



Effects of a combination of *Capsicum* oleoresin and clove essential oil on metabolic status, lactational performance, and enteric methane emissions in dairy cows

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ABSTRACT

Botanical extracts have a potential to modify ruminal fermentation while enhancing metabolism and immunity in dairy cows. The objective of this study was to investigate the effects of a combination of *Capsicum* oleoresin and clove essential oil (botanicals; BTC) on lactational performance, nutrient utilization, enteric methane (CH₄) emissions, and blood parameters in dairy cows. Twenty Holstein cows (12 multiparous and 8 primiparous) averaging (\pm SD) 77 \pm 28 d in milk in the beginning of the study were used in a replicated 4 \times 4 Latin square design experiment with 4 periods of 28 d each. Cows were grouped into squares based on parity, milk yield and days in milk, and assigned to 1 of 4 treatments: control (CON), 150, 300, or 600 mg/cow per day of BTC. Cows received the same basal diet and BTC were top-dressed on the total mixed ration once daily. Dry matter intake, milk production, and milk composition were not affected by BTC supplementation, except for milk fat content that tended to be increased in BTC, compared with CON. Daily CH₄ emission (measured using the GreenFeed system) was linearly decreased by up to 7.5% with increasing doses of BTC. Treatment decreased CH₄ yield (kg of CH₄ \div kg of DMI) and tended to decrease CH₄ intensity (kg of CH₄ \div kg of milk or energy-corrected milk yields) by 5% in BTC, compared with CON. Supplementation of BTC resulted in a quadratic decrease of serum β -hydroxybutyrate in all cows, and a linear decrease of serum insulin concentration in primiparous but not in

multiparous cows. Nutrient utilization and other blood parameters (e.g., blood cells count) were not affected by BTC in the current study. The reduction of enteric CH₄ emission demonstrates a moderate mitigation effect on carbon footprint of milk by BTC supplementation. These results must be further investigated and confirmed in longer-term experiments.

Key words: capsicum, dairy cow, enteric methane, insulin

INTRODUCTION

In recent years, research aimed to identify plant-based feed additives that could enhance animal performance while optimizing the health of dairy cows (Oh et al., 2017b). For instance, increased productivity (e.g., milk yield, ECM, FCM, or ADG) has been reported in dairy (Wall et al., 2014; Oh et al., 2015, 2021) and beef cattle (Westphalen et al., 2021) with dietary supplementation of *Capsicum* oleoresin (CAP), cinnamaldehyde, and clove extracts. *Capsicum* contains bioactive compounds (i.e., capsaicinoids) with a potential to enhance immune status and decrease oxidative stress responses in LPS-challenged dairy cows (Oh et al., 2017a). Moreover, lymphocytes percentage (Oh et al., 2015) and blood monocytes count (Oh et al., 2018a) were decreased in clinically healthy cows. Interestingly, supplementation with low doses of CAP resulted in decreased insulin concentration during a glucose tolerance test (Oh et al., 2017c) and an LPS challenge (Oh et al., 2017a) in dairy cows. The implications of these immune and metabolic responses were subsequently explored by Oh et al. (2021) who described decreased blood BHB concentration prepartum with CAP supplementation, indicating improved energy status of transition dairy cows. Similar effects of capsaicinoids on the insulin axis, glucose, and energy metabolism have also been observed in rodents and humans (Chaiyasit et al., 2009; Kang et al., 2011; Sanati et al., 2018). Other plant extracts can also immunomodulate and improve intestinal barrier in ani-

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mals. Eugenol (i.e., a constituent of clove essential oil) supplementation increased resistance of mice to enteric pathogen infection by stimulating the production of the inner mucus layer (Wlodarska et al., 2015), which is known as a key mucosal barrier against pathogens in the intestines. Taken together, these observations demonstrate the potential of using botanicals to improve metabolic health and intestinal barrier in dairy cattle.

As reported by IPCC (2021), it is unequivocal that emissions of CO₂, CH₄, and N₂O have been increasing since the beginning of the industrial era, and they are contributing to climate change across the globe. Considering the rising demand for animal protein by the growing population worldwide, the use of sustainable practices is crucial to decrease the contribution of the livestock sector to global greenhouse gas (GHG) emissions (Beauchemin et al., 2020). Additionally, enteric CH₄ is not only an environmental concern but also represents a loss of dietary gross energy in ruminants (Johnson and Johnson, 1995). Although research has identified effective GHG mitigation strategies for ruminants (Hristov et al., 2013; Melgar et al., 2020; Cueva et al., 2021), adoption of these strategies by farmers will only take place if accompanied by improved animal performance and farm profitability, or if incentivized by government policies (Hristov et al., 2013).

Nutritional interventions to mitigate enteric CH₄ have been thoroughly investigated and discussed (Hristov et al., 2013; Arndt et al., 2021; Congio et al., 2021), and it is likely that strategies based on supplementation with plant extracts, such as essential oils (EO), may have a higher acceptance by livestock producers compared with, for example, the use of antibiotics. The main reason is that most of these plant extracts are Generally Recognized as Safe (GRAS) compounds by the Food and Drug Administration (US FDA, 2021) and could have an appeal to the consumer. However, research investigating the effect of EO on ruminal fermentation and enteric CH₄ emissions has been mostly based on in vitro batch or continuous culture studies (Tekippe et al., 2012), and results are not always replicated in experiments with dairy cows (Benchaar et al., 2008; Hristov et al., 2012).

The objective of this study was to investigate the effects of a combination of CAP and clove EO (botanicals; BTC) on the lactational performance, enteric CH₄ emission, nutrient excretion, and blood parameters in dairy cows. Our hypothesis was that BTC supplementation would improve ruminal fermentation and metabolic status (e.g., reduced insulin and increased blood glucose concentrations), leading to enhanced nutrient utilization and lactational performance of the cows. We also hypothesized that enhanced animal productivity

would reduce intensity of enteric CH₄ emission contributing for decreased carbon footprint of milk.

MATERIALS AND METHODS

All procedures involving animals carried out in the study were approved by The Pennsylvania State University's Institutional Animal Care and Use Committee.

