Lactational performance, rumen fermentation, nutrient use efficiency, enteric methane emissions, and manure greenhouse gas-emitting potential in dairy cows fed a blend of essential oils

Author(s):
Silvestre, Tatiana; Martins, Leoni F.; Cueva, Sergio F.; Wasson, Derek E.; Stepanchenko, N.; Räisänen, Susanna; Sommai, Sukruthai; Hile, Michael L.; Hristov, Alexander Nikolov

Publication date:
2023-11

Permanent link:
https://doi.org/10.3929/ethz-b-000645829

Originally published in:
Journal of Dairy Science 106(11), https://doi.org/10.3168/jds.2022-23181

J. Dairy Sci. 106:7661–7674
https://doi.org/10.3168/jds.2022-23181

Lactational performance, rumen fermentation, nutrient use efficiency, enteric methane emissions, and manure greenhouse gas-emitting potential in dairy cows fed a blend of essential oils

T. Silvestre,[1] L. F. Martins,[1] S. F. Cueva,[1] D. E. Wasson,[1] N. Stepanchenko,[1] S. E. Räisänen,[1]* S. Sommai,[2] M. L. Hile,[3] and A. N. Hristov[1]†
[1]Department of Animal Science, The Pennsylvania State University, University Park, PA 16802
[2]Department of Animal Science, Khon Kaen University, Thailand 40002
[3]Department of Agricultural and Biological Engineering, The Pennsylvania State University, University Park, PA 16802

ABSTRACT

The objective of this experiment was to investigate the effects of an essential oil (EO) blend on lactational performance, rumen fermentation, nutrient utilization, blood variables, enteric methane emissions and manure greenhouse gas-emitting potential in dairy cows. A randomized complete block design experiment was conducted with 26 primiparous and 22 multiparous Holstein cows. A 2-wk covariate and a 2-wk adaptation periods preceded a 10-wk experimental period used for data and sample collection. Treatments were: (1) basal diet supplemented with placebo (CON); and (2) basal diet supplemented with a blend of EO containing eugenol and geranyl acetate as main compounds. Supplementation with EO did not affect dry matter intake, milk and energy-corrected milk yields, and feed efficiency of cows, compared with CON. Milk fat and lactose concentrations were increased, and milk total solids (TS) concentration and milk fat yield tended to be increased by EO. Multiparous cows supplemented with EO tended to have slightly decreased dry matter and crude protein digestibility compared with CON multiparous cows. There was a tendency for increased ruminal pH by EO, whereas other rumen fermentation variables did not differ between treatments. Daily methane emission was not affected by EO supplementation, but methane emission intensity per kg of milk fat was decreased by 8.5% by EO. Methane emission intensity per kg of milk lactose and milk TS were decreased and methane emission intensity per kg of milk yield tended to be decreased by up to 10% in EO multiparous cows, but not in primiparous cows. The greenhouse gas-emitting potential of manure was not affected by EO supplementation. Compared with CON, fecal nitrogen excretion was increased by EO supplementation in multiparous, but not in primiparous cows, and milk nitrogen secretion (as a % of nitrogen intake) tended to be increased in EO supplemented cows. Blood variables were not affected by EO supplementation in the current study. Overall, dietary supplementation of EO did not affect lactational performance of the cows, although milk fat and lactose concentrations were increased. Most enteric methane emission metrics were not affected, but EO decreased methane intensity per kg of milk fat by 8.5%, compared with the control.

Key words: dairy cow, enteric methane, essential oil, milk production

INTRODUCTION

Intensive research in the past decade resulted in a better understanding of factors driving enteric CH_4 emissions in ruminants (Hristov et al., 2022); however, most of the CH_4 mitigation strategies studied were not effective in vivo and could be limited to short-term effects only (Hristov et al., 2013; Arndt et al., 2022). For instance, essential oils (**EO**) were classified as having an uncertain CH_4 mitigation potential with no long-term effects established (Hristov et al., 2013), and Hegarty et al. (2021) concluded in their review that there is low agreement and medium evidence of the potential of EO to mitigate CH_4 in vivo. The mechanisms by which EO would contribute to CH_4 mitigation are not clear, but some studies suggest that EO could decrease protozoal and methanogen populations in the rumen and shift ruminal fermentation pattern toward increased propionate production (Hegarty et al., 2021). Some reasons for the inconsistency of results are instability and volatility of the active compounds in EO during feed processing and storage, large variability in EO and their active compound types and concentrations, different growth

Received December 21, 2022.
Accepted May 9, 2023.
*Current address: Institute of Agricultural Sciences, ETH Zurich, Switzerland 8092.
†Corresponding author: anh13@psu.edu

and harvesting conditions of the plants used to produce EO, different extraction methods, and differences in doses fed to the animals (Hegarty et al., 2021).

Despite the limitations, a positive and strong argument for continuing the research with EO to mitigate CH_4 emission in livestock is supported by the fact that these compounds may have a higher acceptance by consumers compared with, for example, synthetic CH_4 inhibitors. Botanical preparations and EO are generally recognized as safe (**GRAS**) compounds by the Food and Drug Administration (US Government, 2021) and could have an appeal to the consumer (Wemette et al., 2021). From the producer standpoint, the use of EO could be justified if accompanied by co-benefits, such as enhanced lactational performance, yield of milk components, animal health, or improved efficiency of nutrient utilization. However, more research is needed to determine the long-term effects of EO supplementation, and to better elucidate its interaction with cows' physiological state (e.g., parity and metabolic status) and dietary factors.

In this sense, an EO blend containing a combination of eugenol and geranyl acetate has been reported to successfully decrease enteric CH_4 emissions (Hart et al., 2019) and to increase the performance of dairy cows (Williams et al., 2021; Brambila and Noricumbo-Saenz, 2022). These results were supported by a meta-analysis demonstrating decreased daily CH_4 production (absolute production; −8.8%), CH_4 yield (per kg of DMI; −12.9%), and CH_4 intensity (per kg of fat and protein corrected milk yield; −9.9%), and increased milk yield (+3.6%) and feed efficiency (+4.4%) of dairy cows supplemented with a specific EO blend (Belanche et al., 2020). Some of the data in the Belanche et al. (2020) meta-analysis, however, were generated in pen-based studies with limited number of experimental units and with no individual measurements of DMI (Santos et al., 2010; Hart et al., 2019; Williams et al., 2021; Brambila and Noricumbo-Saenz, 2022). Additionally, out of the 23 studies compiled in Belanche et al. (2020), 13 were unpublished and only 8 studies reported enteric CH_4 emissions data, which could compromise the validity of the meta-analysis conclusions.

Therefore, the objective of this study was to investigate the effects of a EO blend containing geranyl acetate and eugenol on the lactational performance, nutrient utilization, rumen fermentation, blood variables, enteric gas emissions, and manure greenhouse gas (**GHG**) emitting potential (**EP**) in dairy cows. Based on studies published in the literature, we hypothesized that the EO blend would decrease CH_4 emissions and improve lactational performance of dairy cows by modifying ruminal fermentation and enhancing its efficiency. We also hypothesized that enhanced energy utilization in the rumen would contribute to an overall increase in total VFA concentration, blood glucose concentration, and energy metabolism status of the cows.

MATERIALS AND METHODS

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

Animals, Housing, and Experimental Design

This study was conducted with 48 lactating Holstein cows (26 primiparous and 22 multiparous cows) averaging (±SD) 88 ± 27 DIM, 43 ± 10 kg/d milk yield (**MY**), 611 ± 65 kg BW, and 1.8 ± 1 lactations at the beginning of the experiment. Cows were housed at The Pennsylvania State University's Dairy Teaching and Research Center free-stall barn, equipped with a Calan Broadbent Feeding System (American Calan Inc.) for monitoring individual cow DMI.

Cows were enrolled in a randomized complete block design experiment with a 2-wk covariate period followed by a 2-wk adaptation to treatments, and a 10-wk experimental period for data and samples collection (i.e., a total of 14-wk experiment). Parity and DIM data at the beginning of the study were used to block the cows into 24 blocks with 2 cows each. Blocks were adjusted based on MY and CH_4 emission data collected during the covariate period. Cows were then randomly assigned to 1 of 2 dietary treatments as follows: (1) basal diet (**CON**); and (2) basal diet supplemented with 1 g/cow per day AGOLIN 100 (AGOLIN S.A., Bière, Switzerland; **EO**) containing 20% EO, with geranyl acetate and eugenol as main ingredients and the remaining 80% being a carrier (SiO_2 and $CaCO_3$). The basal diet was formulated to meet or exceed NE_L and MP requirements (NRC, 2001) of a lactating Holstein cow with 640 kg of BW, 43 kg/d MY, 3.80% milk fat, and 3.10% milk true protein at 26 kg/d of DMI. The diet was mixed and fed as a TMR once daily at approximately 0800 h and delivered using a Rissler model 1050 TMR mixer (I. H. Rissler Mfg. LLC). The diet was fed ad libitum, targeting 10% refusals, and cows had free access to drinking water. The EO blend was mixed once every 2-wk into a premix containing 75% ground corn, 18% dry molasses, 6.5% soybean meal, and 0.5% EO. Similarly, a CON premix was mixed with the same feed ingredients and composition as for the EO premix, except for the inclusion of 0.5% ground corn replacing EO (i.e., placebo premix). The premixes were fed at 200 g/cow per day and top-dressed twice daily (100 g at each event) at 0800 and 1700 h by mix-

ing with approximately 500 g of TMR. Based on visual observations, cows consumed the mixture of the top-dressed treatments immediately after feeding.

