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Effects of calcium peroxide or biochar-enzyme feed additives on milk production, enteric methane emissions, and ruminal microbiota in Nordic Red dairy cows

J. Vattulainen,[1] A. R. Bayat,[1] T. Stefański,[1] M. Rinne,[1] and I. Tapio[2]*
[1]Animal Nutrition, Production Systems, Natural Resources Institute Finland (Luke), FI-31600 Jokioinen, Finland
[2]Genomics and Breeding, Production Systems, Natural Resources Institute Finland (Luke), FI-31600 Jokioinen, Finland

ABSTRACT

Expanded basic research is needed to discover and develop wider selection of dietary additives that are economically feasible without compromising animal performance or health. The objective of this study was to evaluate the effect of 2 feed additives as methane-mitigating agents in milk production systems. Four multiparous Nordic Red dairy cows were assigned to a 4 × 4 Latin square experiment with four 28-d periods using respiration chambers. The control diet (CON) consisted of grass silage and dietary concentrates mixed at forage-to-concentrate ratio of 65:35 on DM basis. The 3 experimental treatments consisted of the CON diet supplemented with 0.2% of biochar with fibrolytic enzymes and live yeast additive (BFE) or with 0.75% or 1.5% CaO_2 on a DM basis (CaPe1 and CaPe2, respectively). Calcium peroxide (CaPe) was included in the concentrate pellet; the mixture of biochar, fibrolytic enzymes and live yeast was added to the diet during TMR preparation; and diets were fed as TMR 4 times daily. Feeding BFE had minor effect on the parameters evaluated in the experiment. Feeding CaPe resulted in linear reductions in DMI, OMI, CP, ether extract (EE), NDF, and gross energy (GE) intake compared with CON. Yields of milk, ECM, fat, protein, lactose and total solids decreased linearly, but milk composition and SCC were not affected. Apparent total-tract digestibilities of DM, OM, CP, EE, NDF, and GE decreased linearly, whereas excretion of Ca and P in feces increased linearly with increasing CaPe level. We found that CaPe1 tended to decrease the molar proportion of acetate and increased that of propionate, whereas butyrate increased linearly. Dietary CaPe inclusion decreased daily CH_4 production (g/d) linearly by 15.0%, but CH_4 yield (g/kg DM or OM intake) and intensity (g/kg milk or ECM) were not affected. Hydrogen production (g/d) and yield (g/kg DMI) decreased at CaPe1 but plateaued at CaPe2. Feeding CaO_2 increased richness of ciliate protozoa and influenced rumen bacteria and ciliate protozoa community structure. No such effect was observed on archaea or anaerobic fungi. The feed additives BFE and CaPe were not effective CH_4-mitigating agents under the conditions of the present experiment.
Key words: grass silage, feed additive, greenhouse gases, microbiota, respiration chamber

INTRODUCTION

Ruminants have evolved a symbiotic relationship with anaerobic microbes to efficiently digest fibrous plants and convert them into milk and meat, contributing to human food security. However, a natural byproduct of this digestion process is enteric methane (CH_4), which is a short-lived gas with atmospheric lifetime of ~12 years (Wahlen, 1993). Because of the high global warming potential of CH_4, decreasing the emissions of enteric CH_4 from ruminant production is considered as one of the goals in strengthening the sustainability of the livestock sector. In Finland, CH_4 emissions from dairy production represent ~2.5% of the national anthropogenic GHG emissions (Huhtanen et al., 2022), whereas global dairy sector's share of GHG emissions is 2.7% (FAO, 2010).

To date various enteric CH_4 mitigation strategies have been evaluated. Among them, short-term strategies include modification of diets using feed additives that suppress CH_4 production, whereas long-term strategies include genetic selection of livestock for improved efficiency (Hayes et al., 2013; Knapp et al., 2014) combined with precision management that supports animal health and longevity by ensuring that animals have the capacity to produce to their ultimate genetic potential by offering balanced diets (González et al., 2018). To reduce global CH_4 emissions by at least 30% from 2020 levels by 2030 (Malley et al., 2023), the livestock sector should combine both short- and long-term strategies.

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*Corresponding author: ilma.tapio@luke.fi

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Currently, only a limited number of effective feed additives targeted for reducing enteric CH_4 are approved for food production (Hegarty et al., 2021). Thus, there is a need to expand basic research aiming to discover and develop a wider selection of additives that are economically feasible and do not compromise animal performance or health (Morgavi et al., 2023).

Oxidizing agents such as calcium peroxide (CaO_2) are commonly used in agriculture, environmental restoration, and pharmacy. During hydrolysis, CaO_2 produces calcium hydroxide, hydrogen peroxide, oxygen and water. Due to its oxygenation properties, CaO_2 can be used in anaerobic digestors to create microaerobic conditions, where limited oxygen release can stimulate the growth of aerobic or facultatively aerobic microbes, positively affecting the hydrolysis of cellulose (Tsapekos et al., 2017). However, higher CaO_2 doses can have a substantial inhibitory effect on methanogenesis (Wang et al., 2019). Recently, CaO_2 was used as an antimethanogenic feed supplement in a dairy-beef bull experiment that demonstrated 16% to 27% reduction in daily CH_4 production (Roskam at al., 2024). Other oxygen-releasing agents have been successfully tested for significant reductions of gaseous emissions from stored cattle manure slurry (Thorn et al., 2022; Connolly et al., 2023) and in an in vitro rumen simulation system (O'Donnell et al., 2024). These observations suggest that oxidizing agents could potentially be considered as versatile, economically feasible, and environmentally friendly dietary supplements to reduce CH_4 emissions in the beef sector, but to our knowledge, no similar in vivo experiments on dairy cows have been published.

Biochar has demonstrated beneficial impacts across different agricultural applications. When used as a feed additive for ruminants, biochar has shown potential to improve the production performance and health of animals (Nair et al., 2023) or to reduce enteric CH_4 emissions in growing steers (Winders et al., 2019; Nair et al., 2023). Nevertheless, some studies demonstrated no effect of biochar on animal performance, rumen fermentation, or CH_4 emissions (Dittmann et al., 2024), indicating more research is needed on the subject. A combination of biochar with fibrolytic enzymes and live yeast (**BFE**) has shown positive effects on CH_4 mitigation in vitro and milk production in vivo (personal communication, Branko Petrujkic, GoBioFarm Ltd., Iisalmi, Finland). It is expected that the improved diet digestibility via fibrolytic enzyme and provision of optimal conditions for rumen microbiota via biochar could lead to increased milk production in addition to the potential to reduce CH_4 production, resulting in reduced CH_4 intensity (g/kg milk). Therefore, BFE was chosen as an additive to be tested in this study.

The objective of this study was to evaluate the effect of dietary supplementation with BFE and 2 levels of CaO_2 on enteric CH_4 production, milk production, rumen fermentation, and ruminal and fecal microbiota composition in lactating Nordic Red dairy cows. The hypothesis was that CaO_2 will reduce enteric CH_4 production linearly, and BFE will reduce enteric CH_4 and/or affect milk production positively by improving nutrient digestibility.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

The experiment was conducted at the experimental dairy barn of the Natural Resources Institute Finland (Luke; Jokioinen, Finland, 60°49′N, 23°28′E) from October 2023 to February 2024. The experiment was conducted according to Regional State Administrative Agency permission ESAVI/25708/2023 in accordance with the guidelines established by the European Community Council Directive 2010/63/EU for animal experiments and complied with the ARRIVE guidelines (Kilkenny et al., 2010). Four healthy multiparous Nordic Red dairy cows (DIM 58 ± 9.2, BW 637 ± 59.3 kg, and milk yield 38.4 ± 2.6 kg/d at the beginning of the study) were selected based on their calving date, parity, BW, and milk yield and assigned to a 4 × 4 Latin square experiment, which consisted of four 28-d periods. Animals were randomly allocated to the experimental treatments and the treatment sequences were balanced to remove the carryover effects. The first 24 d of each period were used for dietary adaptation where cows were housed in a freestall barn with feed provided in controlled individual feed bins. Cows had constant access to water and salt blocks, were milked twice a day, and received concentrates in the milking parlor (in total 0.5 kg DM/d). During the sampling period (d 24–28), cows were kept in open-circuit respiratory chambers for gas exchange measurements and sample collection. In the chambers, cows were milked twice a day at 0700 and 1700 h.

Experimental diets comprised grass silage and dietary concentrates. Grass silage was made from timothy-meadow fescue swards preserved with formic acid-based additive (AIV 2 Plus Na, Eastman, Oulu, Finland; 5 L/tonne fresh grass). The control diet (**CON**) consisted of grass silage and dietary concentrates mixed at forage to concentrate ratio of 65:35 on DM basis. For the 3 experimental treatments, the CON diet was supplemented with 0.2% of DiGestoChar (BFE; GoBioFarm Ltd., Iisalmi, Finland) or with either 0.75% (**CaPe1**) or 1.5% CaO_2 (**CaPe1** and **CaPe2**, respectively; supplied by GlasPort Bio Ltd., Galway, Ireland). Treatment doses were selected based on consultation with additive providers and in vitro test results (data not presented) using total gas production

measurements by Ankom modules (Ankom Technology, Macedon, NY) and analysis of methane concentration from gas collected after 6 and 24 h of fermentation. The doses used in the in vitro trial were 1.35% and 2.25% for CaO_2, and 0.1% and 0.2% for BFE. The CaO_2 was included in the concentrate pellets, which were produced in one batch at the beginning of the experiment, whereas BFE was mixed with 3 kg of concentrate and added during TMR preparation. All diets were offered 4 times daily at 0700, 1300, 1700, and 1900 h as TMR. The CON and BFE diets were balanced for ME and MP concentrations and Ca:P ratio in line with Luke (2025) feeding recommendations. However, to balance the Ca:P ratio in the CaPe1 and CaPe2 diets, the forage-to-concentrate ratio was marginally adjusted due to the increasing mineral concentration caused by dietary CaO_2 inclusion. Sugar beet pulp and rapeseed meal were kept constant in the diets to avoid any confounding effect from palatability and CP concentration with the treatment effect, respectively. The formulation and chemical composition of the experimental diets are presented in Table 1.