Animals, Experimental Design, and Treatments

The experiment was conducted in the tiestall barn of The Pennsylvania State University's Dairy Teaching and Research Center. A total of 20 lactating Holstein cows (12 multiparous and 8 primiparous) averaging (\pm SD) 2.5 \pm 1.5 lactations, 77 \pm 8 DIM, and 49 \pm 9 kg/d milk yield (MY) at the beginning of the experiment, were used in a replicated 4 \times 4 Latin square design experiment balanced for carryover effects. The experiment consisted of 4 periods with 28 d each, of which 21 d were allowed for adaptation to treatments and 7 d for data and samples collection. Cows were grouped into 5 squares based on parity, DIM, and MY. Cows within square were randomly assigned to 1 of 4 treatments: control (CON), 150, 300, or 600 mg/cow per day of BTC (150, 300, and 600, respectively). The doses were determined based on in vitro studies conducted in our laboratory aimed at evaluating the effect of BTC on CH₄ emissions (unpublished data by A. N. Hristov, The Pennsylvania State University). According to the manufacturer's (AVT Natural North America) specifications, the product contained a mixture of *Capsicum* oleoresin (1.25% total standardized content) and a full-spectrum clove EO, with a total volatile oil content of 5.2% (as-is basis). The remainder of the product (93.5%) was a carrier constituted of fat and used for encapsulation. All cows received the same basal diet (Table 1) once daily at around 0800 h, and the diet was fed ad libitum targeting 10% refusals. The BTC was top-dressed daily, by mixing with approximately 500 g of TMR during feeding. The diet was formulated to meet or exceed the nutrient requirements of a lactating Holstein cow weighing 650 kg BW, producing 48 kg/d MY, with 3.80% fat and 3.20% true protein, at 27 kg/d of DMI (NRC, 2001). Cows were milked twice daily at 0600 and 1800 h and always had free access to drinking water.

In previous research, we observed that the ruminal escape of CAP was up to 33%, depending on the dose (Oh et al., 2016), and that of polyphenols (e.g., eugenol) was up to 73% (Oh et al., 2018b). The ruminal escape fraction of the BTC used in the current experiment was estimated to be 23%, based on a 24-h in situ

Table 1. Ingredient and nutrient composition of the basal diet fed in the experiment

Item	Diet
Feed ingredients, % of DM	
Corn silage ¹	43.4
Alfalfa haylage ²	10.0
Straw/hay mix ³	5.03
Ground corn	8.92
Whole roasted soybeans	3.49
SoyPLUS ⁴	6.02
Canola meal	12.5
Whole cottonseed	4.47
Molasses	4.92
Mineral/vitamin mix ⁵	1.02
Urea	0.22
Nutrient composition, ⁶ % of DM (or as indicated)	
CP	16.1
NDF	31.4
ADF	20.4
NFC ⁷	45.4
Starch	24.2
NE _L balance, ⁷ Mcal/kg	1.54
Ash	4.50
Ca	0.60
P	0.40

¹Corn silage was 40.0% DM and contained (DM basis) 7.0% CP and 34.6% NDF.

²Alfalfa haylage was 47.0% DM and contained (DM basis) 17.2% CP and 49.1% NDF.

³Straw/hay mix was 92.0% DM and contained (DM basis) 9.4% CP and 69.0% NDF.

⁴Heat-treated soybean meal (West Central Cooperative); contained (DM basis) 45.1% CP.

⁵Mineral and vitamin premix (Cargill Animal Nutrition, Cargill Inc.) contained (% as-is basis) limestone, 36.75; dry corn distillers grains with solubles, 29.00; NaCl, 24.85; MgO (54% Mg), 4.15; Bio-Phos, 2.45 (Rootwise Soil Dynamics); zinc sulfate, 0.96; mineral oil, 0.5; vitamin E, 0.37; manganese sulfate, 0.37; copper sulfate, 0.26; ferrous sulfate, 0.16; selenium, 0.13; vitamin A, 0.03; vitamin D₃, 0.013; calcium iodate, 0.008; cobalt carbonate, 0.005.

⁶Unless indicated otherwise, values were calculated using the nutrient analysis (Cumberland Valley Analytical Services Inc., Waynesboro, PA) of individual feed ingredients and their inclusion rates in the diet.

⁷Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment.

incubation experiment (unpublished data by A. N. Hristov, The Pennsylvania State University). Briefly, an in situ experiment was conducted with 3 ruminally cannulated cows using a modification of the procedure described in Lee et al. (2012). Cows were maintained on a basal diet containing feed ingredients typically fed at Penn State's Dairy Teaching and Research Center (corn silage, alfalfa haylage, ground corn grain, canola meal, whole roasted soybeans, and others). Approximately 3 g of BTC were weighed in a 5 × 10-cm, 50-μm porosity polyester bag (Ankom Technology Corp.), soaked in warm water (39°C) for 1 min and incubated in the rumen for 0, 6, 12, and 24 h. The incubation time points were based on Lee et al. (2012) and the bags were incubated in triplicate for each time point and

cow. Bags were inserted into the rumen simultaneously and removed sequentially. When removed from the rumen, bags were washed with cold tap water until the runoff water was clear. Washed bags were then dried in a forced-air oven at 55°C for 72 h; 0-h bags were processed as for the other time points, except for the rumen incubation. The dried bags were weighed, and DM disappearance was calculated based on the original DM of the product, and the dry weights of the incubated BTC sample.

Sample Collections

Feed and TMR Sampling. Weights of feed offered and refusals were recorded daily. Fresh TMR, refusals, and feed ingredient samples were collected twice weekly and stored frozen at -20°C until oven-dried at 55°C for 72 h for further analyses. Dried samples were ground using a Wiley mill (Thomas Scientific) through a 1-mm screen and composited by experimental period (TMR and forage samples) or for the entire experiment (concentrate feeds). Individual feed ingredients were analyzed at Cumberland Valley Analytical Services (Waynesboro, PA) by wet chemistry methods for CP (method 990.03; AOAC International, 2000), amylase-treated NDF (Van Soest et al., 1991; not ash-corrected), ADF (method 973.18; AOAC International, 2000; not ash-corrected), ether extract (method 2003.05; AOAC International, 2006), ash (method 942.05; AOAC International, 2000), minerals (method 985.01; AOAC International, 2000), and calculated NFC (NRC, 2001). Composite TMR samples were analyzed for starch according to Hall (2009) and indigestible NDF (iNDF) as described in Huhtanen et al. (1994) and modified by Lee et al. (2012). Nutrient composition of the basal diet was reconstituted from analyzed values of individual feed ingredients and their inclusion rate in the TMR (Table 1).