Samples Collection

Diet and Feed Ingredients. The amount of feed offered and refused was weighed daily for each cow, and daily as-fed intake was measured during the entire experiment. The DM content of the feed offered and refused were measured weekly and used to calculate daily DMI. Samples of concentrate feeds were collected once weekly, and samples of the forages, TMR, and refusals were collected twice weekly and stored at −20°C. Feed samples were later thawed overnight, dried for 72 h at 55°C in a forced-air oven, and ground in a Wiley mill (Thomas Scientific) through a 1-mm screen for further analyses. Feed samples were composited (on an equal DM basis) and the composite samples were submitted to Cumberland Valley Analytical Services (CVAS, Waynesboro, PA) for wet chemistry analyses of CP (method 990.03; AOAC International, 2000), amylase-treated NDF (**aNDF**; Van Soest et al., 1991), ether extract (method 2003.05; AOAC International, 2006), ADF (method 973.18; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), minerals (Ca and P; method 985.01; AOAC International, 2000), and estimated NFC. Composite TMR and individual feed ingredients samples were analyzed for starch according to Hall (2009), and composite TMR samples were analyzed for indigestible NDF (**iNDF**) as described in Huhtanen et al. (1994) and modified by Lee et al. (2012a). Nutrient composition of the basal diet was reconstituted from analyzed nutrient composition of the individual feed ingredients and their inclusion rate in the TMR (Table 1). Balances of NE_L and MP were estimated based on NRC (2001) using the average DMI, MY, milk composition, and BW of the cows during the experiment.

Milk Production and Composition. Cows were milked twice daily at approximately 0630 and 1800 h, and milk production was recorded daily at each milking (DeLaval milk meter, MM27BC). Milk samples were collected weekly from 4 consecutive milkings (p.m. and a.m.) in wk 2 of the covariate period and during the entire experimental period (experimental wk 5 through 14). Aliquots of the milk samples were placed into 50-mL tubes containing a preservative (2-bromo-2-nitropropane-1,3-diol) and submitted to Dairy One Cooperative Inc. for analysis of milk fat, true protein, lactose, TS, and MUN by infrared spectroscopy (MilkoScan 4000, Foss), and SCC by flow cytometry (Fossomatic models 5000 or FC; Foss Electric A/S). Milk composition data were weighed for the corresponding MY recorded during p.m. and a.m. milkings. The weekly averaged MY data were used to calculate individual milk fat, true protein, lactose, TS, and ECM yields; ECM was calculated according to Sjaunja et al. (1990). Separate unpreserved milk samples were also collected as described above and stored frozen at −20°C

Table 1. Ingredient and chemical composition of the basal diet fed to dairy cows during the experiment

Item	Diet
Ingredient, % of DM or as indicated	
Corn silage[1]	45.7
Alfalfa haylage[2]	9.7
Grass hay[3]	2.8
Corn grain, ground	10.4
Canola meal	11.7
Whole cottonseed	3.3
Roasted soybeans	4.0
SoyPLUS[4]	3.4
Molasses[5]	2.4
Pellets[6]	5.2
Urea	0.2
Mineral and vitamin premix[7]	1.1
Treatment premix with EO[8] or placebo[9]	0.1
Composition, % of DM	
CP[10]	17.3
RDP[11]	10.5
RUP[11]	6.6
NDF[10]	31.5
ADF[10]	18.8
Starch[10]	23.1
Ether extract[10]	4.6
NFC[11]	43.4
NE_L,[11] Mcal/kg DM	1.57
NE_L balance,[11] Mcal/d	−0.1
MP balance,[11] g/d	1
Ca[10]	0.60
P[10]	0.45

[1]Corn silage was 42.0% DM and contained (% of DM): 7.3 CP and 37.5 NDF.

[2]Alfalfa haylage contained (% of DM): 19.2 CP and 36.9 NDF.

[3]Grass hay contained (% of DM): 7.5 CP and 69.3 NDF.

[4]Heat-treated soybean meal (SoyPLUS, Landus Cooperative).

[5]Liquid molasses (Westway Feed Products).

[6]Pellets (Purina Stocker Grower) used as an attractive to cows in GreenFeed units.

[7]Premix (Renaissance Nutrition, Inc.) contained (% of DM or as indicated): 11.6 CP, 4.6 ADF, 17.8 NDF, 16.3 calcium, 0.92 phosphorus, 2.63 magnesium, 1.48 potassium, 15.1 chlorine, 0.42 sulfur, 9.81 sodium, 23 mg/kg cobalt, 651 mg/kg copper, 796 mg/kg iron, 54 mg/kg iodine, 1,190 mg/kg manganese, 13 mg/kg selenium, 1721 mg/kg zinc, 195,000 IU/kg vitamin A, 62,500 IU/kg vitamin D, and 1,864 IU/kg vitamin E.

[8]Premix contained: 75.0% ground corn, 18% dry molasses, 6.5% vegetable oil, and 0.5% EO.

[9]Premix contained: 75.5% ground corn, 18% dry molasses, and 6.5% vegetable oil.

[10]Values calculated using the nutrient analysis of the feed ingredients (Cumberland Valley Analytical Services Inc., Waynesboro, PA) and their inclusion in the diets.

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

until further analysis. These samples were composited on an equal volume basis per cow for the entire experimental period and analyzed for fatty acid (**FA**) profile as described in Rico and Harvatine (2009).

BW and BW Change. Body weights were recorded twice daily when cows exited the milking parlor and walked through a DeLaval automatic weigh system (DeLaval, AWS100). Body weight change was calculated as the difference between the average BW collected during experimental wk 14 and the BW recorded during wk 2 of the covariate period, divided by days in the study.

Apparent Total-Tract Digestibility and Nitrogen Utilization. Spot urine and fecal samples (approximately 300 mL and 500 g per sample, respectively) were collected at 0, 4, 8 and 12 h relative to feeding during 2 consecutive days (i.e., a total of 4 samples/cow) in experimental wk 14 for estimation of N utilization and apparent total-tract digestibility of dietary nutrients. Urine samples were collected by perineal stimulation and added to 2 M H_2SO_4 in a ratio of 60 mL of acid solution per 1,000 mL of urine to reach a pH <3.0. Acidified samples were diluted 1:10 with distilled water, composited on an equal volume basis per cow, and stored at −20°C until further analyses. Composited urine samples were then analyzed for allantoin (Chen et al., 1992), uric acid (Uric Acid Kit 1045; Stanbio Laboratory), creatinine (Creatinine Kit 420; Stanbio Laboratory), and urine urea N (**UUN**; Urea Nitrogen Kit 580; Stanbio Laboratory). Aliquots of the composited urine samples were also freeze-dried (VirTis Ultra 35L; SP Scientific) and analyzed for N using a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.). Daily urinary volume was estimated based on urinary creatinine concentration, assuming a creatinine excretion rate of 29 mg/kg of BW based on unpublished total urine collection data from Hristov et al. (2011). Estimated daily urinary output was used to calculate daily excretions of urine N, UUN, and purine derivatives (allantoin and uric acid). Total excreta N was calculated as the sum of excreted urine and fecal N. Unaccounted N was calculated as the difference between N intake and the sum of total excreta N and milk N. Milk N secretion was estimated as follows: milk N, g/d = [(milk true protein yield, g/d ÷ 6.38) + MUN, g/d].

Fecal samples were collected from the rectum of each cow, oven-dried at 55°C for 72 h, and ground through 1-mm screen in a Wiley mill (Thomas Scientific). Dried and ground fecal samples were composited per cow and analyzed for CP (N × 6.25) as described above, starch according to Hall (2009), and aNDF and ADF using an Ankom 200 fiber analyzer (Ankom Technology Corp.). Ash content was determined by burning the residues of the ADF analysis at 600°C for 6 h, and OM was calculated as the difference between DM and ash contents. Composited fecal samples were analyzed for iNDF concentration after a 12-d in situ ruminal incubation according to Huhtanen et al. (1994), with the exception that 25-μm pore size Ankom filter bags (F57; Ankom Technology Corp.) were used for the rumen incubation (Lee et al., 2012a).

