

Article

Effect of a Combination of Phytogenic Compounds on In Vitro Rumen Fermentation Parameters and In Vivo Lactation Performance and Methane Emissions in Dairy Cows

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Abstract: An in vitro and an in vivo study were conducted to investigate the effects of a blend of cinnamaldehyde, eugenol, and capsicum oleoresin (CEC) on rumen fermentation parameters, animal performance, and methane (CH₄) emissions in dairy cows. Continuous culture fermenters (CCF) were utilized to test one of two treatments: (1) CON; no supplementation and (2) CEC supplemented at 0.0125 g/d. The basal diet consisted of grass hay and concentrate (50:50). Supplementation with CEC increased ($p < 0.01$) total volatile fatty acids (VFA; mM) and decreased ($p = 0.02$) CH₄ concentration compared with CON in vitro. Additionally, protozoa abundance tended ($p = 0.07$) to decrease in CEC compared with CON. The in vivo experiment utilized forty Holstein-Friesian dairy cows (32% primiparous and 68% multiparous) averaging 163 ± 48 days in milk (DIM) and 38 ± 6.2 kg/d of milk yield (MY). Cows were blocked by parity and randomly assigned to one of two treatments: (1) CON; no supplementation and (2) CEC supplemented at 1.2 g/cow/d. The basal diet consisted of grass hay and concentrate (40:60). Individual CH₄ emissions were recorded using the sniffer technique. Dry matter intake (DMI) and eating rate were increased ($p < 0.01$; 3.6% and 5.2%, respectively), while feed efficiency decreased ($p < 0.05$) in CEC compared with CON. Additionally, CEC decreased ($p = 0.02$) CH₄ yield by 16.4% and tended to reduce daily CH₄ production ($p = 0.09$) and CH₄ intensity ($p = 0.08$) by 13.4% and 14.0%, respectively. Supplementing CEC decreased CH₄ concentration in vitro and CH₄ yield in vivo without negatively impacting performance parameters.

Keywords: enteric methane; additives; greenhouse gas; dairy cow; phytogenic compounds



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1. Introduction

Climate change is at the forefront of political and public interest. Thus, greenhouse gas (GHG) emissions arising from anthropogenic activities are under critical review. Livestock emissions account for approximately 32% of global anthropogenic methane (CH₄) emissions [1], and this is largely due to microbial-mediated fermentation by ruminant animals. Unlike carbon dioxide (CO₂) that persists in the atmosphere over centuries, CH₄ has an

atmospheric lifetime of approximately 12 years with 28 times greater Global Warming Potential (GWP₁₀₀) [2]. Though large-scale combustion of fossil fuels remains the largest contributor to climate change, even a moderate reduction of CH₄ in the near term would significantly increase the feasibility of limiting warming to 1.5–2.0 °C [3]. Consequently, there is increasing interest in research to reduce enteric CH₄ emissions from ruminant animals.

A recent meta-analysis identified feed additives as the most promising strategy for reducing enteric methane emissions in ruminants [4]. Due to their antimicrobial properties, plant-derived extracts such as essential oils, tannins, saponins and other flavonoid-containing components are of particular interest. Indeed, a range of essential oils have been reported to decrease CH₄ production in vitro [5]. Previous research has demonstrated that certain essential oils can effectively reduce methane emissions by altering rumen microbial activity [6]. For instance, in an in vitro study evaluating clove oil, eucalyptus oil, garlic oil, organum oil, and peppermint oil, each individual essential oil decreased methane production, with organum oil achieving the greatest reduction of 87% at a dose of 1.0 g/L. However, the study also noted that higher doses could negatively impact feed digestion and fermentation, highlighting the importance of optimizing concentrations to balance methane mitigation with overall rumen function. Additionally, essential oils vary in their effectiveness depending on dose, combination, and interactions with rumen microbiota, with certain formulations showing methane inhibition in vitro but displaying inconsistent results in vivo [7]. For example, studies on cinnamaldehyde supplementation have reported methane reductions of up to 5% in dairy cattle, but higher concentrations may negatively impact fiber digestion and fermentation balance [8].