Production Data Collection and Milk Sampling. Milk yield (Afimilk system) and BW (AfiFarm 3.04E scale system; SAE Afikim) of cows were recorded daily throughout the experiment. Milk samples were collected from 4 consecutive milkings (a.m. and p.m.) over 2 consecutive days during the last week of each experimental period (i.e., a total of 4 samples per cow and period) into 20-mL tubes containing bromo-2-nitropropane-1,3-diol. Samples were analyzed for concentrations of milk fat, true protein, MUN, lactose, TS, and SCC by Dairy One laboratory using Milkoscan models 6000, FT+, or 7 and Fossomatic models 5000 or FC (Foss Electric A/S). Separate unpreserved samples were also collected as described above and stored frozen at -20°C. These samples were composited on an equal-

volume basis per cow and period and analyzed for fatty acid (FA) profile as described by Rico and Harvatine (2013).

Fecal and Urine Sampling. During the last week of each experimental period, 8 spot fecal samples were collected during 3 consecutive days at intervals staggered in time to cover a 24-h feeding cycle: at 0500, 1100, 1700, and 2300 h (d 1), 0800, 1400, and 2000 h (d 2), and 0200 h (d 3). Fecal samples were oven-dried at 55°C for 72 h, ground through a 1-mm sieve in a Wiley mill (Thomas Scientific) and composited per cow and experimental period. Composite fecal samples were then analyzed for total N using a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies Inc.), ADF and NDF using an Ankom 200 fiber analyzer (Ankom Technology Corp.), and starch (Hall, 2009). Indigestible NDF was used as a total-tract digestibility marker (Schneider and Flatt, 1975). For the iNDF analysis, composited fecal samples were oven-dried at 55°C, ground through a 1-mm screen, and incubated for 12 d in the rumen of a lactating rumen-cannulated cow according to Huhtanen et al. (1994), except 25- μ m pore size filter bags (Ankom Technology Corp.) were used for the incubation (Lee et al., 2012). The rumen-cannulated cow used for the iNDF incubation was fed a diet containing corn silage, alfalfa haylage, concentrate feeds, and a mineral and vitamin premix.

Urine samples were collected at the same time points as for fecal samples by perineal stimulation and were filtered through 2 layers of cheesecloth. Aliquots (10 mL) were acidified with 0.6 mL of 2 M sulfuric acid, diluted 1:10 with 90 mL of distilled water, and stored at -20°C until further analyses. Samples were composited by cow and experimental period and analyzed for allantoin (Chen et al., 1992), uric acid (Stanbio Uric acid Kit 1045; Stanbio Laboratory Inc.), urea-N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.), and creatinine (Stanbio Creatinine Kit 420; Stanbio Laboratory Inc.). Composite urine samples were freeze-dried and analyzed for total N using a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies Inc.). Urinary creatinine concentration was used to estimate daily urine output, assuming a creatinine excretion rate of 29 mg/kg of BW determined from total urine collections in lactating Holstein cows (Hristov et al., 2011). Daily excretions of total N, urea-N, and purine derivatives (allantoin and uric acid) were calculated based on the estimated daily urine output.

Blood Sampling. During the last week of each experimental period, blood samples were collected from the coccygeal vein or artery of cows 4 times in 2 consecutive days at 1100 and 1900 h (d 1) and 0700 and 1500 h (d 2). Samples (approximately 10 mL) were

collected into serum-separating vacuum tubes (BD Biosciences). Serum was separated by centrifugation at $1,800 \times g$ at 20°C for 30 min and stored at -80°C until further analyses. Serum samples were composited per cow and experimental period on an equal-volume basis and enzymatic colorimetric methods were used for total FA [total FA-HR(2), kit no. 999-34691, intra- and inter-assay CV: 0.75 and 4.91%, respectively; Wako Diagnostics] and BHB (Autokit 3-HB, kit no. 417-73501, intra- and inter-assay CV: 5 and 10%, respectively; Wako Diagnostics) analyses. The minimum detectable levels of the methods were 0.0014 μ mol/L (oleic acid equivalent) and 3 μ mol/L for total FA and BHB, respectively. Serum insulin was analyzed using an enzyme-linked immunoassay kit (Bovine Insulin ELISA, kit no. 10-1201-01, Mercodia AB). The inter- and intra-assay CV were both <10%. The minimum detection level for insulin was 0.05 μ g/L. Blood chemistry (glucose, creatinine, BUN, total protein, albumin, globulin, alanine transaminase, and alkaline phosphatase) were analyzed using a Catalyst One Chemistry Analyzer (Idexx Laboratories Inc.). A second set of blood samples was collected from the coccygeal vein or artery at the same time points as indicated above for hematology analysis. Samples (approximately 10 mL) were collected into tubes containing EDTA (BD Biosciences), kept refrigerated (4°C), and analyzed on the same day. The analysis included red blood cell count, hemoglobin, hematocrit, and total white blood cell count, including total count of neutrophils, eosinophils, lymphocytes, monocytes, and basophils using an automated hematology analyzer (HemaVet; Drew Scientific).

Enteric Gas Emissions. Enteric gas (CH₄, CO₂, and H₂) emissions were measured using the GreenFeed system (C-Lock Inc.) One GreenFeed unit was used and calibrated at the beginning of each measurement period based on manufacturer's recommendations (https://globalresearchalliance.org/wp-content/uploads/2018/08/GreenFeeds-SOP-_final.pdf; accessed Apr. 11, 2022). Cows were properly trained to use the system before the beginning of the experiment. Cows were identified with a unique radio-frequency identification ear tag for recognition in the GreenFeed unit. Gas measurements were taken 8 times over 3 consecutive days during the last week of each experimental period at 0900, 1500, and 2100 h (d 1), 0300, 1200, and 1500 h (d 2), and 0000 and 0300 h (d 3), following the procedure described by Hristov et al. (2015). Individual breath samples were collected during 5 min sampling events, followed by 2-min intervals for background air collection between cows. Gas samples were collected following the sequence of cows in the tiestall barn, and the sequence of sampling was maintained throughout

Table 2. Dry matter intake, milk production and composition, and BW of dairy cows fed botanicals combination (BTC)