Ruminal Fermentation. Rumen fluid was collected from a subset of 12 cows (6 blocks; i.e., 6 cows per treatment) during experimental wk 13 at approximately 1200 h (i.e., 4 h after feeding), using the stomach tubing technique (de Assis Lage et al., 2020). Approximately 200 mL of initially sampled ruminal fluid was discarded to avoid possible saliva contamination. Whole ruminal contents were filtered through 2 layers of cheesecloth and the filtered ruminal fluid samples were analyzed immediately for pH (59000–60 pH Tester, Cole-Parmer Instrument Company), and aliquots of samples were processed, diluted, acidified, and kept refrigerated until later analyzed for VFA (Yang and Varga, 1989) and NH_3 concentrations (Chaney and Marbach, 1962).

Enteric Gas Emissions. Enteric gas (CH_4, CO_2, and H_2) emissions were measured using the GreenFeed system (C-Lock Inc.). Two GreenFeed units were used and calibrated according to the manufacturer's recommendations (https://globalresearchalliance.org/wp-content/uploads/2018/08/GreenFeeds-SOP-_final.pdf; accessed on Sep. 17, 2022). Cows were properly trained to use the system before the beginning of the experiment and were identified with a unique radio-frequency identification ear tag for recognition in the GreenFeed unit. Cows had free access to both GreenFeed units and a pelletized bait feed (Stocker Grower 14, Purina Animal Nutrition LLC) was used to attract the animals. The weight of the pellets dispensed daily was calculated based on the average weight and number of drops recorded at each individual visit and included in the calculation of individual DMI. Each cow was allowed a maximum of 6 visits in a 24-h period, with a 4-h interval between visits, and no more than 12 feed drops of approximately 32 g of pellets per visit. The average (±SD) number of pellet drops and GreenFeed visits during the experimental period were, respectively, 28 ± 13.9 drops/cow per day and 3 ± 1.6 visits/cow per day. Average calculated daily intake and nutritional composition of pellets were used for the reconstitution of the basal diet (Table 1). Weekly average of emitted CH_4, DMI, MY, ECM, milk fat, milk true protein, lactose, and TS yields were used to calculate weekly averages of CH_4 yield (i.e., g/kg of DMI) and intensity (i.e., g/kg of MY, ECM yield, and milk components yields).

Manure Greenhouse Gas-Emitting Potential. Ammonia, CO_2, CH_4, and nitrous oxide (N_2O) emitting potential of manure from CON and EO cows were

evaluated in vitro. Aliquots of non-acidified urine and fecal samples were collected from a subset of 12 cows (6 blocks; i.e., 6 cows per treatment) during the fecal samples collection. Fecal and urine samples collected from each cow were composited on a wet basis as 1.7:1 feces-to-urine ratio (Lee et al., 2012b) and stored at −20°C for further analysis. Individual composited samples were thawed and re-composited by treatment. The composited treatment samples were split into 2 replicates and the replicated samples were incubated for 14 d following the procedure of Wheeler et al. (2007) to measure the manure GHG EP of treatments.

Blood Variables. Blood samples were collected from coccygeal vein or artery at 0, 4, 8, and 12 h relative to feeding during 1 d on experimental wk 6 and 14. Blood samples (approximately 10 mL) were collected into vacuum tubes containing silica clot-activator (BD Biosciences). Serum was separated by centrifugation at 1,800 × *g* at 20°C for 30 min and stored at −80°C for further analyses. Serum samples were composited per cow and week on an equal-volume basis and samples collected during experimental wk 14 were analyzed for total FA [NEFA-HR(2); Wako Diagnostics], BHB (Autokit 3-HB; Wako Diagnostics), glucose (Stanbio Glucose Liquicolor), and BUN [Stanbio Urea Nitrogen (BUN)].

Statistical Analysis

All data were analyzed using the MIXED procedure of SAS, version 9.4 (SAS Institute Inc.). Data were tested for normality using the UNIVARIATE procedure and outliers were identified by the REG procedure of SAS based on an absolute studentized residual value >3. Log-transformed data were analyzed when the W statistic of Shapiro-Wilk test was less than 0.05 (i.e., SCC). Production and enteric gas emissions data were averaged by week and the average values were used in the statistical analysis. The statistical models for production and enteric gas emissions data (DMI, MY, ECM yield, milk composition and milk components yield, BW, feed efficiency and ECM feed efficiency, CH_4, CO_2, and H_2 production, and CH_4 yield and intensities) included the fixed effects of treatment, week, treatment × week interaction, and the covariate measurement. The effects of parity and treatment × parity interaction were tested and removed from the final models if non-significant ($P > 0.05$). Block and block × treatment were random effects. Week was the repeated term, AR(1) was the covariance structure, and the nested effect of cow (block × treatment) was the subject for all repeated measures models. Blood variables, BW change, rumen fermentation, nutrient digestibility, and N utilization were analyzed with the fixed effect of treatment in the statistical models. Block and block × treatment were random effects. The fixed effects of parity and treatment × parity interaction were also tested and removed from the models if non-significant ($P > 0.05$). Manure GHG EP data were analyzed as repeated measures with the fixed effects of treatment, day, and the treatment × day interaction. Day was the repeated term, AR(1) was the covariance structure, and replicate was the subject for all statistical models. Statistical differences were considered significant at $P \leq 0.05$ and a tendency was declared at $0.05 < P \leq 0.10$. Unless indicated otherwise, data are presented as LSM.

RESULTS AND DISCUSSION

Lactational Performance

Dry matter intake, MY, and feed efficiency (**FE**) were not affected by EO supplementation (Table 2). Milk fat concentration was increased ($P = 0.03$) and milk fat yield tended to be increased ($P = 0.06$) by EO, compared with CON. Milk lactose was higher ($P = 0.04$) and TS concentration tended ($P = 0.07$) to be higher for EO, compared with CON. There were no effects of EO on the other production variables evaluated in the current study, and no treatment × week or treatment × parity interactions were identified for lactational performance variables.

Data from the literature herein discussed correspond to the same EO preparation as the one evaluated in the current study (AGOLIN 100). Effects of supplementation of dairy diets with a blend of geranyl acetate and eugenol have been reported in the literature with varying results. A meta-analysis containing data from 23 published and unpublished studies reported that the EO blend did not affect DMI and milk composition, but increased MY, fat- and protein-corrected milk yield (**FPCM**), and FE of dairy cows (Belanche et al., 2020). That meta-analysis also demonstrated that the EO blend was more effective in longer-term experiments, with differences in MY, FPCM, and FE being more evident after 4 wk of supplementation (Belanche et al., 2020). In agreement with the present study, Elcoso et al. (2019) did not report differences in overall DMI and MY of cows fed EO supplemented diets, compared with CON. However, a treatment × time × parity interaction was reported (Elcoso et al., 2019), whereas multiparous cows fed EO had a higher MY than CON multiparous cows during wk 5 of the study, and primiparous cows fed EO had a higher MY than CON primiparous cows on wk 5, 6, and 8. In the current 10-wk comparison experiment, there was no treatment × week interaction for any production variable (see MY, ECM yield, and FE data in Supplemental Figure S1,

Table 2. Dry matter intake, lactation performance, and BW of dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
DMI, kg/d	27.0	26.8	0.50	0.87
Milk yield, kg/d	37.4	37.4	0.79	0.99
Feed efficiency,[4] kg/kg	1.39	1.39	0.029	0.93
Milk fat, %	3.76	4.07	0.097	0.03
Milk fat, kg/d	1.39	1.50	0.042	0.06
Milk true protein, %	3.11	3.08	0.027	0.50
Milk true protein, kg/d	1.14	1.15	0.023	0.70
Milk lactose, %	4.83	4.86	0.013	0.04
Milk lactose, kg/d	1.80	1.81	0.042	0.87
Total solids, %	12.7	13.0	0.13	0.07
Total solids, kg/d	4.70	4.80	0.098	0.49
MUN, mg/dL	10.4	10.7	0.20	0.35
ECM,[5] kg/d	35.6	36.7	0.80	0.36
ECM feed efficiency,[6] kg/kg	1.31	1.37	0.026	0.12
SCC,[7] $\times 10^3$ cells/mL	3.77 (86.8)	3.65 (63.5)	0.123	0.54
Milk NE_L,[8] Mcal/d	26.3	27.6	0.62	0.16
BW, kg	612	612	6.73	0.96
BW change,[9] g/d	295	103	157	0.46

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 32 for BW change and n = 423 to 448 for all other variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Week effect: $P \leq 0.001$ for all variables. Week × treatment interaction: $P \geq 0.11$ for all variables. Parity × treatment interaction: $P \geq 0.07$ for all variables.