Sample Collection and Measurements

Silage and concentrate samples were taken twice per week and analyzed for DM content throughout the experiment to maintain the forage-to-concentrate ratio. Representative silage and concentrate samples were taken during d 25 to 27 of each experimental period and kept at −20°C until chemical composition analysis. Leftovers were collected and weighed during d 25 to 27 of each experimental period, and representative samples were taken and analyzed for DM content before calculating DM intake. Milk yield and feed intake recorded between d 24 and 28 of each period were used for statistical analysis. In addition, milk samples of 30 mL were collected from 6 consecutive milkings during d 25 to 27, and samples were analyzed for fat, CP, lactose, and urea concentrations, as well as SCC (MilkoScan FT6000, Foss Electric, Hillerød, Denmark).

Oxygen, carbon dioxide, and CH_4 exchanges of the cows were measured over 4 d (starting at 1000 h on d 24 to 1000 h on d 28) using 4 open-circuit respiratory cham-

Table 1. Formulation of experimental diets and their chemical composition

	Treatment[1]			
Item[2]	CON	CaPe1	CaPe2	BFE
Feed ingredient (g/kg DM)				
Grass silage[3]	646	637	623	646
Barley	86.9	85.7	83.8	86.9
Oats	86.9	85.7	83.8	86.9
Molassed sugar beet pulp	70	70	70	70
Rapeseed meal	94.5	94.5	94.5	94.5
Calcium peroxide	0	7.5	15	0
Limestone[4]	6	0	0	6
Monocalcium phosphate	0	10	20	0
Mineral and vitamin premix[5]	10	10	10	10
Forage proportion	646	637	623	646
Forage proportion (excluding minerals)	656	655	652	656
Chemical composition (g/kg DM unless otherwise stated)				
DM	511	519	530	511
OM	925	917	902	925
CP	148	147	144	148
EE	38	39	38	38
NDF	422	414	404	422
Forage NDF	327	322	315	327
GE (MJ/kg DM)	18.0	17.8	17.5	18.0
Ca:P ratio	1.90	1.70	1.82	1.89

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]EE = ether extract; GE = gross energy; Ca:*P* = calcium to phosphorus ratio.

[3]Mean fermentation characteristics of the basal grass silage: pH, 4.15; in DM (g/kg) lactic acid, 41.7; acetic acid, 34.3; propionic acid, 0.73; butyric acid, 1.08; soluble N (g/kg of total N), 599; ammonium N (g/kg of total N), 62.9; gross energy (MJ/kg of DM), 18.1; D-value 688 ± 7.9 (g/kg DM).

[4]Total diet Ca:P ratio (including grass silage) was balanced based on Luke (2023) Ca and P values of the feed ingredients.

[5]Kalkitonkivennäinen, A-Rehu, Seinäjoki, Finland for experimental concentrates; Milking parlor mineral and vitamin premix was Lypsykivennäinen Tiineys+, Hankkija Oy, Riihimäki, Finland; LypsyMelli (Mg) offered ad libitum, Lantmännen Agro, Raisio, Finland.

bers (21.5 m^3). The first day was used for acclimatization to the chamber environment, and gas measurements were recorded over the subsequent 3-d period. The details of gas exchange measurements have been described previously (Bayat et al., 2022). Briefly, concentrations of the gases in the inlet and outlet air were measured by a computer-controlled system using dedicated analyzers (Oxymax, Columbus Instruments) with 3.5-min intervals for each chamber and the reference air. Gas analyzers were calibrated using the authentic standard gases (AGA Ltd.) at the beginning of each measurement period. Air outflow for each chamber was measured by HFM-200 mass flow meter with a laminar flow element capable of measuring up to 3,000 L/min with an accuracy of <1% of full scale and repeatability of 0.05% of full scale (Teledyne Hastings Instruments, Hampton, VA). The air flow was set to 1,500 L/min, and the measured flow was corrected using an integrated thermometer and manometer to standard temperature and pressure (0°C and 101.325 kPa). A recovery test of CO_2 was performed on the chambers after the experiment. Continuous measured versus released CO_2 (7.5 g/min) was compared for at least 75 min after reaching the steady-state condition. The gas recovery ratio was calculated as the ratio between measured and released CO_2 multiplied by 100. The CO_2 recovery was 100.5%, 98.7%, 94.9%, and 92.5% for chamber 1 to 4, respectively, or 96.7% ± 3.49% (mean ± SD). Each cow was kept in the same chamber during all periods to avoid potential confounding effects between the cow and the chamber in the statistical analysis. Thus, the small differences in the recovery rates between chambers did not interfere with comparisons between the experimental diets.

In the chambers, total feces and urine excretions were collected from d 25 to 28, and feces was weighed, thoroughly mixed, subsampled (5%, wt/wt), and stored at −20°C before chemical analysis for determination of nutrient digestibility. Urine was separated from feces via a lightweight harness and flexible tubing attached to the vulva and collected in plastic canisters containing 500 mL of 5 *M* sulfuric acid. Collection vessels were changed at 12-h intervals, and daily samples (5%, wt/wt) were taken and stored at −20°C. Feces and urine samples were pooled over sampling days to provide a representative sample for chemical analysis. The cows were milked inside the chambers using a can milking system (SAC, Kolding, Denmark).

At 1000 h on d 28 of each experimental period, immediately after the cows left the respiratory chambers, samples of rumen liquid (0.5 L) were collected via the esophagus using a Ruminator device (Profs Products, Wittybreut, Germany). In case of saliva presence, contaminated samples were discarded and repeated samples were obtained. Immediately after collection, rumen liquid pH was measured using a portable pH meter, and subsamples were taken for VFA and ammonia-N determination and rumen microbiota analysis as described by Ahvenjärvi et al. (2024).

Chemical Analysis

Frozen feed, and fecal and urinary samples were thawed in room temperature. Feed and fecal samples were dried in a forced-air oven at 50°C and milled using a sample mill with a 1-mm screen (Sakomylly KT-120, Koneteollisuus Oy, Helsinki, Finland) before chemical analysis. Dry matter concentration of milled feed and fecal samples was determined based on weight loss in a forced-air oven at 105°C for 16 h, and grass silage DM concentration was corrected for the loss of volatile compounds (Huida et al., 1986). The official method of AOAC-942.05 was used for analysis of ash concentration. Nitrogen concentration in fresh samples of urine was determined by the Kjeldahl method using $CuSO_4$ as a catalyst, and for dry feed and fecal samples, the Dumas method was applied (Leco FP-428, Leco Corporation, St. Joseph, MI). Starch concentration in feed samples was determined according to Salo and Salmi (1968). The NDF concentration was analyzed in the presence of Na_2SO_3 (Van Soest et al., 1991) using Ankom 220 Fiber Analyzer (Ankom Technology, Macedon, NY) and presented ash-free. For samples containing starch, heat-stable α-amylase was used. Ether extract concentration in feed ingredients was determined according to AOAC official method 920.39 and in feces after hydrolysis with 3 *M* HCl. Gross energy (**GE**) was determined using a Parr 6200 Oxygen Bomb Calorimeter (Parr Instrument Co, Moline, IL) with benzoic acid as a standard. In vitro OM digestibility of grass silage was determined based on pepsin-cellulase solubility according to Nousiainen et al. (2003). The concentration of digestible OM (D-value) in grass silage was estimated using the equation suggested by Huhtanen et al. (2006) for primary growth grass silage: D-value (g/kg of DM) = [1,000 − ash (g/kg of DM)] × [0.077 + 0.86 × OM solubility (g/g of OM)]. Volatile fatty acid concentrations in grass silage and rumen fluid were determined by gas chromatography as described by Huhtanen et al. (1998).