Recently, a commercial blend of cinnamaldehyde, eugenol, and capsicum (CEC) has demonstrated CH₄ reduction capabilities in vivo. The CEC blend decreased CH₄ yield in Holstein [9] and Nordic-Red [10] dairy cows 3.4% and 4.2%, respectively, but it had no impact on animal performance. Inhibiting methanogenesis could theoretically improve energy use efficiency in the rumen by redirecting hydrogen (H₂) towards more valuable fermentation pathways. However, this does not always translate to increased animal performance [11]. Such outcomes may be attributed to a variety of confounding factors such as diet, parity, stage of lactation, or dosage [12]. Additionally, current knowledge on the effects of CH₄ inhibitors on rumen metabolites and microbial abundance is limited [13], highlighting the need for further research. To address this gap, our study uniquely integrates both in vitro and in vivo approaches, enabling a comprehensive assessment of how CEC influences rumen fermentation dynamics, microbial abundance, and CH₄ mitigation across experimental settings. Overall, this study aimed to address (1) the effects of CEC on in vitro rumen fermentation parameters and (2) the effects of CEC on enteric CH₄ emissions and lactation performance of dairy cows. We hypothesized that supplementing CEC blend would (1) decrease CH₄ production and modify rumen microbial abundance and fermentation parameters in vitro and (2) decrease enteric CH₄ emissions and improve lactation performance in vivo. The in vivo study was conducted as a combined efficacy and animal safety trial in which four doses of CEC were evaluated. However, only data from the control and the recommended CEC dose (1.2 g/d/cow) will be presented and discussed in the present study.

2. Results

2.1. Experiment 1 (In Vitro)

Supplementing CEC decreased ($p = 0.02$; Table 1) in vitro CH₄ concentration compared with CON. However, total gas production ($p = 0.12$) and CH₄ production ($p = 0.26$) were not affected by treatment. The abundance of bacteria, archaea, and fungi were not affected by

treatment ($p > 0.05$; Table 2). However, there was a tendency ($p = 0.07$) for CEC to decrease the abundance of protozoa when compared with CON.

Table 1. Effect of CEC on in vitro methane emissions.

	Treatment ¹			<i>p</i> -Value
	CON	CEC	SEM	Trt
Gas Production (mL)	16.5	17.5	0.35	0.12
CH ₄ Concentration (mL/100 mL gas)	0.16	0.14	0.042	0.02
CH ₄ Production ² (mL)	26.8	24.7	0.83	0.26
CH ₄ :VFA ³	0.28	0.24	0.012	0.08

¹ CON = no supplementation; CEC = 0.0125 g/d of Xtract Ruminant (XTRACT RUMINANT, Rolle, Switzerland).

² CH₄ Production = Gas Production × CH₄ Concentration. ³ CH₄:VFA = mL methane/mM total VFA.

Table 2. Effect of CEC on in vitro rumen microbial abundance.

	Treatment ¹			<i>p</i> -Value
	CON	CEC	SEM	Trt
Microbial abundance ²				
Bacteria	8.50	8.42	0.061	0.42
Archaea	5.09	5.08	0.079	0.97
Protozoa	6.34	6.17	0.049	0.07
Fungi	4.51	4.45	0.092	0.69

¹ CON = no supplementation; CEC = 0.0125 g/d of Xtract Ruminant (XTRACT RUMINANT, Rolle, Switzerland).

² Microbial concentration based in qPCR in log10 DNA copies/mg DM.

While molar proportions of individual VFA were unaffected by treatment ($p > 0.05$), total VFA concentration (mM) increased ($p < 0.01$; Table 3) due to CEC supplementation. Additionally, CEC increased ($p = 0.02$) lactic acid concentration (mg/100 mL) when compared with CON.

Table 3. Effect of CEC on in vitro rumen fermentation parameters.

	Treatment ¹			<i>p</i> -Value
	CON	CEC	SEM	Trt
Total VFA, mM	95.6	102.5	0.64	<0.01
Proportions, %				
Acetate	58.2	57.5	1.01	0.63
Propionate	22.0	23.3	1.12	0.45
Butyrate	13.6	13.0	0.33	0.26
Isobutyrate	1.21	1.20	0.051	0.87
Valerate	2.18	2.11	0.055	0.47
Isovalerate	2.80	2.90	0.341	0.85
Acetate:propionate ratio	2.69	2.53	0.139	0.45
Lactic acid, mg/100 mL	29.8	39.7	1.74	0.02
Nitrogen fractions, mg/100 mL ²				
NH ₃ -N	8.71	6.12	1.139	0.18
Amino Acid-N	7.23	13.03	1.589	0.06
Peptide-N	20.88	18.28	3.001	0.57

¹ CON = no supplementation; CEC = 0.0125 g/d of Xtract Ruminant (XTRACT RUMINANT, Rolle, Switzerland).

