emissions from feeding corn silage compared with grass silage have been reported in several studies (Evans, 2018). Vrancken et al. (2019) observed that feeding 15 g/d GCE to Holstein Friesian dairy cows fed a TMR with 55% grass silage and corn silage decreased CH_4 production by 20.7% compared with when cows were not fed GCE. In this trial, a comparatively lesser reduction in enteric CH_4 emissions may be attributed to the proportion of grass silage, indicating that GCE is more effective in CH_4 reduction with lower forage proportion and more starch fermentation in the rumen.

Indeed, antimethanogenic feed additives must exhibit a persistent mitigation effect to ensure successful practical application. It is noteworthy that the CH_4-mitigating effects of feed additives have been mostly investigated in short-term studies, including the experiment reported herein. However, the ability of rumen microbes to adapt to some feed additives could impair their long-term persistent effects on enteric CH_4 reduction (Kumar et al., 2014). Transient effects of CH_4 mitigation have been reported in cattle studies involving supplementation of feed additives such as ionophores (Sauer et al., 1998; Guan et al., 2006), essential oils (Klop et al., 2017) and lipids (Woodward et al., 2006; Muñoz et al., 2021). Although there is limited information about the persistency of the antimethanogenic effects of GCE, Vrancken et al. (2019) observed that CH_4 emission was significantly reduced when GCE was supplemented in the diets of Jersey and Holstein-Frie-

sian cows over a 12-wk period. Thus, further long-term in vivo studies are required to confirm the persistency of GCE supplementation on enteric CH_4 reduction and the rumen microbiome.

Feed Intake and Milk Yield

Intakes of the total diet or the GCE mixed in TMR fed for 2 h were similar to the CTRL, indicating no effect of GCE on palatability. This observation is similar to some of the feed trials conducted with Holstein dairy heifers, cows, or calves fed garlic cloves or powder at a rate of 7 or 10 g/kg of DMI, respectively, which caused no depression in feed intake, even when applied at high dosages (Gholipour et al., 2016; Rossi et al., 2018). Ahmed et al. (2021b), in their study with sheep, also reported no negative effect of GCE supplementation on total feed intake, even at the highest dose of 10 g/kg of DMI.

In this study, ECM and milk yield were similar in CTRL and GCE-supplemented groups. This is contradictory to the observation of Vrancken et al. (2019), who found that feeding GCE led to increased milk yield by Jersey and Holstein Friesian cows. In theory, a decrease in CH_4 production could provide more energy that is partitioned into milk. However, in this study, CH_4 production was reduced by 10.3% and CH_4 intensity by 11.7%, and these effects could be too small to detect an effect on milk yield. However, in recent meta-analyses of CH_4 inhibitors with a larger CH_4 reduction than in this study, a tendency for lower milk yield for 3-nitrooxypropanol (Kim et al., 2020; Yu et al., 2021) or no relationship between reduced CH_4 production and milk yield (Ungerfeld, 2018) was found.

Rumen Fermentation

Rumen pH was not affected by GCE supplementation. This is in accordance with in vitro studies in RUSITEC with garlic oil (Soliva et al., 2011) and in batch culture with GCE (Ahmed et al., 2021a). Moreover, this is consistent with observations reported in previous in vivo studies feeding garlic oil to ewe lambs (Chaves et al., 2008), dairy goats (Kholif et al. (2012), and dairy cows (Yang et al., 2007). The GCE did not affect the daily output of VFA; however, a shift in fermentation pattern was observed.

A tendency for higher propionate proportion and lower acetate-to-propionate ratio in GCE-fed cows was similar to that observed when supplementing Bioflavex (HealthTech BioActives S.L.U.; with main components naringin, neohesperidine, and poncirin) to a feedlot diet fed to Friesian steers, suggesting the effect of citrus extracts in GCE in modulating the activity of rumen microbiota (Seradj et al., 2014). Soliman et al. (2020) also observed higher propionate concentration and lower acetate-to-propionate ratio in Holstein Friesian dairy cows fed a flavonoid-rich fruit and vegetable juice. Enteric CH_4 emissions have been negatively associated with an increase in propionate production and a decrease in the acetate-to-propionate ratio (Wang et al., 2018). After CH_4, propionate is the principal alternative H^+ sink, although H_2 concentration was observed to be similar between both treatments.

The proportion of a minor VFA, caproate, was negatively correlated with both CH_4 production (g/d) and CH_4 yield (g/kg of DMI) in sheep fed alfalfa pellets (Jonker et al., 2019). The lower caproate concentrations we observed in GCE, despite lower CH_4 production and CH_4 yield, are in contrast to the results from the above study. However, Zhu et al. (2015) observed that rapid lactate degradation led to a gradual accumulation of n-caproate in high concentrations in the fermentation pit used for the production of Chinese strong-flavor liquor. It is possible that reduced lactate concentration in the GCE-fed group led to the lower caproate concentration. Because lactate concentration was not measured in this trial, it may not be possible to attribute the lower caproate production in the GCE group to lesser availability of lactate substrate in the rumen.