Microbiota Analysis

Total DNA was extracted from 0.5 mL of rumen liquid and 0.2 to 0.3 g of feces following a protocol described by Rius et al. (2012), with initial bead beating performed 3 times at 6m/s × 1 min in FastPrep (MP Biomedicals, Irvine, CA). Bacterial communities were amplified using universal primers 515F and 806R for the 16S rRNA gene (Caporaso et al., 2011). Anaerobic fungi were determined

using AGF-LSU-EnVs primers that code D2 region of the 28S rRNA gene (Young et al., 2022), and rumen ciliate protozoa were determined using P-SSU-316F and GIC758R primers targeting the 18S rRNA gene (Ishaq and Wright, 2014). The sequencing libraries were prepared and sequenced at Edinburgh Genetics (Edinburgh, United Kingdom) on a Novaseq 6000 platform (500 cycles). Demultiplexing of sequences was performed by the sequencing provider. Sequence read quality control and removal of chimeric reads, as well as clustering of microbiota sequences into amplicon sequence variants (**ASV**), was performed using QIIME v2 (Bolyen et al., 2019) following default settings in DADA2 (Callahan et al., 2016). The ASV with fewer than 10 reads in total or present in only one sample were removed. The reference databases used for taxonomical assignment of ASV were Silva 138.1 (Quast et al., 2013) for bacteria; RIM-DB (Seedorf et al., 2014) for archaea; the Kittelmann et al. (2015) published database for ciliate protozoa; and the AF_LSU v1.0 (https://anaerobicfungi.org) for anaerobic fungi. The fungal database was developed based on data previously published by Hanafy et al. (2020). After quality control, the number of sequencing reads per sample was 196,288–279,727 for rumen bacteria, 15,116–24,387 for archaea, 61,530–176,091 for ciliate protozoa, and 150,375–615,173 for anaerobic fungi. Fecal samples resulted in 176,623–371,496 quality filtered reads for bacteria, 8,827–34,896 for archaea, and 263,137–469,982 for anaerobic fungi. The raw sequence reads were submitted to the National Center for Biotechnology Information Sequence Read Archive under BioProject PRJNA1220600.

Calculations and Statistical Analysis

Total-tract apparent digestibility coefficients were calculated based on the difference between intake of a nutrient and its fecal output divided by the corresponding intake of the nutrient. Intake of ME was calculated as the difference between GE intake and energy excretion in feces, urine, and CH_4. Energy loss as CH_4 was calculated using the factor 55.24 kJ/g (Kriss, 1930). Energy corrected milk yield was calculated using milk fat, protein, and lactose yields according to Sjaunja et al. (1990), and energy secretion (MJ/d) in milk was calculated as 3.14 × ECM yield (kg/d). Heat production was calculated using gas exchanges and urinary N output according to the equation suggested by Brouwer (1965). Nitrogen balance was calculated as the difference between N intake and N excretion in feces, urine, and milk, where milk N was calculated as milk CP/6.38.

Statistical analyses were performed using PROC MIXED of SAS 9.4. (SAS institute Inc., Cary, NC), using period and treatment as fixed effects and cow as a random effect in the model. The treatment means were compared using contrasts as follows: CON versus BFE, and linear and quadratic effects of CaO_2 addition. The effects were considered statistically significant when $P < 0.05$ and tendencies were reported when $P < 0.1$. Diurnal variations of enteric CH_4 and hydrogen emissions were calculated using a mixed model including fixed effects of period, diet, time of day, interactions of period and diet with time of day, and random effect of cow. Time of day was considered as a repeated measurement.

Bacterial community α-diversity of ruminal and fecal samples was estimated using Shannon diversity index and the richness (number of observed ASV) as implemented in the *MicrobiotaProcess* R package (Xu et al., 2023). The data were evenly subsampled to the lowest number of reads per sample in each dataset and significant differences in pairwise comparisons were estimated using nonparametric Wilcoxon test. To evaluate treatment effect on the changes in ruminal and fecal microbial community structure, between sample diversity was calculated as Bray–Curtis dissimilarities following Hellinger transformation and visualized using principal coordinate analysis. The significance of groups was evaluated by distance-based permutational multivariate ANOVA (adonis) and defined at the $P < 0.05$ level after 999 permutations, as implemented in the *vegan* R package (Oksanen et al., 2020). Diet effect on individual microbial genera was evaluated using PROC MIXED as described previously. Before the analysis, all genera below 0.001% abundance in less than 25% of samples were filtered out, number of reads were log base transformed [$\log_2$ (x + 1)] and standardized by data centering. For easier interpretation of the results, microbial genera significantly affected by treatment were converted back to compositional data and presented as relative abundances.

RESULTS

Feed Intake, Milk Production and Diet Digestion

the addition of CaPe linearly reduced ($P < 0.05$) DMI by 16.6% and OMI by 18.6% compared with CON, which was accompanied by linear decreases in CP, EE, NDF, and GE intakes (Table 2). However, the Ca:P ratio decreased linearly ($P < 0.05$), and intakes of Ca and P increased linearly ($P < 0.001$). Inclusion of BFE did not affect ($P \geq 0.99$) DM and nutrient intakes.

Yields of milk, ECM, fat, protein, lactose, and total solids decreased linearly ($P < 0.05$) due to CaPe addition, but milk composition (fat, protein, lactose, total solids, urea) and SCC were not affected ($P \geq 0.11$; Table 3). Use of BFE did not affect ($P \geq 0.32$) milk yield or composition. None of the dietary treatments affected ($P \geq 0.60$) feed efficiency expressed as milk or ECM yield divided by DM or OM intake.

Table 2. Feed and nutrient intakes of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Intake (kg/d unless otherwise stated)	CON	CaPe1	CaPe2	BFE	SEM	Lin[2]	Quad[3]	CON vs. BFE
Total DM	24.7	22.1	20.6	24.7	1.10	0.02	0.61	1.00
OM	22.9	20.2	18.6	22.9	0.99	0.01	0.64	1.00
CP	3.69	3.27	3.00	3.69	0.15	0.01	0.65	0.99
EE[4]	0.93	0.84	0.78	0.93	0.04	0.02	0.81	0.99
NDF	10.3	9.0	8.2	10.3	0.46	0.01	0.62	1.00
Starch	2.67	2.70	2.66	2.68	0.17	0.99	0.86	0.98
GE (MJ/d)	445	394	362	445	19.3	0.01	0.64	0.99
Ca (g/d)	179	236	334	179	19.7	<0.001	0.37	0.99
P (g/d)	94.5	139	183	94.7	9.72	<0.001	0.98	0.99
Ca:P	1.90	1.70	1.82	1.89	0.02	0.04	0.001	0.99

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Linear effect of CaPe inclusion.

[3]Quadratic effect of CaPe inclusion.

[4]EE = ether extract; GE = gross energy; Ca:P = calcium to phosphorus ratio.

Apparent total-tract digestibilities of DM, OM, CP, EE, NDF, and GE decreased linearly ($P < 0.05$) by CaPe addition (Table 4). Apparent bioavailability of Ca was not affected by CaPe addition, but that of P tended to decline ($P = 0.08$). Excretion of both Ca and P in feces increased linearly ($P < 0.001$) in response to CaPe addition. We found no significant differences in DM, CP, EE, Ca, or P digestibilities or fecal concentration of Ca and P between CON and BFE ($P \geq 0.13$), but a tendency for lower digestibility for NDF, GE, and OM with BFE compared with CON ($P \leq 0.09$) was observed.

Rumen pH, ammonia-N and total VFA concentrations were not affected ($P \geq 0.11$) by the treatments (Table 5). The first level of CaPe addition tended to decrease the molar proportion of acetate (quadratic effect $P = 0.08$) and increased that of propionate (quadratic effect $P < 0.05$), whereas butyrate increased linearly ($P < 0.05$) in response to CaPe addition. These changes resulted in significant quadratic effects in the lipogenic to glucogenic VFA ratios so that they were lowest at the first level of CaPe addition ($P < 0.05$). Addition of BFE did not affect molar proportions of ruminal VFA.

Table 3. Milk and ECM yield, milk composition, and milk production efficiency of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item	CON	CaPe1	CaPe2	BFE	SEM	Lin[2]	Quad[3]	CON vs. BFE
Yield (kg/d)								
Milk	33.9	30.6	28.2	33.9	1.87	0.01	0.70	0.99
ECM	37.8	33.8	30.8	38.6	1.44	0.01	0.76	0.70
Fat	1.62	1.44	1.31	1.67	0.07	0.02	0.76	0.62
Protein	1.26	1.12	1.01	1.28	0.06	0.01	0.77	0.70
Lactose	1.55	1.39	1.29	1.54	0.10	0.01	0.65	0.93
Total solids	4.78	4.27	3.90	4.84	0.20	0.01	0.72	0.81
Concentration (g/kg)								
Fat	48.2	47.8	47.5	49.4	3.24	0.53	0.96	0.32
Protein	37.2	36.7	36.0	37.8	0.89	0.10	0.91	0.43
Lactose	45.6	45.4	45.5	45.5	0.75	0.37	0.31	0.46
Total solids	142	140	139	143	3.73	0.22	0.89	0.51
Urea (mg/100 mL)	16.9	16.8	15.9	16.9	1.00	0.26	0.52	0.94
SCC (1000/mL)	35.6	104	90.1	110	69.8	0.22	0.28	0.11
ECM/DMI	1.53	1.54	1.49	1.57	0.05	0.60	0.66	0.60
ECM/OMI	1.65	1.68	1.66	1.69	0.05	0.98	0.73	0.61

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Linear effect of CaPe inclusion.

[3]Quadratic effect of CaPe inclusion.

Table 4. Apparent total-tract digestibility and fecal Ca and P concentrations of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item	CON	CaPe1	CaPe2	BFE	SEM	Lin[2]	Quad[3]	CON vs. BFE
Digestibility (g/kg or otherwise stated)[4]								
DM	718	701	677	707	7.24	<0.001	0.61	0.13
OM	732	719	702	721	7.25	<0.01	0.62	0.09
CP	669	648	620	654	10.7	0.01	0.77	0.26
EE	610	598	556	605	11.3	<0.01	0.12	0.56
NDF	646	620	611	624	12.0	0.01	0.35	0.06
GE (kJ/MJ)	695	680	658	681	7.84	<0.001	0.51	0.07
Ca	299	298	286	262	23.9	0.65	0.82	0.23
P	374	319	320	332	29.0	0.08	0.26	0.15
Fecal concentration (g/kg DM)								
Ca	18.1	25.3	35.6	18.3	1.38	<0.001	0.27	0.90
P	8.48	14.4	18.6	8.69	0.70	<0.001	0.22	0.78

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Linear effect of CaPe inclusion.