Item	Treatment ¹				SEM ²	P-value ³		
	CON	150	300	600		C vs. T	L	Q
DMI, kg/d	27.9	28.2	27.6	27.3	1.25	0.72	0.21	0.76
Milk yield, kg/d	38.9	39.0	38.8	38.4	1.33	0.79	0.48	0.80
Feed efficiency, ⁴ kg/kg	1.41	1.40	1.43	1.42	0.062	0.95	0.73	0.93
Milk fat, %	3.80	3.95	3.91	3.98	0.186	0.06	0.11	0.47
Milk fat yield, kg/d	1.47	1.53	1.51	1.50	0.079	0.29	0.79	0.35
Milk true protein, %	3.01	2.95	2.97	2.97	0.060	0.11	0.45	0.28
Milk true protein yield, kg/d	1.17	1.14	1.15	1.13	0.031	0.24	0.18	0.89
Lactose, %	4.82	4.84	4.82	4.81	0.054	0.91	0.40	0.51
Milk lactose yield, kg/d	1.88	1.89	1.87	1.85	0.070	0.79	0.40	0.77
Total solids, %	12.5	12.6	12.6	12.6	0.23	0.15	0.22	0.58
MUN, mg/dL	10.6	11.3	10.8	11.0	0.33	0.08	0.45	0.44
Log SCC × 10 ³ cells/mL	1.63	1.61	1.59	1.58	0.097	0.49	0.41	0.81
ECM, ⁵ kg/d	37.1	37.7	37.4	36.7	1.49	0.83	0.48	0.40
ECM feed efficiency, ⁶ kg/kg	1.34	1.35	1.37	1.35	0.053	0.56	0.71	0.47
BW, kg	625	627	625	621	20.2	0.59	0.13	1.00

¹Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

²Largest SEM published in table; n = 76 (n represents the number of observations used in the statistical analysis).

³Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity × treatment interaction for any variable ($P \geq 0.25$).

⁴Milk yield ÷ DMI.

⁵Energy-corrected milk (kg/d) = kg of milk × [(38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140] (Sjaunja et al., 1990).

⁶ECM yield ÷ DMI.

the entire experiment. A pelletized bait feed (Stocker Grower 14, Purina Animal Nutrition LLC) was used to attract cows to the GreenFeed unit during gas sampling events. The intake of pellets was calculated based on the weight of pellets in each drop (32 g) and the number of drops during a sampling event. Intake of pellets was included in the daily DMI calculation during sampling days. Dry matter intake, digestible OM intake, MY, and ECM yield during sampling week were used to calculate CH₄ emission yield (i.e., g CH₄ ÷ kg DMI and digestible OM intake) and intensity (i.e., g CH₄ ÷ kg MY and ECM yield).

Statistical Analysis

All data were analyzed with SAS (release 9.4, SAS Institute Inc.). Two cows were removed from the experiment after they were diagnosed with severe mastitis and coxofemoral luxation during experimental period 3. Data collected from these cows on periods 1 and 2 remained in the analysis. Data were analyzed for outliers using the REG procedure and outliers were removed based on an absolute studentized residue value >3. Statistical analyses were performed using the MIXED procedure. Milk yield, DMI, BW, and feed efficiency, from the last 7 d of each experimental period, were analyzed as repeated measures using the AR(1) covariance structure. Statistical models included experimental period,

treatment, parity, and treatment × parity interaction as fixed effects, and day as the repeated term. Square and cow within square were random effects. When non-significant ($P > 0.05$), treatment × parity interaction was removed from the final models. Milk composition, blood chemistry and hematology, nutrient intake and digestibility, urinary excretions, and enteric gas emission data were analyzed without the repeated term in the models. Gas emissions data were averaged across all sampling time points and the averaged values were used for statistical analysis. Orthogonal and polynomial contrasts were used to evaluate BTC treatment versus control and to test for linear and quadratic effects of BTC dose. Statistical differences were considered significant at $P \leq 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$. Data are presented as least squares means.

RESULTS AND DISCUSSION

Production Data

Dry matter intake and MY were not affected by BTC supplementation (Table 2). Milk fat and MUN concentrations tended to be greater ($P = 0.06$ and $P = 0.08$, respectively) for BTC compared with CON. Botanicals had no effects on the other production variables evaluated in the current study.

Table 3. Intake and apparent total-tract digestibility of nutrients in lactating dairy cows fed botanicals combination (BTC)

Item	Treatment ¹				SEM ²	P-value ^{3,4}		
	CON	150	300	600		C vs. T	L	Q
Intake, ⁵ kg/d								
DM	27.7	27.9	27.3	27.1	1.18	0.67	0.23	0.83
OM	25.9	26.2	25.6	25.4	1.11			
NDF	9.71	9.80	9.60	9.52	0.414			
ADF	4.90	4.95	4.85	4.80	0.209			
CP	4.55	4.59	4.50	4.46	0.194			
Starch	6.61	6.67	6.53	6.48	0.282			
Apparent digestibility, %								
DM	69.2	68.6	68.2	68.4	0.43	0.04	0.11	0.11
OM	69.8	69.3	69.1	69.4	0.57	0.33	0.64	0.35
NDF	49.4	49.9	49.9	50.0	0.69	0.32	0.37	0.65
ADF	36.9	35.7	35.9	36.4	1.46	0.58	0.90	0.58
CP	69.7	69.9	69.1	69.6	0.81	0.78	0.77	0.61
Starch	98.7	98.5	98.5	98.5	0.08	0.09	0.21	0.49

¹Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

²Largest SEM published in table; n = 76 for total-tract digestibility of DM, CP, and starch; n = 75 for total-tract digestibility of ADF; n = 77 for all other variables (n represents the number of observations used in the statistical analysis).

³Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity × treatment interaction for any variable ($P \geq 0.10$).

⁴P-values for intake of dietary nutrients are the same as for DMI.

⁵Dry matter intake during the fecal collection, last 3 d of each experimental period.