[4]Milk yield ÷ DMI.

[5]ECM (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).

[6]ECM yield ÷ DMI.

[7]Log-transformed SCC data (untransformed values are shown in parentheses).

[8]Milk NE_L (Mcal/d) = kg of milk × (0.0929 × % fat + 0.0563 × % true protein + 0.0395 × % lactose) (NRC, 2001).

[9]BW change: (average BW during comparison experimental wk 14 – average BW during covariate wk 2) ÷ days in study.

https://scholarsphere.psu.edu/resources/3331091e-2cef-413e-87f9-2e57ec1971ab, Silvestre et al., 2023), demonstrating no short- or long-term effects of EO supplementation on lactational performance of the cows. In fact, there was no treatment effect for any production variable, except for increased (or tendency to increase) milk fat, milk lactose and TS concentrations, and milk fat yield. Dietary supplementation of EO also did not affect DMI and lactational performance parameters of dairy cows supplemented for 22 d in the experiment by Klop et al. (2017a). Nevertheless, long-term lactational performance of cows fed EO was enhanced in a study by Guasch et al. (2016); in that study, milk production was increased and DMI tended to be decreased during the last 31 and 23 d of the experiment, respectively, by EO blend supplementation. Consequently, FE was higher in EO than CON cows for most of the days after 33 d of EO supplementation (Guasch et al., 2016). The modes of action of the EO have not been completely elucidated, and the reasons for differential lactational performance responses remain unclear. It is possible that ruminal effects of EO might interact with diet composition, physiological stage of the cows, and parity. For instance, Elcoso et al. (2019) demonstrated a higher positive effect of EO blend supplementation in primiparous than multiparous cows, which may suggest enhanced nutrient utilization in animals with naturally lower DMI when supplemented with EO. Although there was no treatment × parity interaction for lactational performance variables in the present study, differential responses for DM and CP digestibility, and CH_4 intensities between EO and CON were observed in multiparous but not in primiparous cows, which is in contrast with previously mentioned data (Elcoso et al., 2019). Further discussion regarding variables affected by a treatment × parity interaction can be found later in this manuscript.

Nutrient Intake, Apparent Total-Tract Digestibility, and Nitrogen Utilization

Intake and apparent total-tract digestibility of nutrients were not affected by EO supplementation in the present study (Table 3); however, there was a treatment

Table 3. Intake and apparent total-tract digestibility of nutrients of dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
Intake,[4,5] kg/d				
DM	26.7	26.5	0.99	0.83
OM	25.2	25.0	0.93	
CP	4.6	4.5	0.17	
NDF	10.9	10.8	0.40	
ADF	5.2	5.1	0.19	
Starch	7.0	7.0	0.26	
Digestibility, %				
DM	69.7	69.4	0.33	0.54
Primiparous	68.7	69.5	0.48	0.28
Multiparous	70.7	69.3	0.46	0.06
OM	71.4	71.3	0.37	0.80
CP	75.5	74.7	0.51	0.33
Primiparous	73.9	75.1	0.76	0.28
Multiparous	77.1	74.4	0.69	0.02
NDF	57.6	56.8	0.51	0.20
ADF	43.0	42.4	0.69	0.51
Starch	98.7	98.7	0.10	0.97

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 43 to 44 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity × treatment interaction: $P \leq 0.04$ for DM and CP digestibility; $P > 0.07$ for all other variables.

[4]Intake during digestibility data collection period.

[5]*P*-values for intake of dietary nutrients are the same as for DMI.

× parity interaction for DM ($P = 0.04$) and CP digestibility ($P = 0.02$). Compared with CON, EO tended ($P = 0.06$) to slightly decrease DM and slightly decreased ($P = 0.02$) CP digestibility in multiparous, but not in primiparous cows.

A limited number of studies have evaluated the effects of EO on nutrient digestibility and ruminal fermentation in lactating dairy cows (Santos et al., 2010; Klop et al., 2017b; Elcoso et al., 2019). Digestibility of nutrients in cows supplemented with the same EO blend as the one used in the current experiment did not differ from CON in a pen-based study (Santos et al., 2010). In a study comparing the effects of a continuous supplementation of EO or alternating it with lauric acid supplementation, Klop et al. (2017b) reported no differences in nutrient digestibility in dairy cows, except for enhanced CP digestibility in the last period of the experiment (9 to 10 wk), compared with the pretreatment period. This result aligns with Belanche et al. (2020) hypothesis that the effects of a geranyl acetate and eugenol EO blend supplementation are more pronounced in long-term experiments but does not agree with the data of the current study. Additionally, it should be noted that CP was the only nutrient affected in the Klop et al. (2017b) study. Thus, it is not clear whether EO supplementation could enhance digestibility of nutrients or not, and whether the variable response in DMI reported in the literature could be related to differential responses in digestibility of nutrients by EO supplementation or not. Even though a treatment × parity interaction was detected, and digestibility of DM and CP were decreased in multiparous cows, the difference between CON and EO was small (approximately 2%) and did not appear to be biologically relevant. Nevertheless, decreased DM and CP digestibility could be associated with an inhibition of rumen fermentation, which could be related to the tendency for increased rumen pH as discussed later in this manuscript.

Supplementation with EO did not affect excretions of urine N, UUN, fecal N, and total excreta N (g/d and % of N intake; Table 4); however, there was a treatment × parity interaction, demonstrating a tendency ($P = 0.08$) for increased excretion of fecal N (as g/d and as % of N intake; $P = 0.08$ and $P = 0.02$, respectively) by EO in multiparous, but not in primiparous cows. Additionally, compared with CON, milk N secretion (as % of N intake) tended ($P = 0.07$) to be increased by EO supplementation. Corroborating with apparent total-tract digestibility data discussed above, increased fecal N in multiparous cows is in line with the lower CP digestibility in multiparous cows supplemented with EO relative to CON. The increased milk N secretion (as % of N intake) might be a consequence of numerically increased milk N output and decreased N intake during the urine sampling week (experimental wk 14).

Ruminal Fermentation

Apart from a tendency ($P = 0.10$) for increased ruminal pH in EO cows compared with CON, no other ruminal fermentation parameters were affected by treatment in the current study (Table 5). Considering that some reports, particularly from in vitro studies, have indicated a positive relationship between EO supplementation and improved ruminal fermentation (Hegarty et al., 2021), the increased milk fat and lactose content and tendency for increased milk fat yield observed in the current study could be associated with ruminal effects in EO cows. However, ruminal concentrations of acetate, propionate, and butyrate were not affected by EO supplementation. The rumen sampling technique used in the current experiment could be contributing to the lack of effect of treatment on rumen VFA concentrations, although we have demonstrated that, when properly executed and timed relating to feeding, the technique could produce data comparable to rumen cannula sampling (de Assis Lage et al., 2020). Klop et al. (2017b) did not report any differences in pH and total VFA concentration in dairy cows continu-

Table 4. Nitrogen utilization in lactating dairy cows fed an essential oil blend

Item	Diet[1]		SEM[2]	*P*-value[3]
	CON	EO		
N intake,[4] g/d	736	729	21.1	0.82
Urine output, kg/d	33.0	32.0	1.77	0.72
N excretion or secretion, g/d				
Urine N	229	228	18.2	0.97
UUN[5]	149	125	17.9	0.39
Fecal N	179	181	6.9	0.83
Primiparous	175	154	10.4	0.14
Multiparous	184	209	9.3	0.08
Total excreta N	412	427	21.3	0.62
Milk N	172	189	7.5	0.12
Unaccounted N[6]	134	109	24.2	0.50
As % of N intake				
Urine N	32.4	32.0	2.93	0.93
UUN[5]	21.2	15.7	2.53	0.14
Fecal N	24.5	25.3	0.51	0.33
Primiparous	26.1	24.9	0.76	0.28
Multiparous	22.9	25.6	0.68	0.02
Total excreta N	57.0	59.2	3.06	0.66
Milk N	23.9	25.7	0.71	0.07
Unaccounted N[6]	18.8	15.0	3.44	0.47
Urinary PD[7] excretion, mmol/d				
Allantoin	301	325	42.9	0.75
Uric acid	182	126	24.9	0.16
Total PD	481	451	43.9	0.68

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 43 to 45 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity × treatment interaction: $P \leq 0.05$ for fecal N, g/d and %; $P \geq 0.10$ for all other variables.

[4]Intake during digestibility and urine data collection period.

[5]UUN = urinary urea nitrogen.