[3]Quadratic effect of CaPe inclusion.

[4]EE = ether extract; GE = gross energy.

Gas Production, Energy, and N Metabolism

Dietary CaPe inclusion decreased daily CH_4 production (g/d) linearly ($P < 0.01$) by 15.0%, but CH_4 yield (g/kg DM or OM intake) and intensity (g/kg milk or ECM) were not affected (Table 6). We found no effect of CaPe on CO_2 production (g/d) or yield (g/kg DMI or OMI), but CO_2 yield (g/kg OM digested) increased linearly ($P < 0.05$). The intensity of CO_2 (g/milk) was lowest at the first level of CaPe addition (quadratic effect $P < 0.05$). Hydrogen production (g/d) and yield (g/kg DMI) decreased at the first level of CaPe addition but plateaued thereafter, resulting in significant quadratic effects of CaPe addition ($P < 0.01$). The only effect of BFE on gas production was a higher intensity of CO_2 (g/kg milk) compared with CON ($P < 0.001$). The diurnal variation in CH_4 and hydrogen production is presented in Figures 1 and 2, respectively. Diurnal variation of CH_4 tended ($P = 0.10$) to be affected by the interaction of diet and time of day. Hourly CH_4 production was more constant for CON and BFE, whereas there were more fluctuations for CaPe treatments during feeding times (0700, 1300, 1700, and 1900 h). In general, CH_4 production gradually increased during the daytime, peaking around the last feeding, and gradually declined afterward until feeding in the next morning. Hydrogen production was influenced by the interaction of diet and time of day ($P < 0.01$). Although

Table 5. Rumen fermentation characteristics of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item	CON	CaPe1	CaPe2	BFE	SEM	Lin[2]	Quad[3]	CON vs BFE
pH	6.81	6.54	6.65	6.64	0.09	0.25	0.12	0.21
Ammonia-N (m*M*)	4.05	3.23	3.36	3.01	0.83	0.53	0.62	0.36
Total VFA (m*M*)	110	122	116	118	4.13	0.34	0.11	0.17
Molar proportion (mmol/mol)								
Acetate	648	635	642	642	7.24	0.33	0.08	0.35
Propionate	182	189	176	188	5.49	0.17	0.03	0.22
Butyrate	122	126	130	124	3.39	0.03	0.75	0.55
Isobutyrate	7.18	6.42	7.26	6.78	0.25	0.72	0.01	0.11
Valerate	17.2	18.8	18.4	16.9	0.84	0.25	0.24	0.80
Isovalerate	11.4	11.0	13.8	10.7	1.29	0.06	0.14	0.52
Caproate	11.8	13.9	12.7	11.5	0.88	0.53	0.16	0.77
Ac:Pr	3.57	3.36	3.66	3.44	0.14	0.37	0.02	0.21
AcBu:Pr	4.25	4.03	4.40	4.10	0.16	0.22	0.03	0.26

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Linear effect of CaPe inclusion.

[3]Quadratic effect of CaPe inclusion.

Table 6. Methane, carbon dioxide and hydrogen production of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item	CON	CaPe1	CaPe2	BFE	SEM	Lin[2]	Quad[3]	CON vs. BFE
Methane								
g/d	492	431	419	489	19.7	0.01	0.21	0.88
g/kg DM intake	20.0	19.6	20.2	20.0	0.73	0.62	0.32	1.00
g/kg OM intake	21.6	21.4	22.5	21.6	0.79	0.20	0.27	1.00
g/kg OM digested	29.5	29.7	32.0	30.0	0.92	0.04	0.25	0.62
g/kg Milk	14.6	14.2	15.0	14.5	0.69	0.34	0.08	0.68
g/kg ECM	13.1	12.8	13.6	12.7	0.55	0.19	0.10	0.32
% of GE intake	6.17	6.12	6.42	6.17	0.22	0.20	0.27	0.98
Carbon dioxide								
g/d	13,638	14,487	14,920	12,866	701.1	0.23	0.81	0.46
g/kg DM intake	604	604	618	609	15.9	0.47	0.65	0.81
g/kg OM intake	653	659	685	658	17.2	0.14	0.55	0.06
g/kg OM digested	893	917	977	914	21.0	0.03	0.50	0.50
g/kg Milk	437	408	461	474	8.9	0.02	<0.001	<0.001
g/kg ECM	392	385	434	383	10.4	0.02	0.06	0.57
Hydrogen								
g/d	0.68	0.24	0.18	0.74	0.066	<0.001	0.03	0.55
g/kg DMI	0.03	0.01	0.01	0.03	0.00	<0.001	0.03	0.46

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Linear effect of CaPe inclusion.

[3]Quadratic effect of CaPe inclusion.

H_2 production was in general lower ($P < 0.01$) for CaPe treatments than CON and BFE, it had sharper peaks during feeding times, returning to its baseline shortly after feeding.

The addition of CaPe linearly reduced ($P < 0.05$) GE and ME intakes by 18.7% (Table 7). The proportion of GE lost in feces was linearly increased ($P < 0.01$) by CaPe addition, whereas BFE tended ($P = 0.07$) to increase it. Proportions of GE excreted in urine, CH_4, and milk were not ($P \geq 0.20$) affected by the treatments. However, heat production as a proportion of GE intake tended to increase linearly ($P = 0.09$) due to CaPe addition. Intake of N decreased linearly ($P < 0.01$) whereas N excretion in feces as a proportion of N intake increased linearly ($P < 0.01$) in response to CaPe. However, N excreted in urine and milk and N balance (g/d) were not affected ($P \geq 0.15$) by treatments. Dietary inclusion of BFE did not affect ($P \geq 0.15$) energy and N metabolism.

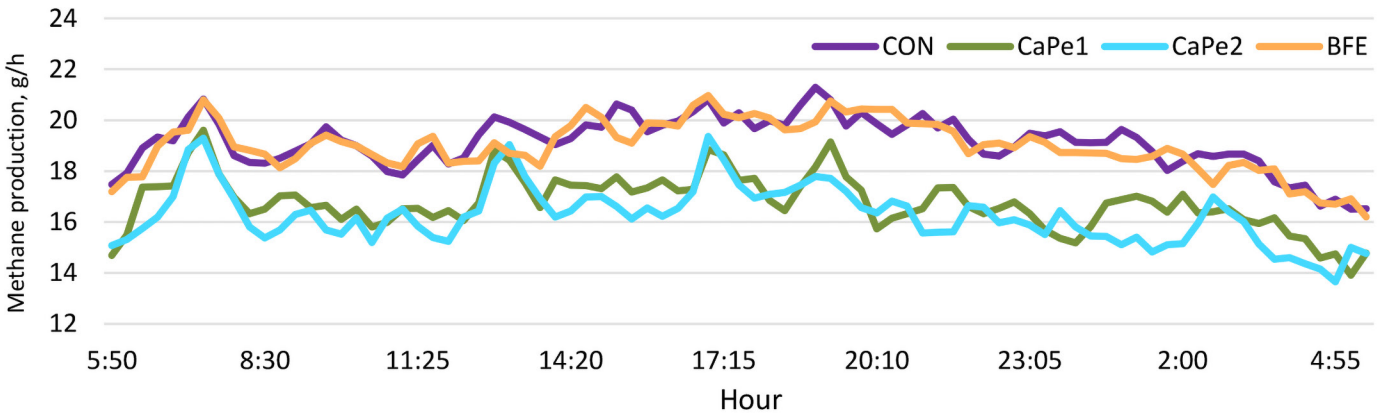

Figure 1. Diurnal variation of methane production of dairy cows fed control diet (CON) and diets containing 2 levels of calcium peroxide (CaPe1 and CaPe2) or biochar with fibrolytic enzymes and live yeast (BFE) additives. The *P*-values for diet, time, and interaction of diet and time were <0.01, < 0.01, and 0.10, respectively.

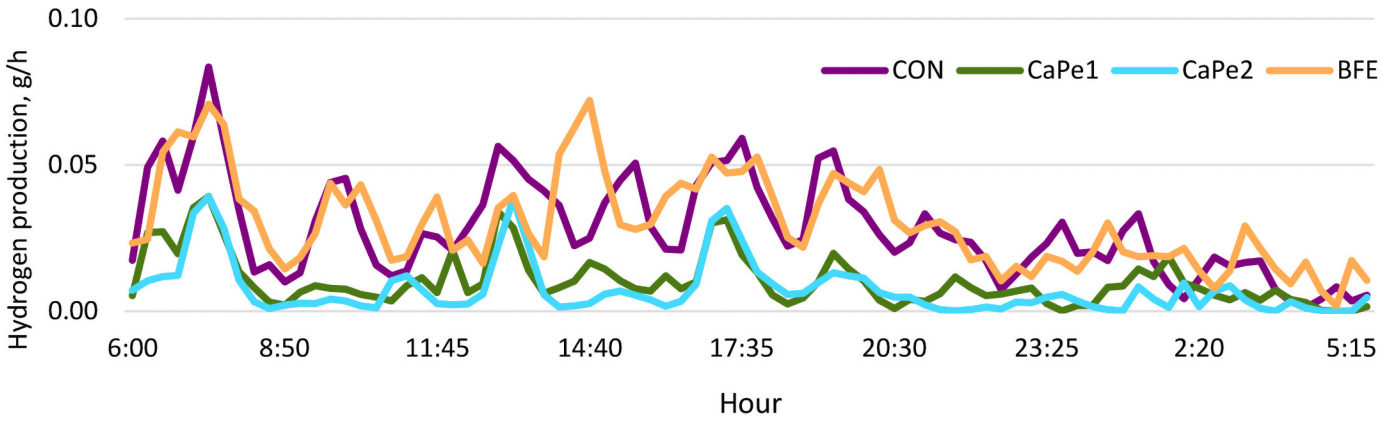

Figure 2. Diurnal variation of hydrogen production of dairy cows fed control diet (CON) and diets containing 2 levels of calcium peroxide (CaPe1 and CaPe2) or biochar with fibrolytic enzymes and live yeast (BFE) additives ($P < 0.01$ for diet, time, and interaction of diet and time).