Contrary to our hypothesis and to results from previous studies evaluating CAP (Oh et al., 2015, 2021) or botanical extracts containing eugenol (Wall et al., 2014; Benchaar et al., 2015), BTC did not improve lactational performance of dairy cows in the present study. It is noted, however, that comparisons with the above studies should be done with caution as the treatment in the current experiment contained both CAP and eugenol and these bioactive compounds were not studied individually. Similar to previous reports (Wall et al., 2014; Oh et al., 2015, 2021), DMI was not affected by BTC in the current study. The tendency for increased milk fat content in response to BTC supplementation may be related to the decreased BHB concentration and increased uptake of ketone bodies by the mammary gland (see later discussion), which could reflect a differential metabolic adaptation and energy partitioning in cows fed BTC.

In agreement with the current data, da Silva et al. (2020) reported that dairy cows fed a blend of EO containing carvacrol, eugenol, and capsaicin tended to increase milk fat concentration by 0.11 percentage units. Energy status of the animal is an important factor to be considered when evaluating milk fat regulation. Increased milk fat synthesis as a consequence of increased BHB uptake by the mammary gland in cows fed a diet supplemented with BTC has not been described yet and needs further investigation. Alternatively, the tendency for increased milk fat could be associated with

increased ruminal acetate and BHB produced from butyrate absorbed in the rumen because these compounds are the major precursors supporting milk fat synthesis (Bauman and Griinari, 2003). However, it is important to point out that ruminal VFA concentrations were not analyzed in the current study. The effect on milk fat in the current experiment was counteracted by a slightly numerical decrease in milk true protein concentration, which resulted in lack of treatment effect on ECM yield.

Apparent Total-Tract Digestibility and Nitrogen Excretions

Intake of dietary nutrients during the digestibility measurement period was not affected by BTC supplementation (Table 3). Apparent total-tract digestibility of DM was decreased ($P = 0.04$) by less than 2 percentage units, and starch digestibility tended to be decreased ($P = 0.09$) by BTC, compared with CON. Additionally, digestibility of other nutrients was not affected by BTC supplementation. The small effect of BTC on DM and starch digestibility, and the lack of effect on digestibility of other nutrients, corroborates with previous studies that supplemented a similar composition of botanical products to dairy (Benchaar et al., 2015; Oh et al., 2015) and beef cattle (Westphalen et al., 2021). Yang et al. (2010) reported that total-tract digestibility of starch in beef heifers tended to decrease linearly with increasing dietary inclusion of eugenol (0,

Table 4. Nitrogen excretion and secretion, and urinary purine derivatives excretion in lactating dairy cows fed botanicals combination (BTC)

Item	Treatment ¹				SEM ²	P-value ³		
	CON	150	300	600		C vs. T	L	Q
N intake, g/d	728	734	720	713	31.0	0.67	0.23	0.83
N excretion or secretion, g/d								
Urine N	175	172	164	177	16.8	0.80	0.92	0.53
Urinary urea-N	135	136	132	139	9.52	0.87	0.60	0.58
Fecal N	221	220	222	220	9.89	0.88	0.87	0.92
Total excreta N	397	393	388	384	21.6	0.60	0.51	0.91
Milk N	183	182	180	176	4.63	0.37	0.11	0.79
As % of N intake								
Urine N	23.8	23.0	22.3	25.1	1.88	0.87	0.59	0.36
Urinary urea-N	18.3	18.7	18.4	19.5	0.99	0.57	0.30	0.65
Fecal N	30.3	30.1	30.9	30.4	0.81	0.78	0.78	0.61
Total excreta N	54.1	53.0	52.9	54.5	1.81	0.78	0.81	0.52
Milk N	25.2	24.4	25.0	24.7	0.87	0.27	0.68	0.56
Unaccounted N ⁴	20.7	21.6	21.1	20.4	2.00	0.89	0.86	0.76
Purine derivatives, mmol/d								
Allantoin	496	538	467	554	65.0	0.68	0.55	0.59
Uric acid	45.9	44.7	39.6	42.4	3.38	0.25	0.29	0.27
Total PD ⁵	544	583	507	596	66.5	0.76	0.61	0.53

¹Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

²Largest SEM published in table; n = 77 (n represents the number of observations used in the statistical analysis).

³Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity × treatment interaction for any variable ($P \geq 0.10$).

⁴Unaccounted N = N intake – (Urinary N + Fecal N + Milk N).

⁵Purine derivatives.

400, 800, and 1,600 mg/d) in their diet. However, effect of BTC on starch digestibility, and perhaps on that of DM, was biologically insignificant in the current study.

Considering the potential of botanical compounds to modify ruminal fermentation (Calsamiglia et al., 2007; Oh et al., 2018a), cows supplemented with BTC may have enhanced ruminal fermentation and potentially microbial protein synthesis, which would lead to decreased urinary N excretion. However, excretions of fecal or urinary N, urinary urea, total excreta N, and urinary purine derivatives were not affected by BTC supplementation (Table 4). These results may be a consequence of a similar pattern of protein and carbohydrates fermentation in the rumen of cows fed CON or BTC, which is in line with the lack of differences in milk N secretion, N use efficiency, and BUN concentration observed in the current study. Digestibility and N excretion data from the current study should be interpreted with caution because spot sampling and markers were used to estimate total fecal and urinary excretions. The spot sampling technique has been validated but results are inherently more variable than total fecal and urine collections (Lee and Hristov 2013; Lee et al., 2019). The latter technique can also produce erroneous results, particularly related to N excretions and balance, if not correctly applied. A recent meta-analysis of 86 dairy cow experiments described that N retention is typically overestimated, mainly due to

unaccounted volatilization losses of N during collection and processing of fecal and urine samples (Spanghero and Kowalski, 2021); these authors reported no statistical difference in estimated N balance between total collection and spot sampling techniques (63 vs. 23 studies, respectively).

Milk Fatty Acid Composition

Only minor changes in milk FA profile were observed in the current study (Table 5). Concentrations of 17:0 increased ($P = 0.02$) and *trans*-5 18:1 tended to decrease ($P = 0.08$) by BTC, compared with CON. Supplementation with BTC decreased ($P = 0.05$) the concentrations of *cis*-6,*cis*-9,*cis*-12 18:3 and *cis*-9,*trans*-11 CLA ($P = 0.05$), compared with CON. Additionally, concentration of 20:2n6 was lower ($P = 0.002$) for BTC, compared with CON, and tended to be quadratically decreased ($P = 0.009$) by BTC dose. Concentration of 20:3n6 also tended to be lower ($P = 0.10$) in BTC, compared with CON.