[6]Unaccounted N = N intake – (urinary N + fecal N + milk N).

[7]PD = purine derivatives.

ously supplemented with EO, and rumen fermentation parameters did not differ between EO and CON cows in the meta-analysis by Belanche et al. (2020). Conversely, propionate concentration was decreased, and butyrate concentration was increased by EO supplementation in the study by Elcoso et al. (2019). Santos et al. (2010) explained the increased milk fat concentration in cows supplemented with EO blend in their study by the potential of eugenol to suppress propionate production and increase acetate:propionate ratio in vivo (Benchaar et al., 2007). However, Santos et al. (2010) did not measure ruminal fermentation variables and a later study by Benchaar et al. (2015) reported no dose-response effect to eugenol on ruminal fermentation of dairy cows. Conversely, Klop et al. (2017a) described a transient increase of propionate and decrease acetate proportions measured in vitro 8 d after starting the EO supplementation to the donor cows, which could be a possible explanation for the increased milk lactose content observed in the current study. Again, it is important to state that the rumen sampling methodology used in our study has its limitations and this could potentially influence the rumen fermentation data; however, compared with samples collected through the rumen cannula, stomach tubing can be feasible when comparing molar proportions of VFA (de Assis Lage et al., 2020), and it can be used to determine differences between treatments when cows are subjected to the same sampling technique. Additionally, VFA concentration may not be representative of VFA production, which is very rarely evaluated in experiments with dairy cattle. The tendency for increased rumen pH described in our study could be a consequence of decreased overall rumen fermentation by EO. It should be noted, however, that DM and CP digestibility were slightly decreased in multiparous cows only, and this may not be associated with the overall effect of treatment on rumen pH of primiparous and multiparous cows. Nevertheless, increased rumen pH was associated with decreased concentration of biohydrogenation intermediates in milk (Bauman and Griinari, 2001) and could help explaining

Table 5. Rumen fermentation in lactating dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
pH	6.59	7.06	0.178	0.10
Ammonia, m*M*	5.05	5.49	1.162	0.78
Total VFA, m*M*	97.9	87.3	9.08	0.42
VFA mol, %				
Acetate	58.2	59.7	1.69	0.47
Propionate	25.0	23.6	1.58	0.53
Butyrate	13.1	13.1	1.07	0.99
Isobutyrate	0.44	0.62	0.088	0.13
Valerate	2.29	1.91	0.302	0.50
Isovalerate	0.94	1.04	0.131	0.64
Acetate:propionate	2.45	2.56	0.325	0.75

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 12 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment.

Table 6. Fatty acid composition of milk fat (g/100 g total fatty acids) in lactating dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
4:0	3.75	3.70	0.091	0.70
6:0	2.13	2.23	0.069	0.38
8:0	1.21	1.30	0.051	0.23
10:0	2.82	3.04	0.124	0.23
12:0	3.35	3.53	0.123	0.32
14:0	10.7	10.8	0.20	0.68
cis-9 14:1	0.97	0.91	0.060	0.47
15:0	1.11	1.16	0.041	0.32
16:0	26.1	27.3	0.45	0.09
cis-9 16:1	1.18	1.28	0.066	0.27
17:0	0.55	0.55	0.008	0.53
18:0	11.3	11.2	0.37	0.80
trans-4 18:1	0.03	0.03	0.001	0.37
trans-5 18:1	0.017	0.020	0.001	0.07
trans-6,8 18:1	0.45	0.38	0.028	0.06
trans-9 18:1	0.33	0.29	0.016	0.12
trans-10 18:1	1.52	0.95	0.22	0.08
trans-11 18:1	1.18	1.25	0.07	0.51
trans-12 18:1	0.63	0.54	0.026	0.15
cis-9 18:1	20.1	19.6	0.36	0.35
cis-11 18:1	0.99	0.95	0.037	0.39
cis-12 18:1	0.47	0.48	0.018	0.79
cis-9,*cis*-12 18:2	3.00	2.93	0.065	0.07
cis-6,*cis*-9,*cis*-12 18:3	0.140	0.139	0.005	0.76
20:0	0.03	0.03	0.002	0.34
cis-11 20:1	0.06	0.05	0.002	0.02
20:2 n-6	0.02	0.02	0.001	0.76
20:3 n-3	0.01	0.01	0.001	0.38
Total *trans* fatty acids	4.49	3.78	0.296	0.12
Σ De novo[4]	26.1	26.8	0.54	0.32
Σ Mixed[5]	27.5	28.5	0.51	0.17
Σ Preformed[6]	42.7	41.1	0.71	0.10

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 44 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity × treatment interaction: $P \geq 0.08$ for all variables.

[4]Σ De novo = sum of 4:0; 6:0; 8:0; 10:0; 12:0; 13:0; 14:0; *cis*-9, 14:1; and 15:0.

[5]Σ Mixed = sum of 16:0; and *cis*-9, 16:1.

[6]Σ Preformed = sum of 17:0; *cis*-9, 17:1; 18:0; *trans*-4, 18:1; *trans*-6,8 18:1; *trans*-9, 18:1; *trans* 10, 18:1; *trans*-11, 18:1; *trans*-12, 18:1; *cis*-9, 18:1; *trans*-15, 18:1; *cis*-11, 18:1; *cis*-12, 18:1; *cis*-9, *cis*-12, 18:2; *cis*-6,*cis*-9,*cis*-12, 18:3; 20:0; *cis*-9,*cis*-12,*cis*-15, 18:3; *cis*-11, 20:1; 20:2 n-6; 22:0; 20:3 n-6; 20:3 n-3; *cis*-13, 22:1; 20:4 n-6; *cis*-14,*cis*-16, 22:2; 20:5 n-3; 24:0; 24:1 n-9; 22:4 n-3; and 22:5 n-3.

the increased milk fat content observed in the current study, as discussed in the following section.

Fatty Acid Composition of Milk Fat

Only minor changes in milk FA profile (as a % of milk fat) were observed in the current study (Table 6). Concentrations of 16:0 and *trans*-5 18:1 tended to increase ($P = 0.09$ and 0.07, respectively), whereas concentration of *trans*-10 18:1 and the sum of preformed FA tended to decrease ($P = 0.08$ and 0.10, respectively) by EO, compared with CON.

Decreased concentration of *trans*-10 18:1 could indicate a lower risk of milk fat depression (**MFD**), which aligns with the observed higher milk fat concentration in EO cows. Increased concentrations of *trans*-10, 18:1 and other biohydrogenation intermediates (e.g., *trans*-10,*cis*-12 CLA) in duodenal digesta and milk are usually associated with decreased rumen pH and can be used as markers of MFD in dairy cows (Bauman and Griinari, 2001, 2003; Harvatine et al., 2009). Although we cannot conclusively state that CON cows experienced MFD, it is plausible to postulate that the increased milk fat concentration by EO supplementation was a consequence of decreased production of biohydrogenation intermediates in the rumen following a tendency for increased rumen pH observed in this study. Klop et al. (2017b) also reported minor differences in milk FA profile of dairy cows continuously supplemented with EO, compared with the pretreatment period. However, proportions of 15:0 *iso*, 15:0 *anteiso*, and 17:0 *anteiso* in milk fat were reduced in their study after starting EO supplementation. Odd- and branched-chain FA in milk are associated with changes in fibrolytic and amylolytic bacteria populations in the rumen (Vlaeminck et al., 2006), and changes in the concentration of milk *iso* and *anteiso* forms of FA could be associated with changes in CH_4 emissions in dairy cows (van Gastelen and Dijkstra, 2016). *Iso* and *anteiso* forms of FA were not evaluated in the present study, and no significant differences between treatments in odd- and branched-chain FA were observed, which aligns with the lack of effects of EO on CH_4 emission (discussed below).

Table 7. Enteric gas emissions in lactating dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
CH_4 g/d	381	380	9.16	0.93
CH_4 per DMI, g/kg	14.1	14.2	0.31	0.79
CH_4 per dDM,[4] g/kg	21.3	21.9	0.51	0.46
CH_4 per dOM,[5] g/kg	22.0	22.6	0.52	0.47
CH_4 per milk yield, g/kg	10.5	10.4	0.24	0.60
Primiparous	10.4	10.9	0.36	0.30
Multiparous	10.6	9.8	0.36	0.07
CH_4 per ECM[6] yield, g/kg	10.9	10.5	0.25	0.26
CH_4 per fat yield, g/kg	284	259	7.2	0.02
CH_4 per protein yield, g/kg	341	334	8.4	0.56
CH_4 per lactose yield, g/kg	218	214	5.3	0.57
Primiparous	214	228	7.60	0.15
Multiparous	222	201	8.03	0.04
CH_4 per total solids yield, g/kg	82.8	80.0	1.93	0.29
Primiparous	81.5	84.3	2.73	0.43
Multiparous	84.2	75.7	2.95	0.04
CO_2, g/d	13,064	13,040	253	0.93
H_2, g/d	1.56	1.42	0.061	0.12

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 406 to 423 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Week effect: $P \leq 0.001$ for all variables. Week × treatment interaction: $P \geq 0.12$ for all variables. Parity × treatment interaction: $P \leq 0.05$ for CH_4 per milk yield, CH_4 per lactose yield, and CH_4 per solids yield; $P \geq 0.06$ for all other variables.