Rumen Microbiota

Dietary feed additives did not stipulate significant changes in bacterial α-diversity, but influenced bacterial community structure. Bray–Curtis dissimilarities demonstrated significant (adonis $P = 0.03$) differences between CON and CaPe2 treatments (Figure 3A). In comparison to CON, CaPe linearly reduced the relative abundances of *Gastranaerophilales*, *Butyrivibrio*, *[Eubacterium] cellulosolvens* and *[Eubacterium] ruminantium* groups, *Rhodospirillales* sp., and *Succinivibrionaceae* UCG-002, but linearly increased *Bacteroidales* BS11, RF16 and p-251-o5 groups, *Prevotellaceae* sp., *Prevotellaceae* UCG-004, several members of the *Firmicutes* bacilli class (*Anaeroplasma*, *Erysipelatoclostridiaceae* UCG-004, *Erysipelotrichaceae* sp., *Solobacterium*, and *RF39*), several members from the *Lachnospiraceae* family (*Coprococcus*, *Lachnospiraceae* UCG-008, *Marvinbryantia*, *Moryella*), and *Pirellulaceae* p-1088-a5 gut group (Table 8).

Archaeal community was dominated by *Methanobrevibacter gottschalkii* clade (average 76.6%), followed by *Methanosphaera* sp. ISO3-F5 (10.4%), *Methanobrevibacter ruminantium* (8%), and *Methanosphaera* sp. (1.9%). Members from *Methanomassiliicoccaceae* family were observed at low abundances. Among them,

Table 7. Energy and nitrogen metabolism of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item	CON	CaPe1	CaPe2	BFE	SEM	Lin[2]	Quad[3]	CON vs. BFE
Energy								
GE intake (MJ/d)	445	394	362	445	19.3	0.01	0.64	0.99
ME intake (MJ/d)	255	221	193	250	11.7	0.01	0.81	0.72
Proportion of energy intake (kJ/MJ)								
Feces	305	320	342	319	7.8	<0.01	0.51	0.07
Urine	59.6	58.7	60.1	58.7	1.03	0.70	0.38	0.56
Methane	61.7	61.2	64.2	61.7	2.24	0.20	0.27	0.98
Milk	267	271	267	273	8.7	0.97	0.73	0.62
Heat	332	340	352	335	9.6	0.09	0.83	0.78
Milk energy/ME intake	470	480	500	490	20	0.16	0.97	0.36
Energy balance	−10.7	−18.9	−31.2	−19.3	5.41	0.03	0.77	0.29
Nitrogen (N)								
N intake (g/d)	590	523	480	590	24.3	0.01	0.65	0.99
Proportion of N intake (g/kg)								
Feces	331	352	380	346	10.7	<0.01	0.77	0.26
Urine	321	294	302	301	11.7	0.16	0.15	0.15
Milk	334	336	330	341	10.2	0.79	0.74	0.61
N balance (g/d)	8.79	10.5	−5.80	8.53	8.35	0.25	0.40	0.98

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Linear effect of CaPe inclusion.

[3]Quadratic effect of CaPe inclusion.

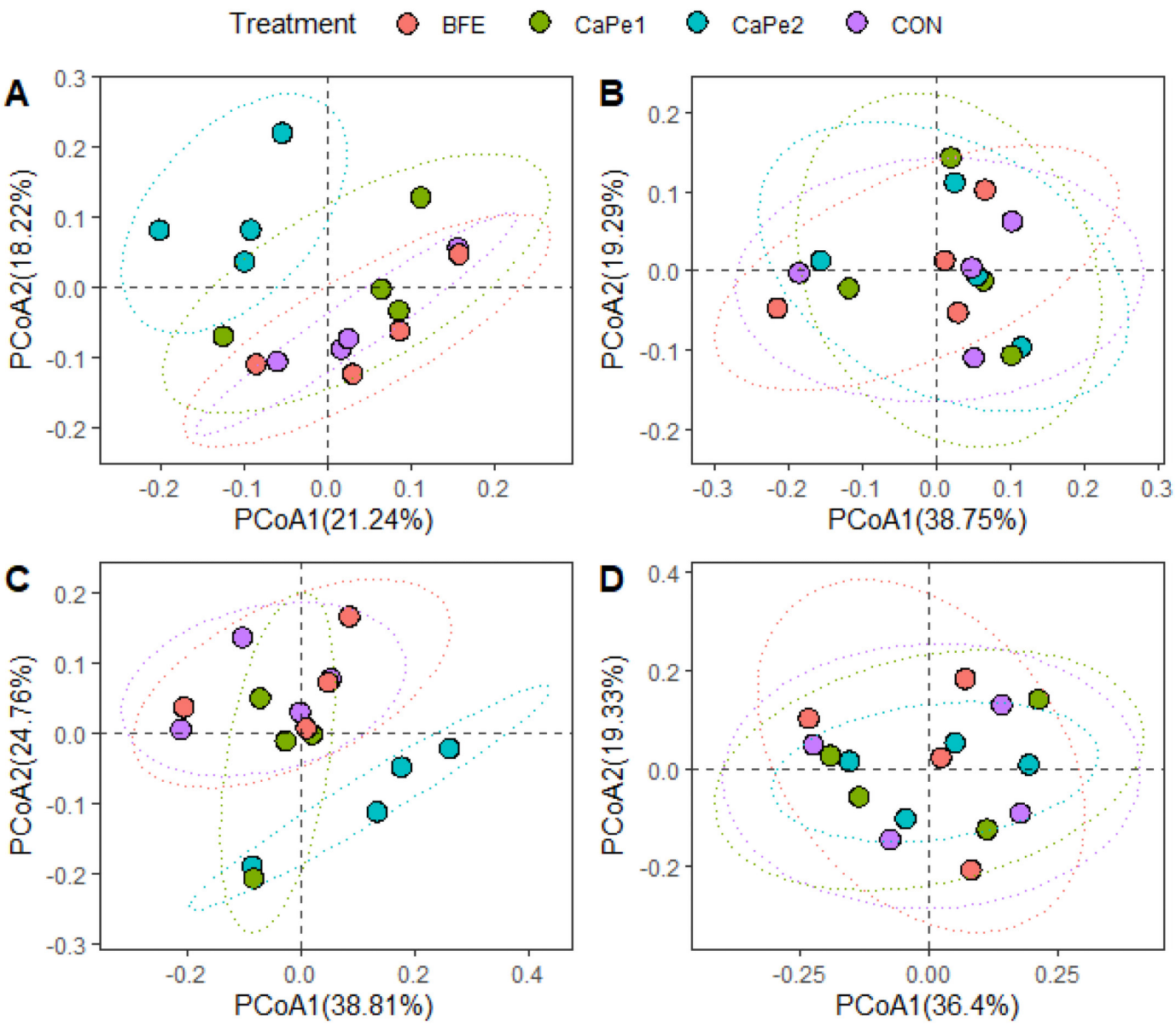

Figure 3. Rumen bacterial (A), archaeal (B), ciliate protozoan (C), and anaerobic fungal (D) principal coordinate analysis (PCoA) visualization of Bray–Curtis dissimilarities for dairy cows when offered dietary treatments: control diet (CON), biochar with fibrolytic enzymes and live yeast (BFE), and treatments containing 2 levels of calcium peroxide (CaPe1 and CaPe2).

Methanomassiliicoccus Group 10 sp. (1.5%) and *Methanomassiliicoccus Group 12* sp. ISO4-H5 (0.7%) were the more abundant groups. Alpha diversity analysis showed a tendency (P = 0.053) for lower archaeal richness in CaPe1 treatment as compared with CON but similar trend was not observed in CON–CaPe2 or CON–BFE comparisons. In this experiment archaeal community structure was not influenced by the treatments (adonis P = 0.82; Figure 3B).