The evaluation of milk FA profile can be used as a proxy to understand ruminal fermentation and metabolism in dairy cows. For instance, impaired ruminal fermentation is related to increased biohydrogenation intermediates with a potential to induce milk fat depression (Rico and Harvatine, 2013). Increased concentration of preformed FA in milk, in contrast, can

Table 5. Fatty acid composition of milk fat (g/100 g of total fatty acids) in lactating dairy cows fed botanicals combination (BTC)

Item	Treatment ¹				SEM ²	P-value ³		
	CON	150	300	600		C vs. T	L	Q
4:0	4.95	4.84	4.78	4.71	0.220	0.37	0.30	0.78
6:0	2.43	2.45	2.43	2.40	0.091	0.89	0.56	0.76
8:0	1.31	1.31	1.32	1.29	0.049	0.82	0.62	0.73
10:0	2.97	2.96	3.00	2.96	0.108	0.98	0.93	0.76
12:0	3.38	3.33	3.42	3.38	0.118	0.99	0.82	0.90
14:0	10.9	10.9	11.0	10.9	0.24	0.80	0.99	0.56
<i>cis</i> -9 14:1	0.87	0.78	0.87	0.84	0.053	0.35	0.98	0.66
15:0	0.99	1.01	1.04	1.01	0.046	0.38	0.61	0.33
16:0	26.8	27.2	27.1	27.2	0.57	0.24	0.36	0.65
<i>cis</i> -9 16:1	1.21	1.15	1.26	1.24	0.087	0.84	0.28	0.92
17:0	0.48	0.51	0.50	0.50	0.013	0.02	0.19	0.15
18:0	11.6	11.9	11.6	11.7	0.71	0.64	0.95	0.78
<i>trans</i> -4 18:1	0.02	0.02	0.02	0.03	0.002	0.82	0.71	0.70
<i>trans</i> -5 18:1	0.017	0.015	0.014	0.013	0.0013	0.08	0.08	0.46
<i>trans</i> -6,8 18:1	0.36	0.34	0.34	0.34	0.015	0.12	0.25	0.41
<i>trans</i> -9 18:1	0.30	0.29	0.29	0.29	0.013	0.13	0.36	0.30
<i>trans</i> -10 18:1	0.84	0.80	0.76	0.90	0.157	0.79	0.44	0.16
<i>trans</i> -11 18:1	1.13	1.06	1.10	1.07	0.053	0.17	0.38	0.57
<i>trans</i> -12 18:1	0.58	0.57	0.57	0.56	0.017	0.23	0.24	0.84
<i>cis</i> -9 18:1	19.4	19.4	19.4	19.6	0.50	0.96	0.77	0.86
<i>cis</i> -11 18:1	1.19	1.24	1.23	1.22	0.667	0.31	0.77	0.39
<i>cis</i> -12 18:1	0.47	0.45	0.46	0.44	0.012	0.25	0.16	0.85
<i>cis</i> -9, <i>cis</i> -12 18:2	2.88	2.87	2.80	2.93	0.063	0.83	0.61	0.26
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12 18:3	0.031	0.025	0.030	0.025	0.0018	0.05	0.09	0.96
20:0	0.13	0.13	0.13	0.13	0.006	0.83	0.72	0.90
<i>cis</i> -9, <i>trans</i> -11 CLA	0.57	0.52	0.55	0.53	0.028	0.05	0.24	0.39
<i>cis</i> -12, <i>trans</i> -10 CLA	0.001	0.004	0.001	0.002	0.002	0.51	0.97	0.91
20:2n6	0.034	0.022	0.023	0.026	0.003	0.002	0.15	0.009
20:3n6	0.021	0.015	0.012	0.010	0.005	0.10	0.11	0.45
Total <i>trans</i> -fatty acids	3.82	3.62	3.65	3.74	0.231	0.19	0.78	0.17
Σ SFA	67.8	68.4	68.2	68.0	0.59	0.34	0.85	0.36
Σ MUFA	28.0	25.8	26.0	26.2	0.58	0.93	0.60	0.68
Σ PUFA	3.75	3.62	3.72	3.73	0.072	0.43	0.81	0.44
Σ De novo ⁴	28.9	28.6	29.0	28.5	0.55	0.71	0.70	0.86
Σ Mixed ⁴	28.1	28.5	28.5	28.6	0.64	0.27	0.32	0.67
Σ Preformed ⁴	41.7	41.8	41.7	41.9	0.81	0.87	0.78	0.90
Σ OBCFA ⁵	3.28	3.30	3.36	3.29	0.071	0.36	0.76	0.16

¹Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

²Largest SEM published in table; n = 77 (n represents the number of observations used in the statistical analysis).

³Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity × treatment interaction for any variable ($P \geq 0.10$).

⁴De novo fatty acids (FA; <C16) are synthesized by the mammary gland; preformed FA (>C16) originate primarily from extraction from plasma; and mixed fatty acids (C16) originate from both sources.

⁵Odd- and branched-chain FA; sum of C11:0, *iso* C13:0, ante-*iso* C13:0, C13:0, *iso* C14:0, *iso* C15:0, ante-*iso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, ante-*iso* C17:0, C17:0, and C17:1 *cis*-9.

indicate excessive mobilization of adipose tissue and greater negative energy balance (Dórea et al., 2017). None of these conditions, however, were observed in the current study. Few studies have evaluated the effects of CAP and eugenol on milk FA profile of dairy cows. A study by Oh et al. (2015) only reported minor changes in milk FA profile (e.g., decreased concentration of *cis*-9,*trans*-11 CLA) and increased milk fat yield in cows fed with CAP, which is in line with the tendency for increased milk fat content described in the current study. Similarly, Benchaar et al. (2015) also described minor changes in milk FA profile of

cows fed increasing doses of eugenol (0, 25, 50, and 75 mg/kg of feed DM). It is interesting to note that, from all FA herein described, decreased concentration of *cis*-9,*trans*-11 CLA in milk fat has been also reported by others (Oh et al., 2015). The enzyme Δ^9 -desaturase plays an important role in the endogenous synthesis of *cis*-9,*trans*-11 CLA, which is the major CLA in dairy products. The substantial differences among individual cows in Δ^9 -desaturase activity and its contribution to milk fat CLA reported by Peterson et al. (2002) may help explain differences between treatments in the current experiment. Additionally,