² NH₃-N = ammonia; Amino Acid-N = small peptides and amino acids; Peptide-N = large peptides.

Amino Acid-N tended to be greater ($p = 0.06$) due to CEC supplementation, while Peptide-N ($p = 0.57$) and NH₃-N ($p = 0.18$) were not affected by treatment.

2.2. Experiment 2 (In Vivo)

2.2.1. Enteric Methane Emissions

Supplementation with CEC decreased ($p = 0.02$; Table 4) CH₄ yield by 16.4% compared with CON. Additionally, supplementation with CEC tended to decrease CH₄ production ($p = 0.09$) by 13.4% and CH₄ intensity ($p = 0.08$) by 14% when compared with CON. No weekly effect was observed ($p > 0.05$) for any of the CH₄ measurements.

Table 4. Effect of CEC on methane exhalation of lactating dairy cows.

	Treatment ¹			<i>p</i> -Value ²		
	CON	CEC	SEM	Trt	Week	T × W
CH ₄ production, L/d	636	551	29.5	0.09	0.17	0.54
CH ₄ intensity, L/kg of Milk	17.1	14.7	0.79	0.08	0.12	0.45
CH ₄ yield, L/kg of DMI	25.6	21.4	1.22	0.02	0.13	0.49

¹ CON = no supplementation; CEC = cows received 1.2 g/d of (XTRACT RUMINANT, Rolle, Switzerland).

² Trt = effect of treatment; Week = effect of week; T × W = interaction between treatment and week of study.

2.2.2. Animal Performance

The addition of CEC increased ($p < 0.01$; Table 5) DMI (kg/d) and eating rate (g/min) by 3.6% and 5.2%, respectively. There were no differences ($p > 0.05$) in milk yield and milk composition parameters between CON and CEC cows. However, there was a weekly effect ($p < 0.01$) for body weight (BW), milk yield, milk fat, milk protein, milk solids nonfat, and energy-corrected milk (ECM). As expected, BW increased over time whereas milk yield, milk fat, and ECM decreased over time. Milk solids nonfat decreased in the first 3 weeks and increased during the rest of this study, while the opposite was observed for milk protein. A treatment by week interaction ($p < 0.01$) was observed for BW, with BW being greater in CEC cows in the first 3 weeks but decreasing during weeks 5–7 compared with CON cows. Feed efficiency (relative to ECM) decreased ($p < 0.05$) in CEC cows.

Table 5. Effect of CEC on production characteristics of lactating dairy cows.

	Treatment ¹			<i>p</i> -Value ²		
	CON	CEC	SEM	Trt	Week	T × W
DMI, kg/d	24.8	25.7	0.12	<0.01	0.87	0.57
Eating rate, g/min	115	121	1.1	<0.01	0.99	0.95
BW, kg	769	769	17.4	0.99	<0.01	<0.01
Milk yield, kg/d	38.7	38.6	1.04	0.96	<0.01	0.93
Milk fat, %	3.58	3.61	0.02	0.47	<0.01	0.96
Milk fat, kg/d	1.39	1.39	0.04	0.92	<0.01	0.89
Milk protein, %	3.21	3.23	0.02	0.57	<0.01	0.39
Milk protein, kg/d	1.24	1.25	0.03	0.94	<0.01	0.89
Milk lactose, % ³	4.89	4.99	0.04	0.26	0.06	0.33
Milk solids nonfat, % ³	8.85	8.99	0.05	0.19	<0.01	0.75
Milk SCC, × 10 ³ /mL ³	4.86	5.38	0.20	0.20	0.40	0.69
ECM, kg/d ⁴	39.5	39.6	1.02	0.96	<0.01	0.90
Feed efficiency, % ⁵	1.61	1.55	0.02	0.05	0.64	0.99

Abbreviations: DMI = dry matter intake; BW = body weight; Milk SCC = milk somatic cell count; ECM = energy-corrected milk. ¹ CON = no supplementation; CEC = cows received 1.2 g/d of Xtract Ruminant (XTRACT RUMINANT, Rolle, Switzerland). ² Trt = effect of treatment; Week = effect of week; T × W = interaction between treatment and week of study. ³ Determined at 13, 26, 41, and 56 d of study. ⁴ Calculated as $0.3246 \times \text{milk yield (kg)} + 12.86 \times \text{fat (kg)} + 7.04 \times \text{protein (kg)}$ (NRC 2001 [14]). ⁵ Calculated as ECM/DMI.