At the genus level, the ciliate protozoan community was dominated by *Epidinium* (average 29.5%), *Entodinium* (26.9%) and *Isotricha* (25%). Less abundant genera were represented by *Ostracodinium* (7%), *Eudiplodinium/Eremoplastron* (3.4%), *Dasytricha* (2.1%), *Eudiplodinium* (1.9%), and *Anoplodinium/Diplodinium* (1.7%). Alpha diversity analysis demonstrated a significant increase in richness in CaPe2 as compared with the CON diet (P = 0.029). No significant differences were observed in other pairwise comparisons. Also, Bray–Curtis dissimilarities showed CaPe2 tendency for treatment effect on ciliate protozoan community structure (adonis P = 0.056; Figure 3C). In comparison to CON, CaPe linearly increased the

Table 8. Rumen microbiota relative abundance (%) of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item[2]	CON	CaPe1	CaPe2	BFE	SEM	Lin[3]	Quad[4]	CON vs. BFE
Actinobacteriota								
Bifidobacteriaceae spp.	0.096	0.000	0.023	0.070	0.0300	0.10	0.02	0.87
Olsenella	2.17	2.33	1.73	2.25	0.477	0.03	0.07	0.92
Bacteroidota								
Bacteroidales BS11 gut group	0.102	0.113	0.134	0.113	0.0429	0.04	0.89	0.98
Bacteroidales RF16 group	1.886	2.189	3.523	1.839	0.446	0.02	0.56	0.95
Prevotellaceae spp.	0.656	0.893	1.69	0.766	0.1660	<0.001	0.26	0.25
Prevotella	20.4	20.9	17.0	20.4	0.8795	<0.01	0.05	0.74
Prevotellaceae UCG-004	0.220	0.244	0.391	0.244	0.0280	<0.01	0.18	0.38
Prevotellaceae YAB2003 group	1.27	1.38	0.427	1.23	0.3197	0.01	0.04	0.60
Bacteroidales p-251-o5	0.477	0.506	1.05	0.491	0.1921	<0.01	0.05	0.94
Cyanobacteria								
Gastranaerophilales	0.999	0.608	0.174	1.15	0.0721	<.0001	0.07	0.43
Fibrobacterota								
Fibrobacter	0.962	0.738	1.01	0.854	0.0799	0.59	0.01	0.25
Firmicutes								
Anaeroplasma	0.227	0.231	0.300	0.216	0.0212	0.01	0.31	0.95
Erysipelatoclostridiaceae UCG-004	0.409	0.441	0.697	0.384	0.0441	0.01	0.18	0.77
Solobacterium	0.107	0.152	0.220	0.114	0.0275	<0.01	0.67	0.66
Erysipelotrichaceae spp.	0.055	0.076	0.129	0.078	0.0131	<0.01	0.60	0.04
RF39	1.55	1.91	2.19	1.66	0.194	0.01	0.60	0.48
Clostridia UCG-014	1.49	1.68	0.917	1.49	0.2185	0.01	0.02	0.93
Defluviitaleaceae UCG-011	0.084	0.089	0.120	0.082	0.009	0.02	0.62	1.0
Butyrivibrio	0.514	0.421	0.322	0.439	0.0438	0.01	0.56	0.31
Coprococcus	0.092	0.104	0.136	0.087	0.0096	<0.01	0.72	0.87
Lachnospiraceae UCG-008	0.119	0.120	0.149	0.111	0.0102	0.02	0.38	0.59
Marvinbryantia	0.180	0.205	0.312	0.168	0.0237	<0.01	0.37	0.86
Moryella	0.342	0.343	0.549	0.349	0.0348	<0.01	0.07	0.76
[Eubacterium] cellulosolvens group	0.199	0.184	0.064	0.199	0.0522	0.02	0.17	0.83
[Eubacterium] ruminantium group	0.794	0.743	0.591	0.802	0.0613	0.03	0.34	0.99
[Ruminococcus] gauvreauii group	0.624	0.700	0.766	0.590	0.0554	<0.01	0.28	0.47
Oscillospiraceae spp.	0.087	0.085	0.124	0.097	0.0115	0.01	0.1	0.31
[Eubacterium] coprostanoligenes group	0.267	0.345	0.337	0.280	0.0216	0.01	0.02	0.39
Anaerovoracaceae spp.	0.094	0.109	0.134	0.082	0.0142	<0.01	1.0	0.23
Succiniclasticum	3.83	4.08	3.38	4.10	0.241	0.03	0.05	0.48
Planctomycetota								
Pirellulaceae p-1088-a5 gut group	0.157	0.266	0.355	0.194	0.0185	<0.001	0.15	0.06
Proteobacteria								
Rhodospirillales spp.	0.374	0.157	0.055	0.228	0.0645	<0.01	0.40	0.41
Succinivibrionaceae UCG-002	1.97	1.30	0.621	2.74	0.6548	0.01	0.83	0.39
Verrucomicrobiota								
WCHB1–41	1.17	0.885	1.44	1.01	0.2161	0.06	0.01	0.73
Trichostomatia								
Entodinium sp.	11.3	19.2	31.3	14.2	2.98	0.01	0.67	0.26
Entodinium longinucleatum	0.184	1.21	5.47	1.12	1.252	<0.01	0.12	0.05
Epidinium sp.	0.954	1.27	0.362	0.706	0.1492	<0.01	0.02	0.07
Epidinium sp.	32.0	35.8	19.8	27.2	5.23	0.01	0.01	0.56
Isotricha sp.	19.8	7.9	1.9	16.5	3.19	<0.001	0.48	0.46
Neocallimastigomycota								
Buwchfawromyces	9.77	5.61	0.83	7.70	5.196	0.05	0.65	0.34
Neocallimastigaceae sp.	5.09	5.04	7.25	5.32	1.984	0.03	0.26	0.73

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Only significantly ($P < 0.05$) affected microbial genera are presented.

[3]Linear effect of CaPe inclusion.

[4]Quadratic effect of CaPe inclusion.

relative abundances of *Entodinium* sp., and *Entodinium longinucleatum*, but decreased *Isotricha* sp. The effect on *Epidinium* sp. was quadratic ($P < 0.05$), with CaPe1 increasing and CaPe2 decreasing abundance as compared with CON (Table 8).

The total fungal community was dominated by *Neocallimastix* (average 33.3%), *Piromyces* (28.3%), *SK3*

(11.7%) and *Cecomyces* (8.4%), followed by less abundant *Buwchfawromyces* (6%), *Neocallimastigaceae* sp. (5.7%), *Orpinomyces* (2.9%), and AL8 (2.1%). The remaining 6 genus level taxonomical groups were observed at minor relative abundance. Dietary treatments did not affect fungal α-diversity or fungal community structure (Figure 3D).

Fecal Microbiota

The fecal bacterial community was represented by 307 genera level taxonomical groups, but only 27 of them were detected at a relative abundance >1%. Among the more abundant genera from *Bacteroidota* phylum were *Bacteroides* (average 6.3%), *Bacteroidales RF16* group (2%), members of *Prevotellaceae* family (8.2%; *UCG-003*, *UCG-004*, *UCG-001*, and *Prevotella*), and members from *Rikenellaceae* family (8.2%; *Rikenellaceae RC9* gut group, *Alistipes*). More abundant genera from the *Firmicutes* phylum were *Christensenellaceae R-7* group (5.7%), *Oscillospiraceae UCG-005* (4.8%), members of the *Lachnospiraceae* family (5.4%; unclassified sp., *NK3A20* group, *Acetitomaculum*), *[Eubacterium] coprostanoligenes* group (3.1%), *Monoglobus* (1.5%), *Romboutsia* (1.6%), *RF39* (1.6%), and *Ruminococcus* (1.1%). Representatives of other phyla were *Treponema* (1.1%), *Akkermansia* (1.2%), and *Olsenella* (1.2%). Dietary treatments did not affect fecal bacterial α-diversity, but compared with CON, CaPe linearly reduced the relative abundance of *Bacteroidales RF16* group, *Erysipelatoclostridium*, *Lactobacillus*, *Howardella*, and *Oscillospirales* sp. On the other hand, CaPe linearly increased the relative abundance of *Barnesiellaceae* sp., *Bacteroidales F082*, *Catenisphaera*, *Solobacterium*, *[Eubacterium] hallii group*, *[Ruminococcus] gauvreauii group*, *Anaerovoracaceae* sp., *Family XIII UCG-001*, and *Rhodospirillales* sp. (Table 9). The BFE diet had a significantly ($P < 0.01$) higher relative abundance of *Paludibacter* and *Ruminococcaceae* sp. as compared with CON.