[4]dDM = intake of digestible DM, calculated as: DM intake, kg/d × DM digestibility.

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

[6]See Table 2 footnote for ECM formula.

Enteric Gas and Manure Greenhouse Gas Emissions

Contrary to our hypothesis, EO supplementation did not decrease enteric gas emissions (Table 7) neither in the short nor in the long term (see treatment × week interactions for CH_4 production, yield, and intensity; Figure 1). When CH_4 emission was expressed on a milk fat yield basis, EO decreased ($P = 0.03$) CH_4 intensity by 8.5%, compared with CON, as a result of the tendency for increased milk fat yield. There was a treatment × parity interaction for CH_4 emissions expressed on a kg of MY ($P = 0.05$), milk lactose yield ($P = 0.01$), and milk TS yield ($P = 0.04$) basis. Compared with CON, EO tended to decrease ($P = 0.08$) and decreased ($P = 0.04$) CH_4 intensity expressed on a kg of MY, and on a kg of milk lactose and TS yield, respectively, by up to 10% in multiparous cows, but not in primiparous cows.

The effects of supplementation of a blend containing geranyl acetate and eugenol on enteric CH_4 emissions are inconsistent in the literature. For instance, a tendency for decreased CH_4 production and yield, but no differences on CH_4 intensity, was reported by Castro-Montoya et al. (2015) when evaluating the supplementation of EO to late-lactation Holstein cows fed at 95% of ad libitum intake during an 8-wk experiment. It is important to note that, in that study, EO were compared with the emissions of the cows during a pretreatment period of 2-wk (i.e., baseline), and all cows were fed the EO during the remaining 6-wk of experiment (i.e., treatment); therefore, decreased CH_4 production and yield could be associated with a natural decline in DMI observed in late-lactation cows. Klop et al. (2017a) investigated the adaptation of dairy cows to feed additives with a potential to reduce CH_4 emissions (i.e., EO and lauric acid) using the in vitro gas production technique. According to the authors, asymptotic gas and CH_4 productions from rumen fluid of cows receiving the EO supplemented diet differed from CON cows on d 8, but not on d 15 and 22, which indicated an adaptation of the rumen to the EO supplementation. In a following up study, Klop et al. (2017b) hypothesized that EO effects on CH_4 production were transient and that continuous feeding of EO with weekly rotation of a different mitigation agent (e.g., lauric acid) could result in a persistent CH_4 decline. These authors reported a 7% decrease in CH_4 production, and 11% and 12% decrease in CH_4 yield and intensity, respectively, in cows during the first period of EO supplementation, compared with the pretreatment period (i.e., no supplementation of EO); however, differences in CH_4 emissions were not sustained through the rest of the experiment (Klop et al., 2017b). Decreased CH_4 production and intensity

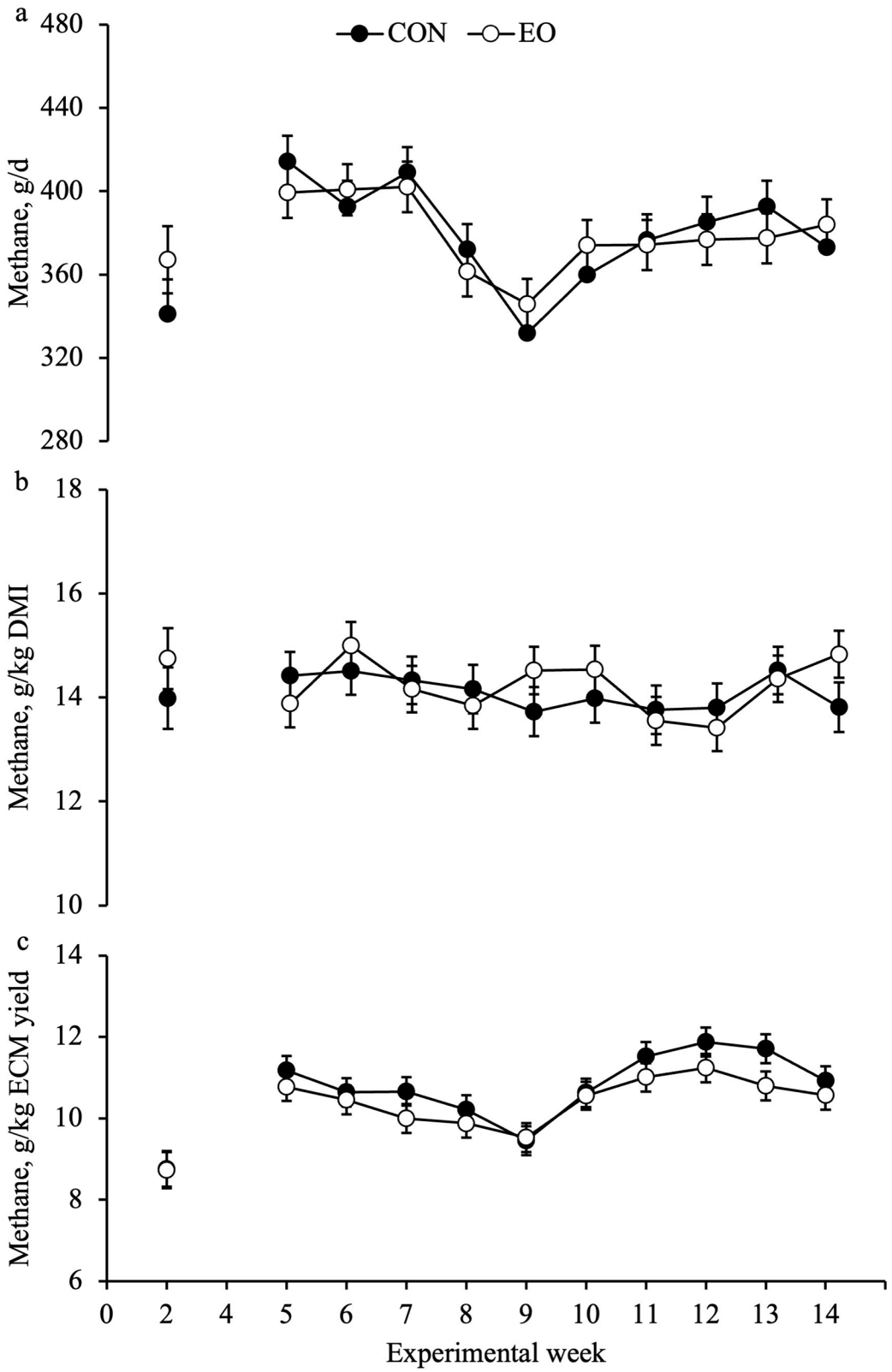

Figure 1. Methane production (a), methane yield (b), and methane intensity (c) of dairy cows fed an essential oil blend (EO). CON = basal diet; EO = basal diet supplemented at 1 g/cow per day EO. Error bars represent SEM.

Table 8. Manure emissions in lactating dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
Flux, mg/d				
CH_4	0.26	0.28	0.019	0.54
CO_2	106	123	9.4	0.29
NH_3	33.6	33.6	5.70	1.00
N_2O	ND[4]	ND		

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 44 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Day effect: $P < 0.001$ for all variables. Day × treatment interaction: $P > 0.10$ for all variables.

[4]ND = not detected.

Table 9. Blood metabolites concentration in lactating dairy cows fed an essential oil blend

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	EO		
BHB, μM	509	527	50.8	0.81
Total fatty acids, μM	171	171	7.8	1.00
BUN, mg/dL	11.5	11.2	0.52	0.60
Glucose, mg/dL	56.0	56.4	3.90	0.94

[1]Treatments were CON = control and EO = essential oil blend fed at 1 g/cow per day.

[2]Largest SEM published in table; n = 43 to 46 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity × treatment interaction: $P > 0.10$ for all variables.