3. Discussion

This study aimed to provide a comprehensive evaluation of the effects of CEC on rumen fermentation, CH₄ emissions, and animal performance by integrating both in vitro and in vivo methodologies. We hypothesized that supplementing CEC would decrease

CH₄ production and modify rumen microbial abundance and fermentation parameters in vitro and decrease enteric CH₄ emissions and improve lactation performance in vivo. Although the in vitro and in vivo trials were not run in parallel with the same animals and diets, they were both conducted with the same type of animal and compound and utilized diets consisting primarily of grass hay, and concentrate. Thus, in vitro fermentation and microbial abundance data will be used to support the discussion of the in vivo results, providing insight into potential mechanisms of CEC effects on the cow.

3.1. Experiment 1 (In Vitro)

Total in vitro gas production was not affected by CEC supplementation, but there was a decrease in CH₄ concentration and a numerical decrease in CH₄ production. Supplementation with plant extracts tends to decrease gas production due to the antimicrobial effects that can alter rumen fermentation, of which gas is a byproduct [15,16]. The mechanism of action of most plant extracts is related to their ability to disrupt microbial cell membranes leading to decreased ATP synthesis and eventually cell death [5]. For example, eugenol may cause leakage of the bacterial cell content, while cinnamaldehyde may disrupt the transmembrane and affect cell stability [17]. The overall difference in the effects of different plant extracts on rumen microbial fermentation may be the result of different sensitivities of specific microbial populations to these compounds. Although plant extracts are effective against Gram-positive and Gram-negative bacteria, the outer membrane of Gram-negative bacteria provides a degree of protection, making them less sensitive to the presence of essential oils [5]. A study in lambs fed a high-concentrate diet revealed significant differences in rumen microbial beta-diversity between lambs supplemented with CEC and a control group [18]. Supplemented lambs had greater relative abundance of Synergistota at the phyla level and *Megasphaera* at the genus level with both positively correlating to increased butyrate production. In the current study, bacteria, archaea, and fungi abundance were not affected by CEC supplementation, but protozoa abundance tended to decrease. Another potential mechanism of CEC to decrease CH₄ is by suppressing the activity of H₂-producing microbes, therefore reducing the availability of the main substrate necessary for CH₄ production in the rumen [19]. Previous studies show that CH₄ concentrations decrease in defaunated animals likely due to the decrease in H₂ available for methanogenesis [20,21]. Therefore, the decrease in protozoa abundance due to supplementation of CEC in vitro could decrease the H₂ available for methanogenesis. Although H₂ was not measured in the present study, a decrease in H₂ emissions due to CEC supplementation has been observed previously in vivo [9,10]. Additionally, certain protozoa consume lactate and engulf starch granules very rapidly competing with amylolytic bacteria that tend to produce lactate in the rumen [21]. Thus, a decrease in protozoa abundance could lead to an increase in lactate concentration or VFA, which agrees with the results in the current study. However, the link remains speculative, and future research incorporating functional and transcriptomic analyses such as measurements of hydrogen flux and mcrA gene expression would be needed to confirm the proposed mechanism.

Nitrogen metabolism in the rumen can generally be divided into protein degradation and microbial protein synthesis. In the present study, NH₃-N was numerically decreased, and AA-N significantly increased. Consistent with our findings, previous studies [18,22,23] also reported a reduction in ruminal NH₃-N concentrations following supplementation with the same CEC blend. This could suggest CEC inhibits deamination or directly decreases abundance of proteolytic and/or hyper-ammonia producing bacteria [24].