Dietary treatments did not induce changes in archaeal α-diversity or archaeal community structure. Fecal archaeal community was largely dominated by *Mbb. gottschalkii* clade (average 81.2%), followed by *Methanosphaera sp.* ISO3-F5 (6.8%), *Mbb. ruminantium* clade (5.2%), *Methanocorpusculum* sp. (4.1%), and *Methanosphaera* sp. (1.9%). In addition *Methanomassiliicoccus Group 10* sp., *Group 12* sp. ISO4-H5, and *Group 9*

Table 9. Fecal microbiota relative abundance (%) of lactating dairy cows fed diets containing different feed additives

	Treatment[1]					*P*-value		
Item[2]	CON	CaPe1	CaPe2	BFE	SEM	Lin[3]	Quad[4]	CON vs. BFE
Actinobacteriota								
Parvibacter	0.261	0.126	0.199	0.315	0.0601	0.38	0.02	0.68
Bacteroidota								
Bacteroides	6.83	5.82	6.28	6.17	0.306	0.19	<0.001	0.01
Bacteroidales RF16 group	2.24	1.90	1.63	2.15	0.200	<0.001	0.98	0.52
Barnesiellaceae sp.	0.342	0.402	0.460	0.312	0.0279	0.01	0.68	0.19
Bacteroidales F082	0.245	0.292	0.433	0.254	0.0415	<0.01	0.68	0.49
Paludibacter	0.436	0.330	0.356	0.595	0.0334	0.04	0.10	<0.01
Paludibacteraceae sp.	0.301	0.397	0.257	0.315	0.0322	0.22	0.02	0.88
Prevotella	1.77	0.929	1.33	1.43	0.191	0.09	0.01	0.18
Firmicutes								
Erysipelatoclostridium	0.195	0.167	0.123	0.166	0.0205	<0.01	0.17	0.17
Catenisphaera	0.080	0.112	0.136	0.061	0.0130	0.03	0.45	0.48
Solobacterium	0.135	0.212	0.351	0.162	0.0384	<0.01	0.79	0.27
Lactobacillus	0.386	0.308	0.291	0.320	0.0255	0.02	0.57	0.11
Howardella	0.115	0.062	0.053	0.135	0.0126	<0.001	0.28	0.27
[Eubacterium] hallii group	0.172	0.231	0.325	0.215	0.0271	<0.01	0.90	0.13
[Ruminococcus] gauvreauii group	0.294	0.335	0.431	0.306	0.0538	0.01	0.58	0.80
Oscillospirales spp.	0.129	0.074	0.050	0.102	0.0283	0.03	0.84	0.55
Ruminococcaceae sp.	0.364	0.537	0.333	0.588	0.0898	0.87	0.07	0.05
Negativibacillus	0.277	0.276	0.226	0.266	0.0299	0.04	0.09	0.90
Anaerovoracaceae sp.	0.159	0.185	0.221	0.167	0.0181	0.01	0.53	0.42
Family XIII UCG-001	0.076	0.090	0.127	0.077	0.0139	0.01	0.90	0.57
Proteobacteria								
Rhodospirillales spp.	0.150	0.158	0.348	0.094	0.0404	0.03	0.20	0.28

[1]CON = control diet; CaPe1 = diet containing 7.5 g/kg calcium peroxide; CaPe2 = diet containing 15 g/kg calcium peroxide; BFE = control diet containing 2 g/kg DM DiGestoChar (GoBioFarm Ltd., Iisalmi, Finland).

[2]Only significantly ($P < 0.05$) affected bacterial genera are presented.

[3]Linear effect of CaPe inclusion.

[4]Quadratic effect of CaPe inclusion.

sp. ISO4-G1 were observed at the relative abundance <0.05%.

Dietary treatments did not affect anaerobic fungi α-diversity and did not stimulate significant changes in community structure. Fecal anaerobic fungal community was represented by the same genera as in the rumen, but with *Piromyces* (average 30.4%), *Cecomyces* (28.8%) and SK3 (18.4%) being the predominant groups. Less abundant fungi in feces were *Neocallimastigaceae* sp. (9.4%), *Neocallimastix* (6.9%), and AL8 (3.5%), whereas *Buwchfawromyces* and *Orpinomyces* decreased in abundance to <1% when compared with their abundance in rumen.

DISCUSSION

Effects of Calcium Peroxide Addition

There is an urgent need to find effective CH_4-mitigating measures to improve the sustainability of ruminant production. The use of CaO_2 has potential to manipulate the rumen environment, but limited data are available about its effects on enteric CH_4 production and other animal parameters. A recently published in vivo study using growing bulls (Roskam et al., 2024) and an in vitro study reported by Graham et al. (2025) showed positive prospective results. However, preliminary results from an in vivo trial using sheep failed to demonstrate significant reduction in enteric CH_4 production (Pugh et al., 2024), which was also the case in our experiment.

Although daily CH_4 production decreased on CaPe treatments in our study, this was a reflection of reduced feed intake, and we found no differences between CON and CaPe treatments within CH_4 yield (g/kg DMI or g/kg OMI) or intensity (g/kg milk or g/kg ECM). Reduced feed intake was also linked to the negative effect of CaPe on milk production, as conversion of feed into milk (milk yield/DMI and ECM/DMI) remained similar as in CON. Our observations contrast with Roskam et al. (2024), who reported a reduction of 20% to 27% CH_4 yield (g/kg DMI) and 22% to 32% intensity (g CH_4/ kg ADG) in beef cattle, mainly due to no negative CaO_2 effect on DM intake in low (1.35% CaO_2) and high (2.25% CaO_2) treatments, where CaO_2 was included into pelleted concentrate, and no significant treatment effect on animal performance and efficiency traits. However, when a higher dose of CaO_2 was fed as coarse ration, DM intake in beef cattle was significantly reduced.

Several aspects of CaO_2 feeding may affect these outcomes. The level of inclusion is one important factor. The concentrations used in the current dairy cow experiment were 0.75% and 1.50% of diet DM, whereas Roskam et al. (2024) used higher doses for growing bulls (1.35% and 2.25% of diet DM). However, in our study, the effect of CaO_2 concentration on dairy animal responses was not linear for several parameters, and finding the optimal dose would require further experimentation. In general, a very high inclusion rate of CaO_2 is not favorable due to diluting energy and nutrient concentrations of the diets and the need for adjustment of the Ca:P ratio. An imbalance in the Ca:P ratio can cause metabolic or skeletal disorders in dairy cows, which may be a challenge in formulation of commercial diets.

The format and frequency of CaO_2 administration may play a role in explaining its efficacy. Roskam et al. (2024) offered CaO_2 as powder or in a pelleted form. The powdered form of CaO_2 decreased DMI, but it was not affected by the pelleted form. In our trial, CaO_2 was included in a pelleted concentrate mixture, but it nevertheless negatively affected intake. Based on subjective observations, the cows marginally sorted against the concentrate pellets containing CaO_2, which can be interpreted as an indication of reduced palatability. The leftovers were only analyzed for DM content, and calculating the proportion of silage (88%, 79%, 75%, and 86%) and concentrate in the leftovers based on silage, concentrate, and leftover DM contents for CON, CaPe1, CaPe2, and BFE, respectively, indicated marginal sorting against CaO_2 containing concentrates. Different pelleting techniques and encapsulation of CaO_2 could be evaluated in future trials to improve the palatability of CaO_2-containing feeds. Unlike for some other CH_4 mitigating agents, the high temperature and pressure associated with pelleting has no effect on the viability and function of CaO_2 (Roskam et al., 2024).

Another difference between the beef trial and our dairy experiment was feeding frequency. The beef cattle were fed CaO_2-containing concentrate twice daily with a 7-h break in between the meals and separately from forage. In our experiment, the CaO_2-containing concentrate pellets were mixed into a TMR and fed 4 times daily. Because CaO_2 is metabolized in the rumen into H_2O_2 before breaking down to oxygen and water, it potentially creates oxidative stress to the rumen and to rumen microbes, which might depress appetite (Demeyer, 1982). In a beef trial (Roskam et al., 2024) special boluses were used to measure oxidation-reduction potential (**ORP**) and demonstrated that ORP increased 2 to 3 h after feeding in diets with CaO_2 but returned to the control level before the afternoon feeding of CaO_2 containing concentrates. Graham et al., (2025) showed that ORP remained elevated even 8 h after the addition of CaO_2–containing feed in an in vitro system. Therefore, it is plausible to hypothesize that a constant supply of CaPe in every meal as a component in the TMR in our experiment was keeping the rumen environment outside the optimal ORP level, with negative effects on appetite, feed intake, and digestibility. It is possible that administration of CaO_2-containing

concentrates at intervals greater than 5 h would provide better conditions for the rumen environment and should be tested in future experiments with dairy cattle. This would make CaO_2 supplementation attractive for farms that are using separate concentrate feeding (e.g., in grazing systems), unlike 3-nitrooxypropanol (**3-NOP**), which needs to be administered continuously (Vattulainen et al., 2024) and is thus feasible only for farms using a TMR feeding.

Links between elevated ORP values and reduced digestibility are supported by the currently available data. In our study, CaPe linearly decreased the digestibilities of DM, OM, CP, EE, NDF, and GE, similarly with the observations on reduced DM, OM, and NDF digestibilities in beef cattle (Roskam et al., 2024), and reduced NDF, OM, and CP digestibilities in an in vitro RUSITEC system (Graham et al., 2025).

The effects of CaO_2 on rumen fermentation were dose dependent. In this study, no significant increases in pH or reductions in ammonia-N or total VFA were observed, which is contrary to the observations in beef trial. Molar proportions of butyrate increased, and isovalerate tended to increase linearly, whereas propionate and isobutyrate behaved quadratically. We hypothesize that rumen microbial community structure and activity is sensitive to CaO_2 concentration, where shifts in fermentation pathways may occur due to different thresholds for CaO_2.

In this experiment, the supplementation of CaO_2 led to a linear decrease in hydrogen production (g/d) and yield (g/kg DMI). The diurnal pattern of gas production demonstrated a hydrogen spike immediately after feeding, with reduced values in CaPe treatments. The same pattern was observed in the beef trial by Roskam et al. (2024), with a significant reduction by 32% to 36%. With other CH_4-inhibiting feed additives, such as 3-NOP and *Asparagopsis*, the reduction of methanogenesis is linked with an increase in hydrogen production (van Gastelen et al., 2020, Romero et al., 2024). This indicates that CaO_2 alters hydrogen pathways and might have alternative hydrogen sinks in rumen fermentation differently to 3-NOP. Longer chain saturated VFA synthesis is one of the potential sinks for extra hydrogen. Because only major VFA were measured in our trial, it would be interesting to elucidate the rumen metabolome to determine if longer chain fatty acids are present in CaO_2 treatments. This topic would warrant further metabolome research.