(per kg of MY and ECM yield) were reported, and more extensive mitigation effect (i.e., numerically evaluated by CH_4 intensity over time) was detected after 21 d of EO supplementation in the study by Hart et al. (2019). The results of the former study, however, should be interpreted with caution because they were not statistically analyzed. Short- and long-term effects of EO supplementation have been discussed by Elcoso et al. (2019) and Belanche et al. (2020). For instance, estimated CH_4 production was lower in dairy cows supplemented with EO in wk 4, 6, and 8, but no differences were detected in experimental wk 2 in the study by Elcoso et al. (2019). Daily CH_4 production, yield, and intensity were decreased by 8.8%, 12.9%, and 9.9%, respectively, relative to CON treatments, in dairy cows after 28 d of EO supplementation (Belanche et al., 2020). In agreement with Belanche et al. (2020), EO supplementation was responsible for a 28 g decrease in daily CH_4 emission in cattle when compared with a CON group in a more recent meta-analysis evaluating 3 treatments means comparisons (Honan et al., 2022). It is important to note, however, that CH_4 yield was not affected by EO when considering the entire data set described in the meta-analysis by Belanche et al. (2020), which corroborates with our data. Additionally, cows in the current study were supplemented twice daily during a 12-wk period (i.e., a 10-wk comparison period) in a robust experimental design, and data herein presented do not support a treatment × time interaction for the mitigation effect of EO supplementation. The treatment × parity effect by EO supplementation observed for CH_4 intensity in multiparous cows in the current study could be related to the tendency for decreased digestibility of DM in that group of cows, and to a potentially decreased rumen fermentation as previously discussed. It is important to note, however, that the magnitude of the CH_4 intensity decrease was higher than the decrease in DM digestibility (7.5% vs. 2%, respectively).

Manure EP for CH_4, CO_2, and NH_3 were not affected by EO, and N_2O emission was undetectable for both treatments in the current study (Table 8). To the best of our knowledge, the current study is the first to report GHG EP of manure from lactating dairy cows fed this EO blend. The lack of N_2O emission was not surprising as Hristov et al. (2011) indicated that longer-term measurements are needed to detect N_2O emission from cattle manure in vitro. Using the head chamber methodology for 12 h gas sampling, Carrazco et al. (2020) did not detect any differences in enteric CH_4, CO_2, and N_2O production and yield between cows supplemented with EO blend and CON, but the researchers reported decreased NH_3 production, and decreased NH_3, NO_2, and CH_4 intensities. Overall, the lack of differences in manure GHG EP observed in the current study is in line with the lack of differences in rumen fermentation, enteric CH_4 emissions, and digestibility of nutrients.

Blood Variables

Blood concentrations of BHB, total FA, BUN and glucose were not affected by EO supplementation (Table 9). The slight increase in milk lactose concentration was not supported by an increase in blood glucose in EO cows. Additionally, the lack of differences in blood BHB and total FA concentrations is an indication that EO supplementation did not affect the overall energy balance of the cows in the present study. Host-mediated effects of phytonutrients have been described in the literature (Oh et al., 2017), and differential blood BHB and insulin concentrations have been reported in primiparous and multiparous cows fed increasing doses of a blend of *Capsicum* oleoresin and clove oil (Silvestre et al., 2022). Even though the EO blend used in this study contained eugenol (which is the main active compound

in clove oil), based on literature data evaluating this specific preparation (Benchaar et al., 2015), we did not expect to observe post-ruminal effects of EO. The lack of treatment effect on BUN concentration agrees with the ruminal NH_3 concentration, MUN, and UUN data, indicating that EO did not affect the overall efficiency of N utilization in the current study. Similarly, Carrazco et al. (2020) did not observe any effects of EO on BUN concentration in dairy cows.

CONCLUSIONS

Treatment did not affect DMI, feed efficiency, or lactational performance of the cows. The EO blend increased milk fat concentration, which resulted in decreased enteric CH_4 emission intensity per unit of milk fat. A treatment × parity interaction was observed and CH_4 intensity per unit of MY tended to be decreased by EO in multiparous, but not in primiparous cows. The GHG emitting potential of manure was not affected by EO supplementation. Contrary to our hypothesis, EO supplementation did not modify rumen VFA concentration or profile, and the concentration of blood energy markers was not affected by treatments in this study. Changes in ruminal pH could help explaining the increased milk fat concentration, and the decreased digestibility of DM and CP in multiparous cows could explain the tendency for decreased CH_4 intensity (per MY) and increased fecal N excretion observed with EO supplementation in multiparous cows, respectively. Overall, a blend of EO containing geranyl acetate and eugenol appeared to have a potential to decrease enteric CH_4 intensity by increasing milk fat in lactating dairy cows.

ACKNOWLEDGMENTS

This work was supported by the USDA (Washington, DC) National Institute of Food and Agriculture Federal Appropriations under Project PEN 04539 and Accession Number 1000803. Partial funding for the study was also provided by Agolin S.A (Bière, Switzerland). The authors thank the staff of The Pennsylvania State University Dairy Teaching and Research Center (University Park, State College, PA) for their conscientious care and management of the experimental cows and for technical assistance during the study. The authors have not stated any conflicts of interest.

REFERENCES

AOAC International. 2000. Official Methods of Analysis. 17th ed. AOAC International, Arlington, VA.

AOAC International. 2006. Official Methods of Analysis. 18th ed. AOAC International, Arlington, VA.

Arndt, C., A. N. Hristov, W. J. Price, S. C. McClelland, A. M. Pelaez, S. F. Cueva, J. Oh, A. Bannink, A. R. Bayat, L. A. Crompton, J. Dijkstra, M. A. Eugène, D. Enahoro, E. Kebreab, M. Kreuzer, M. McGee, C. Martin, C. J. Newbold, C. K. Reynolds, A. Schwarm, K. J. Shingfield, J. B. Veneman, D. R. Yáñez-Ruiz, and Z. Yu. 2022. Full adoption of strategies to mitigate enteric methane emissions by ruminants and how they can help to meet the 1.5°C climate target by 2030 but not 2050. Proc. Natl. Acad. Sci. USA 119:e2111294119. https://doi.org/10.1073/pnas.2111294119.

Bauman, D. E., and J. M. Griinari. 2001. Regulation and nutritional manipulation of milk fat: Low-fat milk syndrome. Livest. Prod. Sci. 70:15–29. https://doi.org/10.1016/S0301-6226(01)00195-6.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23:203–227. https://doi.org/10.1146/annurev.nutr.23.011702.073408.

Belanche, A., C. J. Newbold, D. P. Morgavi, A. Bach, B. Zweifel, and D. R. Yáñez-Ruiz. 2020. A meta-analysis describing the effects of the essential oils blend Agolin ruminant on performance, rumen fermentation, and methane emissions in dairy cows. Animals (Basel) 10:620. https://doi.org/10.3390/ani10040620.

Benchaar, C., F. Hassanat, and H. V. Petit. 2015. Dose-response to eugenol supplementation to dairy cow diets: Methane production, N excretion, ruminal fermentation, nutrient digestibility, milk production, and milk fatty acid profile. Anim. Feed Sci. Technol. 209:51–59. https://doi.org/10.1016/j.anifeedsci.2015.07.027.

Benchaar, C., H. V. Petit, R. Berthiaume, D. R. Ouellet, J. Chiquette, and P. Y. Chouinard. 2007. Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. J. Dairy Sci. 90:886–897. https://doi.org/10.3168/jds.S0022-0302(07)71572-2.

Brambila, R., and J. Noricumbo-Saenz. 2022. Evaluation of Agolin ruminant, an essential oil blend, as a feed additive for cows at two levels of production. Open J. Anim. Sci. 12:380–389. https://doi.org/10.4236/ojas.2022.123029.

Carrazco, A. V., C. B. Peterson, Y. Zhao, Y. Pan, J. J. McGlone, E. J. DePeters, and F. M. Mitloehner. 2020. The impact of essential oil feed supplementation on enteric gas emissions and production parameters from dairy cattle. Sustainability (Basel) 12:10347. https://doi.org/10.3390/su122410347.

Castro-Montoya, J., N. Peiren, J. W. Cone, B. Zweifel, V. Fievez, and S. De Campeneere. 2015. In vivo and in vitro effects of a blend of essential oils on rumen methane mitigation. Livest. Sci. 180:134–142. https://doi.org/10.1016/j.livsci.2015.08.010.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130–132. https://doi.org/10.1093/clinchem/8.2.130.

Chen, X. B., Y. K. Chen, M. F. Franklin, E. R. Orskov, and W. J. Shand. 1992. The effect of feed intake and body weight on purine derivative excretion and microbial protein supply in sheep. J. Anim. Sci. 70:1534–1542. https://doi.org/10.2527/1992.7051534x.

de Assis Lage, C. F., S. E. Räisänen, A. Melgar, K. Nedelkov, X. Chen, J. Oh, M. E. Fetter, N. Indugu, J. S. Bender, B. Vecchiarelli, M. L. Hennessy, D. Pitta, and A. N. Hristov. 2020. Comparison of two sampling techniques for evaluating ruminal fermentation and microbiota in the planktonic phase of rumen digesta in dairy cows. Front. Microbiol. 11:618032. https://doi.org/10.3389/fmicb.2020.618032.