3.2. Experiment 2 (In Vivo)

Similar to previous observations [9,10], CEC supplementation decreased CH₄ yield (L/kg DMI) and tended to decrease CH₄ production (L/d) and CH₄ intensity (L/kg milk) by 16.4%, 13.4%, and 14%, respectively. Although some of these effects did not meet the threshold for statistical significance, the consistent direction and magnitude of the responses suggest biologically meaningful reductions in methane emissions. Even modest reductions align with mitigation targets and could contribute to improved environmental sustainability when implemented at scale [25]. The magnitude of inhibition in the current study is greater than previously reported, which could be attributed to differences in diet, animal type, duration of study, or method of CH₄ measurement. Efficacy of anti-methanogenic additives, like *Asparagopsis* spp. and 3-NOP, has been linked to nutrient composition of the diet and, more specifically, to the levels of NDF [26]. The diet in the present study had a lower NDF (and higher NFC) than the previous studies conducted with CEC, which may explain the difference in CH₄ response. Additionally, the previous studies used GreenFeed (GF) units over a period of 12 weeks to determine CEC effects on CH₄, while the current study used a “sniffer” technique for a duration of 8 weeks. These different approaches in experimental design are highlighted in the recent review outlining recommendations for testing anti-methanogenic additives [26]. Authors note that long-term studies should be conducted to provide information on persistence of CH₄ mitigation effects, but short-term studies can sufficiently provide evidence for the efficacy of CH₄ mitigating additives. No treatment by time interactions were observed for CH₄ parameters in the present study or previous studies [9,10], suggesting a stable response to CEC. The challenge remains to identify essential oils that selectively inhibit rumen methanogenesis across a range of diets, with persisting effects and without depressing rumen fermentation and animal productivity. Overall, the consistent CH₄ inhibition response by CEC suggests it could be a beneficial CH₄ inhibitor in the dairy industry.

Supplementing CEC in the current study improved feed intake in dairy cows, confirming previous work by van Gastelen et al. [9]. Indeed, aromatic herbs, spices, and various plant extracts are often claimed to improve the flavor and palatability of feed, thus increasing voluntary feed intake, resulting in improved performance [5]. When dairy cows were fed capsicum extract, a strong correlation between water consumption and dry matter intake was observed [27]. However, despite the increase in DMI in the current study, MY was not improved and feed efficiency decreased in CEC cows compared with CON. This reduction in feed efficiency may reflect altered nutrient partitioning or metabolic shifts associated with increased intake. The additional nutrients may have been directed toward maintenance energy, thermogenesis, or other physiological processes not directly contributing to milk synthesis [28]. This could in part explain the treatment x week interaction observed for BW where CEC cows exhibited greater BW during the first three weeks. Essential oils and plant extracts can influence rumen fermentation and metabolic activity, potentially affecting how nutrients are utilized. This interpretation aligns with findings from a meta-analysis evaluating several essential oil combinations that did not observe a significant impact on feed efficiency or milk yield, concluding that diet and supplementation period may influence the impact of plant extracts on performance [29]. For instance, a blend of eugenol, coriander essential oil, and geranyl acetate supplemented to dairy cows on a diet containing no silage had a reduction in feed efficiency [30], but the same blend fed to dairy cows on a diet containing corn-silage had an increase in feed efficiency [31].

4. Materials and Methods

The in vitro experiment (Experiment 1) was conducted in continuous culture fermenters to assess the effects of CEC on rumen fermentation parameters and microbial abundance. The in vivo experiment (Experiment 2) was conducted on Holstein dairy cows to assess the effect of CEC on CH₄ emissions and animal performance.

4.1. Experiment 1 (In Vitro)

4.1.1. Experimental Design

The experiment was conducted at Estación Experimental del Zaidín (EEZ-CSIC) in Spain. The experimental diet consisted of a 50:50 grass hay/concentrate (Table 6). Each fermenter was fed with 16 g of fresh matter per day of the basal diet ground at 1 mm, divided into two equal portions given at 09:00 and 14:00 h, respectively. The vessels were inoculated with pooled rumen fluid, and the flow through rate was maintained by continuous infusion of buffer [32] at a rate of 40 mL/h. The stomach tubing technique [33] was used to collect rumen fluid 3 h after feeding from 3 different dairy cows. Two vessels per treatment were randomly allocated to (1) CON (no additive) or (2) CEC (0.0125 g/d) and were incubated in two consecutive periods of 15 days ($n = 4$). The dose selected was based on the dose given in vivo (1.2 g/cow/d) by assuming the volume of a typical rumen (80 L) [34,35] and adjusting to the volume used in the fermenters (1 L). The first 5 days served as adaptation for the rumen microbial environment and in vitro conditions [36], and on day 6, treatments were applied. Fermentation vessels were sampled on day 14 and 15 two hours after morning feeding and divided into subsamples for analysis of VFA concentration, nitrogen fractions, and microbial abundance.

Table 6. Ingredient and nutrient composition (DM basis) of the in vitro experimental diets ¹.