Effects of Biochar With Fibrolytic Enzyme and Live Yeast Addition

In the current study, supplementation with 0.2% BFE did not affect diet digestion, animal performance, or CH_4 production compared with CON. Biochar has been identified as a feed additive with CH_4 mitigation potential (Hegarty et al., 2021), but results are controversial. The variable responses to biochar supplementation may be attributed to differences in biochar source material, production methods, inclusion rates, and variations in basal diets (Schmidt et al., 2019). It is possible that the beneficial effect of biochar would be more pronounced with lower quality feeds (Erickson et al., 2011). In this study, the diets were based on high-quality grass silage (D-value 734 g/kg DM); therefore, at the tested inclusion rate, there might have been no suitable conditions for improvement in diet digestion, as visible in lack of BFE effect on rumen microbial composition, rumen fermentation parameters, and production traits. In addition, NDF and GE digestibilities tended to decrease on the BFE supplemented diet, which contradicts findings in other studies (Winders et al., 2019; Ni et al., 2024).

Even if CH_4 mitigation could not be achieved, biochar may have other beneficial properties that warrant further research. Biochar can be explored as an electron mediator, as it can be temporarily oxidized or reduced by microbes (Schmidt et al., 2019). We hypothesize that biochar could be tested in combination with CaO_2 to elucidate potential benefits in supporting rumen microbiome to adjust to elevated ORP values.

Effects of Calcium Peroxide on Rumen and Fecal Microbiota

We elucidated for the first time the dietary CaO_2 effect on all 4 microbial groups in the rumen (bacteria, archaea, ciliate protozoa, and anaerobic fungi), and our results suggest that bacteria and ciliate protozoa were most affected.

In the rumen environment, CaO_2 reacts with water to produce calcium hydroxide and hydrogen peroxide as intermediate products. Calcium hydroxide, through reaction with carbon dioxide, can create calcium carbonate, which in the acidic environment of the rumen will release calcium and carbonate ions. The released calcium could be absorbed and utilized by the cow for various physiological functions, such as milk synthesis, or the excess may be excreted in feces. Gut bacteria have specific enzymes to release dietary bound calcium, although addition of calcium to the diet, such as in the form of calcium propionate, had little effect on the rumen bacterial community composition (Zhang et al., 2022). On the other hand, Liu et al. (2020) studied goats for gut microbiota responses to digestibility of different dietary calcium amounts and noted significant increase in relative abundance of *Christensenellaceae R-7* group, *Ruminococcus*

2, *Quinella*, *Ruminococcaceae* UCG-004, *Mogibacterium*, Family XIII UCG-002, or *Ruminococcaceae* UCG-007 in the higher calcium digestibility group. All of these genus level groups were present also in our data, but most of them did not show significant abundance changes in response to CaPe treatments. Therefore, the role of the rumen microbiota in the utilization of calcium requires further investigation.

The second intermediate compound, hydrogen peroxide, can induce oxidative stress with damaging effects on host and microbial cells (Pang et al., 2024). Rumen microorganisms have antioxidant defense mechanisms in the form of enzymes that reduce or break down hydrogen peroxide into water and oxygen (Mann et al., 2018). It is expected that the extra O_2 introduced into the system may elevate ORP outside of the favorable range for the growth and activity of rumen anaerobic microorganisms, as has been observed in the beef trial using ORP measuring boluses (Roskam et al., 2024). Rumen samples in our study were collected 3 h after the morning feeding and fall within the time frame of potentially elevated ORP, suggesting that we might have captured microbial ecosystem under oxidative stress conditions. Supplementation of CaPe increased abundance of several bacilli (*Anaeroplasma*, *Erysipelatoclostridiaceae* UCG-004, *Erysipelotrichaceae* sp., *Solobacterium*, *RF39*). Interestingly, the relative abundance of *Bacillus* was 15 times higher during anaerobic fermentation of waste activated sludge treated with CaO_2 (Li et al., 2015), and various bacilli responded to CaO_2 pretreatment in waste composting trial (Hu et al., 2025). *RF39* and several members from *Erysipelotrichales* are uncultured orders within the bacilli. They have been shown to populate the human gut (Nayfach et al., 2019), and metagenome sequencing demonstrates that many of these members have hydrogen peroxide catabolism genes (Wang et al., 2020). The observed increase in bacilli could reflect their enzymatic capacity to manage reactive oxygen species.

Supplementation with CaPe increased *Entodinium* abundance as compared with CON at the expense of significant decreases in *Epidinium* and *Isotricha*. Demeyer (1982) experimented with increasing amounts of CaO_2 in sheep and demonstrated a defaunating effect of CaO_2, with *Entodinium* reappearing as the first ciliate protozoa. A 3-fold increase in abundance of *Entodinium* in the CaPe2 treatment suggests its potential tolerance to reactive oxygen species. In general, ciliate protozoa are aerotolerant anaerobes. Experiments with *Polyplastron multivesiculatum* and *Eudiplodinium maggii* demonstrated their ability to consume O_2 up to the inhibitory threshold specific for each species (Ellis et al., 1989), whereas *Entodinium caudatum* and *Epidinium caudatum* could be cultured successfully in aerobic media supplemented with selected antioxidants (Park and Yu, 2018). Furthermore, Park et al. (2021) sequenced the macronuclear genome of *E. caudatum* and found presence of genes encoding oxygen-scavenging enzymes, indicating that *E. caudatum* can tolerate O_2.

We hypothesize that ciliate protozoa could be responsible, at least partially, for the reduction in hydrogen production observed with CaPe treatments. Protozoa can produce hydrogen through hydrogenosomes, but their function can be affected by the physiological levels of O_2 in the rumen. Prins and Prast (1973) showed that in the presence of small amounts of O_2, production of hydrogen by *Isotricha* spp. decreased. Ellis et al. (1989) demonstrated that in the presence of O_2, *E. maggii* produced 8 times more hydrogen, whereas similar exposure of *P. multivesiculatum* to O_2 decreased hydrogen by 18%. This is suggested to be due to the modulation of the hydrogen-evolving system either directly by O_2 or by reactive oxygen species.

In this experiment, methanogens were not significantly affected by CaO_2, suggesting that the minor decrease in CH_4 production in CaPe treatments resulted from the overall decrease in feed digestion and reduced availability of methanogenic substrates. Hydrogen emission was reduced in the CaPe treatments, and therefore our prior hypothesis was to see *Mbb. ruminantium* at higher relative abundance, as it is suggested to thrive better under low levels of hydrogen partial pressure due to the absence of the methyl coenzyme-M reductase II (Mcr II) system in their genome (Morgavi et al., 2013). However, the average abundance of *Mbb. gottschalkii* reached 76%, whereas *Mbb. ruminantium* clade was only detected at ~8% abundance. A potential explanation could be that ORP more strongly affected the particular microbial interactions between *Mbb. ruminantium* and other bacteria or ciliate protozoa involved in hydrogen metabolism and transfer than in the case of *Mbb. gottschalkii*.

We elucidated the effect of dietary CaO_2 on the fecal microbiome due to the increased Ca and P concentrations in feces and reduced digestibility in rumen. *Bacteroidales RF16* group, *Prevotella*, *Solobacterium*, *[Ruminococcus] gauvreauii* group, and *Rhodospirillales* spp. were a few of the genera significantly affected by CaPe in both rumen and feces, but the remaining affected genera were low abundance and feces specific. Liu et al. (2020) observed diverse bacterial genera responding to digestibility of calcium across different parts of the gastrointestinal tract in goats, supporting lack of overlap in our data. However, the function of these genera in the colon requires further investigation.

CONCLUSIONS

Calcium peroxide, especially when used at a higher level (1.5% DM), reduced feed intake and milk yield

without affecting feed efficiency. Daily CH_4 and CO_2 production were reduced by both levels of CaO_2 but CH_4 and CO_2 yields (g/kg DMI) or intensities (g/kg ECM) were not different from the control, indicating, that the reduction in intake was the main reason for reduced CH_4 and CO_2 emissions. Feeding CaO_2 influenced rumen bacteria and ciliate protozoa communities more than archaea or anaerobic fungi. To overcome palatability issues, future research with dairy cows could test feeding CaO_2 pelleted concentrates separately from silage instead of feeding them as TMR, by allowing a break of several hours between the CaO_2-concentrate offerings. The biochar with fibrolytic enzyme and live yeast product at the inclusion rate of 0.2% did not influence feed intake, milk and ECM yields, feed efficiency, CH_4 and CO_2 emissions, rumen fermentation, or gut microbiota composition.

NOTES

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Nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; ASV = amplicon sequence variant; BFE = biochar with fibrolytic enzymes and live yeast; CaPe = calcium peroxide; CaPe1 = diet supplemented with 0.75% CaO_2 on a DM basis; CaPe2 = diet supplemented with 1.5% CaO_2 on a DM basis; CON = control diet; EE = ether extract; GE = gross energy; Lin = linear effect; ORP = oxidation-reduction potential; PCoA = principal coordinate analysis; Quad = quadratic effect.

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ORCIDS

J. Vattulainen, https://orcid.org/0009-0001-6268-2014
A. R. Bayat, https://orcid.org/0000-0002-4894-0662
T. Stefański, https://orcid.org/0000-0001-5553-9941
M. Rinne, https://orcid.org/0000-0001-6323-0661
I. Tapio https://orcid.org/0000-0002-0752-9551