Elcoso, G., B. Zweifel, and A. Bach. 2019. Effects of a blend of essential oils on milk yield and feed efficiency of lactating dairy cows. Appl. Anim. Sci. 35:304–311. https://doi.org/10.15232/aas.2018-01825.

Guasch, I., G. Elcoso, B. Zweifel, and A. Bach. 2016. 1480 Effects of a blend of essential oils on milk yield and feed efficiency of lactating cows. J. Anim. Sci. 94(suppl_5):718–719. https://doi.org/10.2527/jam2016-1480.

Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method rec-

ommended for AOAC collaborative study. J. AOAC Int. 92:42–49. https://doi.org/10.1093/jaoac/92.1.42.
Hart, K. J., H. G. Jones, K. E. Waddams, H. J. Worgan, B. Zweifel, and C. J. Newbold. 2019. An essential oil blend decreases methane emissions and increases milk yield in dairy cows. Open J. Anim. Sci. 9:259–267. https://doi.org/10.4236/ojas.2019.93022.
Harvatine, K. J., Y. R. Boisclair, and D. E. Bauman. 2009. Recent advances in the regulation of milk fat synthesis. Animal 3:40–54. https://doi.org/10.1017/S1751731108003133.
Hegarty, R. S., R. A. Cortez Passetti, K. M. Dittmer, Y. Wang, S. Shelton, J. Emmet-Booth, E. Wollenberg, T. McAllister, S. Leahy, K. Beauchemin, and N. Gurwick. 2021. An evaluation of emerging feed additives to reduce methane emissions from livestock. Edition 1. A report coordinated by Climate Change, Agriculture and Food Security (CCAFS) and the New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) initiative of the Global Research Alliance (GRA). https://hdl.handle.net/10568/116489.
Honan, M., X. Feng, J. M. Tricarico, and E. Kebreab. 2022. Feed additives as a strategic approach to reduce enteric methane production in cattle: Modes of action, effectiveness, and safety. Anim. Prod. Sci. 62:1303–1317. https://doi.org/10.1071/AN20295.
Hristov, A. N., C. Lee, T. Cassidy, M. Long, K. Heyler, B. Corl, and R. Forster. 2011. Effects of lauric and myristic acids on ruminal fermentation, production, and milk fatty acid composition in lactating dairy cows. J. Dairy Sci. 94:382–395. https://doi.org/10.3168/jds.2010-3508.
Hristov, A. N., A. Melgar, D. Wasson, and C. Arndt. 2022. Symposium review: Effective nutritional strategies to mitigate enteric methane in dairy cattle. J. Dairy Sci. 105:8543–8557. https://doi.org/10.3168/jds.2021-21398.
Hristov, A. N., J. Oh, J. L. Firkins, J. Dijkstra, E. Kebreab, G. Waghorn, H. P. S. Makkar, A. T. Adesogan, W. Yang, C. Lee, P. J. Gerber, B. Henderson, and J. M. Tricarico. 2013. Special topics: Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci. 91:5045–5069. https://doi.org/10.2527/jas.2013-6583.
Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Anim. Feed Sci. Technol. 48:211–227. https://doi.org/10.1016/0377-8401(94)90173-2.
Klop, G., S. van Laar-van Schuppen, W. F. Pellikaan, W. H. Hendriks, A. Bannink, and J. Dijkstra. 2017a. Changes in in vitro gas and methane production from rumen fluid from dairy cows during adaptation to feed additives in vivo. Animal 11:591–599. https://doi.org/10.1017/S1751731116002019.
Klop, G., J. Dijkstra, K. Dieho, W. H. Hendriks, and A. Bannink. 2017b. Enteric methane production in lactating dairy cows with continuous feeding of essential oils or rotational feeding of essential oils and lauric acid. J. Dairy Sci. 100:3563–3575. https://doi.org/10.3168/jds.2016-12033.
Lee, C., A. N. Hristov, T. W. Cassidy, K. S. Heyler, H. Lapierre, G. A. Varga, M. J. De Veth, R. A. Patton, and C. Parys. 2012a. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 95:6042–6056. https://doi.org/10.3168/jds.2012-5581.
Lee, C., A. N. Hristov, C. J. Dell, G. W. Feyereisen, J. Kaye, and D. Beegle. 2012b. Effect of dietary protein concentration on ammonia and greenhouse gas emitting potential of dairy manure. J. Dairy Sci. 95:1930–1941. https://doi.org/10.3168/jds.2010-4141.
NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press.
Oh, J., E. H. Wall, D. M. Bravo, and A. N. Hristov. 2017. Host- mediated effects of phytonutrients in ruminants: A review. J. Dairy Sci. 100:5974–5983. https://doi.org/10.3168/jds.2016-12341.
Rico, D. E., and K. J. Harvatine. 2013. Induction of and recovery from milk fat depression occurs progressively in dairy cows switched between diets that differ in fiber and oil concentration. J. Dairy Sci. 96:6621–6630. https://doi.org/10.3168/jds.2013-6820.
Santos, M. B., P. H. Robinson, P. Williams, and R. Losa. 2010. Effects of addition of an essential oil complex to the diet of lactating dairy cows on whole tract digestion of nutrients and productive performance. Anim. Feed Sci. Technol. 157:64–71. https://doi.org/10.1016/j.anifeedsci.2010.02.001.
Silvestre, T., L. F. Martins, S. F. Cueva, D. E. Wasson, N. Stepanchenko, S. E. Räisänen, S. Sommai, M. L. Hile, and A. N. Hristov. 2023. Supplemental material: Lactational performance, rumen fermentation, nutrient use efficiency, enteric methane emissions and manure greenhouse gas emitting potential in dairy cows fed a blend of essential oils. ScholarSphere. https://scholarsphere.psu.edu/resources/3331091e-2cef-413e-87f9-2e57ec1971ab.
Silvestre, T., S. E. Räisänen, S. F. Cueva, D. E. Wasson, C. F. A. Lage, L. F. Martins, E. Wall, and A. N. Hristov. 2022. Effects of a combination of *Capsicum* oleoresin and clove essential oil on metabolic status, lactational performance, and enteric methane emissions in dairy cows. J. Dairy Sci. 105:9610–9622. https://doi.org/10.3168/jds.2022-22203.
Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setälä. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. Pages 156–157 in 27th Session of the International Commission for Breeding and Productivity of Milk Animals, Paris, France. Wageningen Academic Publishers.
US Government. 2021. Executive Order on Tackling the Climate Crisis at Home and Abroad. Accessed Sep. 26, 2022. https://www.whitehouse.gov/briefing-room/presidential-actions/2021/01/27/executive-order-on-tackling-the-climate-crisis-at-home-and-abroad/.
van Gastelen, S., and J. Dijkstra. 2016. Prediction of methane emission from lactating dairy cows using milk fatty acids and mid- infrared spectroscopy. J. Sci. Food Agric. 96:3963–3968. https://doi.org/10.1002/jsfa.7718.
Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.
Vlaeminck, B., V. Fievez, A. R. J. Cabrita, A. J. M. Fonseca, and R. J. Dewhurst. 2006. Factors affecting odd- and branched-chain fatty acids in milk: A review. Anim. Feed Sci. Technol. 131:389–417. https://doi.org/10.1016/j.anifeedsci.2006.06.017.
Wemette, M., A. Greiner Safi, A. K. Wolverton, W. Beauvais, M. Shapiro, P. Moroni, F. L. Welcome, and R. Ivanek. 2021. Public perceptions of antibiotic use on dairy farms in the United States. J. Dairy Sci. 104:2807–2821. https://doi.org/10.3168/jds.2019-17673.
Wheeler, E. F., P. A. Topper, N. E. Brown, and G. A. Varga. 2007. Multiple-chamber steady-state gas emission detection from dairy manure slurry. Proceedings of the International Symposium on Air Quality and Waste Management for Agriculture, Broomfield, CO. ASABE Publication Number 701P0907cd.
Williams, P., J. Clark, and K. Bean. 2021. Evaluation of Agolin, an essential oil blend, as a feed additive for high producing cows. Open J. Anim. Sci. 11:231–237. https://doi.org/10.4236/ojas.2021.112018.
Yang, C. M. J., and G. A. Varga. 1989. Effect of three concentrate feeding frequencies on rumen protozoa, rumen digesta kinetics, and milk yield in dairy cows. J. Dairy Sci. 72:950–957. https://doi.org/10.3168/jds.S0022-0302(89)79188-8.