Ingredients, % DM	Forage	Concentrate
Alfalfa hay	50.0	
Barley grain		29.5
Corn grain		10.0
Sunflower cake		10.0
Urea		0.5
Nutrients		
Dry matter, %	91.6	89.4
Crude protein, %	7.1	17.7
NDF, %	59.8	31.4
ADF, %	34.8	10.4
Ether extract, %	1.4	2.5

¹ CON = no supplementation; CEC = 0.0125 g/d of Xtract Ruminant (XTRACT RUMINANT, Rolle, Switzerland).

On day 16, contents from each vessel were used in a batch-culture in vitro trial [37] to determine total gas production and CH₄ concentration over a 24 h period as previously described by Martínez-Fernández et al. [36]. Briefly, the contents from each fermenter were filtered through two layers of cheesecloth and bubbled with CO₂. Serum bottles (120 mL) were filled with 60 mL of fermenter content and incubated with 500 mg of the same diet. Three replicates and a blank control were used for each fermenter and treatment. The serum bottles were sealed with rubber stoppers and aluminum caps and incubated at 39 °C in a water bath. At 24 h after inoculation, the total gas volume was measured in each bottle and a sample of the gas was collected in a graduated syringe, transferred to a 5 mL vacuum tube (Venoject, Terumo Europe N.V., Leuven, Belgium) and kept at room temperature before measurement of CH₄ concentration by gas chromatography.

4.1.2. Fermentation Parameter Analyses

Concentrations of individual VFA (acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate) were determined by a gas chromatography system coupled with a flame ionization detector (Auto-system Perkin-Elmer Corp., Shelton, CT, USA) as previously described [38]. Lactate and ammonia concentrations were measured using the colorimetric methods described previously [39,40].

Samples taken from the fermentation vessels on d 14 and d 15 of incubation were analyzed for nitrogen content and fractionated into large peptides (Pep-N; between 3 and 10 AA), small peptides and amino acids (AA-N; <2 and 3 AA), and ammonia (NH₃-N). The amino acid and peptide nitrogen fractions were analyzed as described by Busquet et al. [41]. Briefly, a sample of filtered fermenter fluid was added to 10% (wt/vol) sodium tungstate and 1.07 N sulfuric acid, incubated at 5 °C for 4 h, and then centrifuged at 9000 × *g* for 15 min. The supernatant was analyzed for tungstic acid precipitable nitrogen (TA-N) by the Kjeldahl procedure [42]. Next, trichloroacetic (TCA) solution was added to filtered fermenter fluid, incubated at 5 °C for 4 h, and then centrifuged at 9000 × *g* for 15 min. The supernatant was analyzed for TCA acid precipitable nitrogen (TCA-N) by the Kjeldahl procedure. Results were used to calculate (1) large peptide N (LPep N; between 3 and 10 AA) = [TCA-N] – [TA-N]; and (2) small peptide (between 2 and 3 AA) plus amino acid N (Spép + AA N) = [TA-N] – [ammonia N] (in mg/100 mL) [43].

The *in vitro* CH₄ concentration was determined by gas chromatography with a Hewlett–Packard 5890 Series II gas chromatograph (Waldbronn, Germany). A sample of 0.5 mL of gas was injected into the chromatograph with a 1 mL Sample-Lock syringe (Hamilton, Reno, NV, USA). The amount of CH₄ produced in each fermenter was calculated by multiplying the total gas produced by the concentration of CH₄ obtained [36].

4.1.3. Real-Time qPCR Analysis

Total genomic DNA was extracted from samples taken from the fermenter on d 14 and d 15 and abundance of protozoa, bacteria, fungi, and archaea were determined by qPCR as described in [36]. Briefly, samples were freeze-dried and thoroughly mixed by physical disruption with a bead beater (Mini-bead Beater-8) for 1 min before using a QIAGEN QIAamp DNA stool mini kit (Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions apart from a higher temperature (95 °C) used in the lysis incubation step. The DNA samples were used as templates for quantifying the copy numbers of 16S rRNA (for bacteria), the methyl coenzyme M reductase A (*mcrA*) gene (for methanogenic archaea), and 18S rRNA (for protozoa). Primer sets used were as follows: forward: 5'-GTG-STGCAYGGYTGTCTCA-3' and reverse: 5'-ACGT-CRTCCMCACCTTCCTC-3' for total bacteria ([44]; Table 7) and forward: 5'-GCTTTTCGWTGGTAGT-GTATT-3' and reverse: 5'-CTTGCCCTCYAATCGT-WCT-3' for protozoa [45]. The primer sets for detection and enumeration of methanogenic archaea (*mcrA*) were forward: 5'-TTCGGTGGATCD-CARAGRGC-3' and reverse: 5'-GBARGTCGWAWC-CGTAGAATCC-3' [46]. Real-time PCR analyses were performed with an iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). One microliter of DNA extract was added to amplification reaction mixtures (25 µL) containing 0.2 µL of each primer (10 µM) and 12.5 µL of iQ SYBR Green Supermix (BioRad Laboratories). Cycling conditions were 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 55 s, and 72 °C for 1 min. The absolute amount of DNA for each microbial group, expressed as the number of DNA copies per gram of fresh matter, was determined via a standard curve.