Table 6. Enteric gas emissions¹ in lactating dairy cows fed botanicals combination (BTC)

Item	Treatment ²				SEM ³	P-value ⁴		
	CON	150	300	600		C vs. T	L	Q
CH ₄ , g/d	389	365	367	360	11.8	0.01	0.04	0.22
CH ₄ per DMI, g/kg	14.0	13.1	13.4	13.3	0.53	0.05	0.27	0.24
CH ₄ per dOM, ⁵ g/kg	21.5	20.8	20.7	20.8	1.05	0.39	0.48	0.66
CH ₄ per milk yield, g/kg	10.1	9.54	9.60	9.67	0.494	0.08	0.35	0.18
CH ₄ per ECM yield, g/kg	10.5	9.94	9.93	10.0	0.57	0.08	0.29	0.19
CO ₂ , g/d	12,166	11,996	12,264	12,046	241	0.71	0.78	0.71
CO ₂ per DMI, g/kg	442	432	450	449	19.8	0.87	0.21	0.91
H ₂ , g/d	1.06	1.12	0.98	0.99	0.069	0.69	0.23	0.96

¹Enteric gas emissions were measured using the GreenFeed system (C-Lock Inc.).

²Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

³Largest SEM published in table; n = 76 (n represents the number of observations used in the statistical analysis).

⁴Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity × treatment interaction for any variable ($P \geq 0.57$).

⁵dOM = intake of digestible OM, calculated as dOM = OM intake, kg/d × OM digestibility.

vaccenic acid (*trans*-11 C18:1) is described as the main ruminal precursor for *cis*-9,*trans*-11 CLA synthesis in the mammary gland (Kay et al., 2004) and it was numerically decreased ($P = 0.17$) by BTC in the current study. Da Silva et al. (2020) also reported a numerical decrease in milk concentration of vaccenic acid in dairy cows fed a blend of botanicals including CAP and eugenol, which is in line with our data. The mechanisms by which BTC may reduce Δ^9 -desaturase activity was not investigated in the current study and should be addressed in future research. Although we speculate that the tendency for increased milk fat content in BTC cows would be associated with an increased uptake of BHB by the mammary gland, no differences for de novo-synthesized FA were observed between BTC and CON.

Enteric Gas Emissions

Daily CH₄ emission and CH₄ yield were decreased ($P \leq 0.05$) by BTC, compared with CON (Table 6). As BTC supplementation level increased, daily CH₄ production decreased linearly ($P = 0.04$) by up to 7.5%. The CH₄ emission per kilogram of digestible OM intake was not affected by treatments, although a numerical decrease of 4% was observed. Methane emission intensity (per MY or ECM) tended to be decreased ($P = 0.08$) by BTC, compared with CON. Supplementation with BTC had no effect on CO₂ emission and yield and daily H₂ emission.

To the best of our knowledge, the current experiment is the first study to report enteric CH₄ emissions of lactating dairy cows fed a blend of CAP and clove EO. The effects of BTC on CH₄ yield and intensities in the

current study were a result of decreased daily CH₄ production (g/d) and similar DMI, MY, and ECM among treatments. It is important to note that the decrease in DM digestibility in the current study (less than 2%) was smaller than the decrease in CH₄ yield (5 to 6%), which indicates that CH₄ inhibition was not only a result of decreased digestibility. The lack of statistical significance for CH₄ emission per kilogram of digestible OM intake was likely a result of intrinsic variability associated with the marker methodology used for estimation of digestibility of nutrients.

The effects of individual or combinations of EO on enteric CH₄ emissions in ruminants have been studied (Hristov et al., 2013; Benchaar et al., 2015; Beauchemin et al., 2020), and several in vitro studies have investigated the CH₄ mitigation potential of EO and their bioactive compounds (Calsamiglia et al., 2007; Benchaar and Greathead 2011; Tekippe et al., 2012). For instance, Chaves et al. (2008) reported that cinnamon leaf oil (containing 760 g/kg eugenol) decreased CH₄ production by 72% while increasing the molar proportion of butyrate (but decreasing propionate) in vitro. These authors suggested that cinnamon oil could be used as an alternative to ionophores due to its inhibition of ruminal methanogens. It must be pointed out, however, that in vitro data are rarely representative of in vivo responses (Hristov et al., 2012). For instance, in an experiment with mid-lactation dairy cows, Benchaar et al. (2015) did not find any effects of eugenol (inclusion up to 75 mg/kg of feed DMI) on daily CH₄ emission or emission yield. Although the effect of BTC on CH₄ in the current experiment is encouraging, it needs to be confirmed in following-up in vivo studies across a range of diets and inclusion rates.

Table 7. Blood chemistry of lactating dairy cows fed botanicals combination (BTC)

Item	Treatment ¹				SEM ²	P-value ³		
	CON	150	300	600		C vs. T	L	Q
BHB, μM	995	884	858	928	51.5	0.06	0.48	0.05
Total FA, μM	130	120	130	160	0.017	0.74	0.16	0.38
Insulin (overall), pmol/L	178	195	186	170	19.7	0.77	0.57	0.42
Primiparous	232	219	182	142	26.7	0.09	0.01	0.99
Multiparous	141	179	193	184	26.3	0.06	0.16	0.17
Glucose, mg/dL	59.2	60.2	58.9	60.6	2.17	0.74	0.67	0.82
BUN, mg/dL	11.2	11.4	11.5	11.2	0.47	0.62	0.94	0.39
Creatinine, mg/dL	0.62	0.63	0.63	0.65	0.030	0.65	0.49	0.91
Total protein, g/dL	7.77	7.69	7.60	7.75	0.240	0.64	0.94	0.48
Albumin, g/dL	2.72	2.73	2.71	2.76	0.046	0.74	0.53	0.72
Globulin, g/dL	5.04	4.94	4.87	4.98	0.219	0.52	0.79	0.45
Alanine transferase, U/L	63.3	65.7	64.3	63.9	2.35	0.48	0.98	0.49
Alkaline phosphatase, U/L	46.6	46.3	45.1	45.3	3.60	0.64	0.58	0.78

¹Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

²Largest SEM published in table; n = 74 for insulin, n = 76 for alkaline phosphatase, n = 77 for all other variables (n represents the number of observations used in the statistical analysis).

³Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity \times treatment interaction for any variable ($P \geq 0.17$), except for insulin ($P = 0.02$).

Blood Chemistry

In the current study, serum BHB concentration tended to be decreased ($P = 0.06$) by BTC compared with CON, and it was decreased by the low and medium BTC doses but not affected at the high BTC dose ($P = 0.05$, a quadratic effect). There was no effect of treatment on blood, total FA and glucose concentrations (Table 7). A treatment \times parity interaction ($P = 0.02$) was detected for serum insulin concentration. Insulin was linearly decreased ($P = 0.01$) in BTC primiparous cows, but not in multiparous cows. Decreased insulin concentration in primiparous cows receiving BTC corroborates with Oh et al. (2017c) and may be explained by lower glucose uptake by insulin-dependent tissues such as skeletal muscle and adipose tissue, as a result of insulin resistance (De Koster and Opsomer, 2013). Contrary, another explanation for decreased insulin levels in the current experiment is that insulin sensitivity of insulin-dependent tissues might be increased by BTC; however, insulin sensitivity was not determined in the current study. If the later hypothesis is true, it could also explain the lower concentration of BHB in BTC cows (i.e., reduced lipolysis and increased lipogenesis). Circulating concentrations of total FA and BHB can be used as energy balance markers, with total FA being related to fat mobilization and BHB related to liver oxidation and ketone bodies formation (Duffield et al., 2009; Ospina et al., 2010). These data are in line with a previous study conducted in our laboratory (Oh et al., 2021), which reported improved energetic status of transition cows supplemented with CAP during the

prepartum period (i.e., reduced BHB concentration). Despite the physiological difference in lactation stage, mid-lactation versus transition period in the study by Oh et al. (2021), decreased BHB concentration in both studies indicates that BTC supplementation (specifically CAP) has a potential to enhance the metabolic status of dairy cows mediated by changes in insulin and, perhaps, the somatotrophic axis. The mechanisms behind the changes in metabolic markers remain unclear; however, they may be linked to the capsaicin intestinal receptor TRPV1 stimulating the release of neuropeptides such as calcitonin gene-related peptide, which is known to directly influence the insulin axis in other species (Oh et al., 2017b).

It is interesting to note that, in the current study, responses to BTC supplementation appeared to be both gut-mediated responses (changes in blood metabolites) and rumen-mediated responses (changes in enteric CH₄ emissions). This might be possible due to the relatively high level of ruminal escape of the active compounds in BTC allowing the supplement to induce both ruminal and postruminal effects. Future research in our laboratory will further explore these responses and evaluate their consistency.

Blood Cell Count

Blood cell count, hemoglobin concentration, and hematocrit percentage were not affected by BTC supplementation (Table 8), and the count of total white and red blood cells were within normal levels for cattle (Roland et al., 2014). Although some studies

Table 8. Blood cell counts in lactating dairy cows fed botanicals combination (BTC)

Item	Treatment ¹				SEM ²	P-value ³		
	CON	150	300	600		C vs. T	L	Q
White blood cells, 10 ³ /μL	7.87	8.04	7.81	7.87	0.439	0.91	0.86	0.96
Neutrophils	3.20	3.21	3.48	3.17	0.300	0.74	0.99	0.42
Lymphocytes	3.76	4.11	3.78	3.86	0.485	0.33	0.94	0.54
Monocytes	1.11	1.30	1.12	1.10	0.127	0.49	0.56	0.40
Eosinophils	0.33	0.32	0.28	0.30	0.039	0.27	0.30	0.31
As % of total								
Neutrophils	39.0	36.3	39.4	39.4	2.31	0.77	0.61	0.69
Lymphocytes	43.7	45.5	43.7	43.8	2.12	0.74	0.84	0.75
Monocytes	13.2	14.2	13.5	13.1	0.85	0.67	0.62	0.47
Eosinophils	4.02	4.00	3.48	3.69	0.458	0.32	0.26	0.40
Neutrophil:lymphocyte	0.99	0.86	0.99	0.97	0.098	0.59	0.81	0.69
Red blood cells, 10 ⁶ /μL	5.83	5.76	5.94	5.76	0.170	0.91	0.68	0.20
Hemoglobin, g/dL	9.40	9.34	9.57	9.31	0.163	0.95	0.61	0.11
Hematocrit, %	27.4	27.2	27.9	27.1	0.483	0.96	0.57	0.13

¹Treatments were as follows: CON = control, 0 mg/cow per day of BTC; 150 = 150 mg/cow per day of BTC; 300 = 300 mg/cow per day of BTC; 600 = 600 mg/cow per day of BTC.

²Largest SEM published in table; n = 72 for white blood cells, n = 73 for lymphocytes counts, n = 76 for all other variables (n represents the number of observations used in the statistical analysis).

³Contrasts: C vs. T = control vs. BTC treatments; L = linear effect of BTC; Q = quadratic effect of BTC. There was no effect of parity or a parity × treatment interaction for any variable ($P \geq 0.12$).

reported decreased lymphocytes percentage (Oh et al., 2015) and decreased blood monocytes count (Oh et al., 2018a) by EO supplementation, the lack of effect of BTC on blood cells count is in line with a previous study that supplemented the diet of lactating dairy cows with a rumen-protected CAP (Oh et al., 2017a). Similarly, rumen-protected CAP supplementation did not affect blood cell count in beef cattle (Westphalen et al., 2021). *Capsicum* and bioactive compounds extracted from clove EO have been described as potential molecules to modulate the immune system by leading to anti-inflammatory, antioxidant, or immune enhancement properties mediated by the connection with ion channels (i.e., TRPV1, TRPV3, and TRPA1; Vennekens et al., 2008; Oh et al., 2017a); however, effects of BTC on immune cells were not observed in the current study.

CONCLUSIONS

Contrary to our hypothesis, a combination of *Capsicum* oleoresin and clove EO did not affect lactational performance of mid-lactation dairy cows, despite some indications of improved metabolic status illustrated by tendencies for decreased serum BHB and insulin (primiparous cows only). Although nutrient utilization was not affected by BTC supplementation, enteric CH₄ production was linearly decreased by up to 7.5% with increasing doses of BTC. The moderate decrease in enteric CH₄ yield suggests a potential of BTC to reduce the carbon footprint of milk, but this effect needs to be confirmed.

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