Microbial Population

The abundance of bacteria was not affected by GCE, which is in agreement with previous studies where the flavonoid naringin (Oskoueian et al., 2013) or garlic oil (Patra and Yu, 2015) were used in in vitro experiments using rumen fluid from male or lactating dairy cattle. *Succinivibrionaceae* is the dominant family among rumen *Proteobacteria* (Tapio et al., 2017) and is known to play an important role in succinate production through hydrogen utilization. This not only enables competition with hydrogenotrophic methanogens for substrate, but succinate is also a precursor for propionate production (McCabe et al., 2015; Bailoni et al., 2021), with an estimated 70 to 100% of the propionate produced via the succinate pathway (Van Lingen, 2017). Ramayo-Caldas et al. (2020) observed that *Succinivibrionaceae* are associated with improved feed efficiency, reduced CH_4 emissions, and higher propionate concentration in lactating Holstein cows. This supports the significant abundance of the *Succinivibrionaceae* family and a tendency to higher propionate concentration and lower CH_4 production in GCE-fed cows in this trial. Conversion of succinate to propionate was further aided by the significantly higher abundance of the *Succiniclasticum* genus in the GCE. According to van Gylswyk (1995), *Succiniclasticum* is characterized as a gram-negative

and nonmotile species, unable to ferment carbohydrates, amino acids, or mono-, di-, and tricarboxylic acids other than succinate to propionate.

The relative abundance of strict anaerobic *Methanobrevibacter* genus was lower with GCE. This resulted in a comparable decrease in enteric CH_4 emissions, considering that the dominant methanogens in the rumen fluid were related to the genus *Methanobrevibacter* (Danielsson et al., 2012). This is in line with Eger et al. (2018), who showed reduced abundance of several OTU of the *Methanobrevibacter* genus in an in vitro experiment with GCE.

The abundance of rumen ciliate protozoa is a function of energy and nitrogen availability and the quality of diet in the rumen (Vogels et al., 1980). For example, the *Ostracodinium* genus possesses high cellulolytic activity, and their increased relative abundance is known to be associated with high-forage diets (Dehority and Odenyo, 2003; Bailoni et al., 2021). According to Vogels et al. (1980), the somatic association between ciliate protozoa and methanogenic bacteria can be observed in all species of the genera *Epidinium*, *Entodinium*, and *Ostracodinium* in addition to a few others; however, the differences observed in their frequency of association are not concretely understood. Further, the association of methanogens with subspecies of one species of ciliate protozoan may differ substantially, and therefore the difference in their numbers in control and treatment groups cannot be categorically elucidated. For example, the range of association of methanogens to subspecies *longinucleatum* of *Entodinium* species is 5 to 10%, whereas this association with *caudatum* subspecies is 20 to 50% (Vogels et al., 1980). The occurrence of *Isotrich* ciliates in high numbers in GCE compared with CTRL may not imply any differences because, according to Vogels et al. (1980), no externally associated methanogens have been observed to be associated with *Isotrich* ciliates. Alternatively, Belanche et al. (2015) in their experiment with 8 Texel-crossbred fauna-free sheep observed that post-inoculation *holotrich*-faunated sheep exhibited a 22% population of *Isotricha* spp. and produced 11.8 times more CH_4 per cell than the total protozoa population observed in fully faunated sheep. In a recently published meta-analysis reporting data from 79 in vivo experiments, Dai et al. (2022) observed a positive association between isotrichids and CH_4 emissions. The meta-analysis also illustrated that a positive association exists between CH_4 emissions from entodiniomorphids and dairy cows but not with small ruminants and beef cattle. Ranilla et al. (2007), in an in vitro trial, compared 4 protozoa species, *Entodinium caudatum*, *Isotricha intestinalis*, *Metadinium medium*, and *Eudiplodinium maggii*, from monofaunated and conventional wethers. The trial demonstrated that compared with *Isotricha* spp., *E. caudatum* increased CH_4 production and protein degradation, displaying a negative effect on rumen fermentation for the host.

The overall differences in ciliate protozoa populations among the CTRL and GCE-supplemented groups indicate the rumen-modifying potential of the GCE supplement. This is in line with the observations of Wanapat et al. (2008), who observed a difference in ciliate protozoa numbers in incubated rumen fluid of Holstein Friesian crossbred steers fed diets supplemented with various levels of garlic powder.

CONCLUSIONS

Feeding GCE to dairy cows for 18 d reduced CH_4 production and intensity without compromising feed intake or milk production of dairy cows. The GCE supplement modified rumen fermentation and microbiota, as shown by a tendency for higher propionate concentration and lower acetate-to-propionate ratio, a higher relative abundance of the *Succinivibrionaceae* family of bacteria acting as a hydrogen sink, and a lower relative abundance of the CH_4-producing *Methanobrevibacter* genus. Hence, supplementation of dairy cattle with GCE could be an effective strategy for enteric CH_4 mitigation in dairy cows.

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