Table 7. Primers used for qPCR.

Target	Forward Primer	Reverse Primer	Reference
16S rRNA	GTG-STGCAYGGYTGTCGTCA	ACGT-CRTCCMCACCTTCCTC	[44]
18S rRNA	GCTTTCGWTGGTAGTGATT	CTTGCCCTCYAATCGTWCT	[45]
mcrA	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAAATCC	[46]

4.2. Experiment 2 (In Vivo)

4.2.1. Animals, Design, and Diets

All experimental procedures were approved and supervised by the Animal Care Committee of IRTA (Barcelona, Spain, expedient number: CEEA 307/2022). The experiment was conducted at Blanca from the Pyrenees in Spain. Forty lactating Holstein dairy cows (32% primiparous and 68% multiparous) with an initial milk production of 38.0 ± 6.2 kg/d, live BW of 712 ± 75 kg, and 163 ± 48 DIM (mean $\pm$ SD) were blocked by parity and randomly split in 2 treatments ($n = 20$) following a complete randomized design. All cows were housed in a single pen and received the same total mixed ration (TMR) ad libitum during the adaptation period (10 d) consisting of 40% hay and 60% concentrate on a DM basis (Table 8). During the experimental period (8 weeks), dietary treatments were applied consisting of (1) CON—control with no supplementation or (2) CEC—supplementation of 1.2 g/cow/d of XTRACT Ruminant, X60-7065, a specific blend of essential oil compounds manufactured by ADM (ADM International Sàrl, Rolle, Switzerland). The blend is composed of 9.5% eugenol, 5.5% cinnamaldehyde, and 3.5% capsicum oleoresin encapsulated in hydrogenated oil (81.5%). Half of the daily CEC dose (0.6 g per portion and 1.2 g/d in total) was added to 2 kg of soybean meal and incorporated in the TMR during morning and evening feeding. The same amount of soybean meal without CEC was fed to control cows.

Table 8. Ingredient and nutrient composition (DM basis) of the experimental diet.

Item	Ingredient
Ingredients, % DM	
Alfalfa hay	7.57
Fescue hay	14.42
Rye grass hay	18.03
Straw	0.77
Soybean meal ¹	11.68
Barley grain	1.51
Corn grain	21.09
Wheat grain	10.75
Soybean hulls	10.31
Palm oil	2.05
Calcium carbonate	0.82
Magnesium oxide	0.29
Salt	0.33
Mineral vitamin premix ²	0.37
Nutrients	
Crude protein, %	16.1
NDF, %	34.5
ADF, %	23.8
Ash, %	7.5
Net energy, Mcal/kg ³	1.68
Ether extract, %	4.03
Non-fiber carbohydrate, %	37.9

¹ CON = no supplementation; CEC = cows received 1.2 g/d of XTRACT Ruminant, X60-7065, Rolle, Switzerland via 2 kg of soybean meal. ² Contained: 81.6 mg/kg of Zn; 11.5 mg/kg of Cu; 57.6 mg/kg of Mn; 9.86 mg/kg of Co; 1.92 mg/kg of I; 0.34 mg/kg of Se; 58 mg/kg of S; 120,000 IU/kg of vitamin A; 28,800 IU/kg of vitamin D; and 1920 IU/kg of vitamin E. ³ Net energy was estimated using NRC (2001) [14] equations.

4.2.2. Sample Collection and Chemical Analyses

Daily feed intake was recorded for individual cattle using electronic feed bins (MooFeeder, MooSystems, Cortes, Spain) which only opened in response to the respective

dietary treatments. Samples of TMR were obtained weekly, composited for subsequent analysis of nutrient content, and stored at $-20\text{ }^{\circ}\text{C}$. Individual body weights were recorded twice daily upon exiting the milking parlor using electronic scales. Individual milk yield was recorded using electronic milk meters (AfiMilk, Afikim Ltd., Kibbutz Afikim, Israel) at every milking. Milk fat and milk protein contents were also determined after every milking using the AfiLab system (Afikim Ltd., Kibbutz Afikim, Israel). Calibration was performed by analyzing individual milk samples from every milk meter in the local DHI laboratory (Allic, Barcelona, Spain) and then adjusting the meters to match the result from the laboratory.

Moisture concentration of the forage, concentrate (Experiment 1), and TMR (Experiment 2) was determined by heating at $103\text{ }^{\circ}\text{C}$ for 24 h, ash content by heating at $550\text{ }^{\circ}\text{C}$ for 4 h, N concentration following the method (988.05) of AOAC International [47] adapted for an automatic distiller Kjeldahl (Kjeltec Auto 1030 Analyzer, Tecator, Hoganas, Sweden) with copper sulfate/selenium as a catalyst instead of copper sulfate/titanium dioxide, NDF concentration using sodium sulfite and heat-stable α -amylase, and ether extract as described in method 920.39 of AOAC International [47] with petroleum ether used for extraction. Energy-corrected milk was calculated as $\text{ECM (kg/d)} = 0.3246 \times \text{milk yield (kg)} + 12.86 \times \text{fat (kg)} + 7.04 \times \text{protein (kg)}$ [14]. Feed efficiency was calculated as the amount of ECM divided by the amount of DMI [14].

4.2.3. Gas Emissions

Methane and CO_2 recordings were performed using a sniffer technique with NDIR (Guardian NG Edinburg Instruments Ltd., Livingston, UK) as described by Bach et al. [30] and validated by Rey et al. [48]. Methane production was calculated using carbon dioxide as a tracer gas, estimating daily heat producing units for each animal based on BW and ECM as described by Madsen et al. [49], and heat was converted into liters of carbon dioxide following Pedersen et al. [50]. With this system, the typical CV for CH_4 production (using CH_4/CO_2 ratio) is $<10\%$ and repeatability $>80\%$ [51]. Individual CH_4 exhalation was determined on all cows exiting the milking parlor two days a week, alternating days (i.e., Monday and Wednesday week 1; Tuesday and Thursday week 2), for 20 min a day (10.02 ± 1.83 min in the morning and evening) resulting in 32 sets of 10 min measures for every cow in the study. Five measurement stations consisting of a feed bin and two electronic meters each (one for CH_4 and one for CO_2) were used. These stations were placed at the exit of the milking parlor, and cows were offered 100 g of soybean meal to ensure that their head was placed inside the recording station. The stations sampled air at 1 L/min from the front of the cow's head and pumped it into the electronic gas analyzer to continuously measure CH_4 and CO_2 at 1 s intervals and persisted in a database. Baseline CH_4 and CO_2 concentrations were calculated as mean CH_4 and CO_2 concentrations before starting the measurements and subtracted from the subsequent measured data. Before the start of the study, four measures of CH_4 and CO_2 exhalations were collected for each animal on two separate collection days and later included as covariates in the statistical analysis [30]. Twice daily, in the morning and in the afternoon, animals and housing facilities were inspected to check the general health status, to ensure constant feed and water supply, as well as to ensure adequate temperature and ventilation.

4.2.4. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (9.2, SAS Institute Inc., Cary, NC, USA). Experiment 1 included the fixed effects of treatment, period, and their interaction, with the random effect of fermenter vessel. Experiment 2 included the fixed effects of treatment, week, and their interaction, with the random effects of animal and block (parity).

All daily values were averaged by animal and week, and weekly means were used for data analysis. The experimental unit was the animal. Week was used as a repeated measure following a first order auto-regressive covariate structure, which yielded the lowest Bayesian information criterion. For CH₄ and CO₂ exhalation data, the same model was used but including the basal exhalations for each animal as a covariate. Least square means $\pm$ standard error of means (SEM) are reports with significance declared at $p \leq 0.05$ and tendency assumed for $0.05 < p < 0.10$.

5. Conclusions

Supplementing CEC in vitro increased VFA concentration and decreased CH₄ concentration and protozoa abundance. When supplemented in vivo, CEC increased DMI by 3.6%, but milk yield and lactation characteristics were not affected. Notably, CEC decreased CH₄ yield (L/kg DMI) by 16.4% and tended to decrease CH₄ production and intensity by 13.4 and 14%, respectively. Further research evaluating persistence of CH₄ reduction due to CEC supplementation and potential dietary interactions are warranted.

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