Nutrient digestibility, ruminal fermentation, and milk yield in dairy cows fed a blend of essential oils and amylase

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ABSTRACT

Two experiments were carried out to evaluate a blend of essential oils (EO) combined with amylase as an alternative to ionophores and its potential for reducing the use of antibiotics in the dairy industry. In experiment 1, 8 rumen-cannulated Holstein cows (576 ± 100 kg of body weight, 146 ± 35 d in milk, and 35.1 ± 4.0 kg/d of milk yield at the start of the experiment) were assigned to a 4×4 Latin square experiment with 21-d periods to determine the influence of feed additives on total apparent digestibility of nutrients, ruminal fermentation, N utilization, microbial protein synthesis, blood glucose and urea concentrations, and milk yield and composition in dairy cows. Treatment sequences assigned to cows in each block included no feed additives (control; CON); monensin (MON) added at 13 mg/kg of diet dry matter (DM); a blend of EO supplemented at 44 mg/kg of diet DM; and EO treatment combined with α -amylase at 330 kilo novo units/kg of diet DM (EOA). Differences among treatments were studied using orthogonal contrasts as follows: CON versus feed additives (MON, EO, and EOA), MON versus EO and EOA, and EO versus EOA. No differences were detected in nutrient intake and digestibility in cows. In general, feed additives decreased ruminal $\text{NH}_3\text{-N}$ concentration of cows, notably when diet was supplemented with MON. Furthermore, feed additives increased ruminal concentrations of acetate, butyrate, and branched-chain fatty acids. Cows fed treatments containing EO and EOA exhibited lower pH, higher $\text{NH}_3\text{-N}$, and a trend to greater total volatile fatty acid concentration in the ruminal fluid compared with cows fed MON. Treatments containing EO increased ruminal butyrate concentration compared with MON. No treatment $\times$ time interaction effect was observed on

ruminal fermentation measurements. Cows fed diets supplemented with feed additives had greater efficiency of N transfer into milk (milk N:N intake), whereas cows fed EOA exhibited greater N transfer into milk than those fed EO. Treatments had no effect on milk yield and composition, but feed additives increased the milk yield efficiency (milk yield divided by dry matter intake), whereas treatments containing EO had similar milk yield efficiency compared with MON. For experiment 2, 30 multiparous Holstein cows (574 ± 68 kg of body weight, 152 ± 54 d in milk, and 30.9 ± 4.1 kg/d of milk yield at the start of the experiment) were enrolled to a randomized complete block design experiment. The MON, EO, and EOA treatments were randomly assigned to cows within blocks ($n = 10$), and feed additives were provided throughout a 9-wk period. No differences were found in nutrient intake and digestibility, but cows fed EOA tended to exhibit greater dry matter intake than those fed EO. Blood metabolites and milk production were not affected by treatments. However, cows fed MON or EOA had greater milk protein content than those cows fed treatments containing EO. Feeding EO with or without amylase had similar response to feeding MON in terms of feed intake and milk yield, with a small negative effect on milk protein yield when feeding EO alone. Feed additives increased the concentrations of acetate, butyrate, and branched-fatty acids in ruminal fluid, whereas treatments containing EO had greater ruminal butyrate and $\text{NH}_3\text{-N}$ concentrations. Therefore, either EO or EOA can replace MON in diets of dairy cows while maintaining performance.

Key words: α -amylase, antimicrobial, ionophore, plant secondary metabolite

INTRODUCTION

The increasing wealth of emerging economies has promoted the global demand for an adequate and trustworthy supply of meat and milk, which is expected to double by 2050 (Alexandratos and Bruinsma, 2012).

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Despite the necessity of increments in food production, farmers face multiple socioeconomic and environmental challenges, such as pressure to decrease greenhouse gas production, competition for human-edible feedstuff, and a tendency toward a decrease in use of antibiotics and growth promoters in livestock. In this regard, several reviews have highlighted the potential of feed enzymes and plant extracts to improve nutrient utilization in ruminants as alternatives to ionophore antibiotics (Meale et al., 2014; McGrath et al., 2018).

Monensin (**MON**) is the major ionophore antibiotic used in diets of dairy cows either to improve feed efficiency or to decrease the risk of metabolic disorders such as acidosis and ketosis (Duffield et al., 2008; Gandra et al., 2010). However, use of MON as a feed additive was discontinued by the European Union (Regulation 1831; European Commission, 2003) based on the “precautionary principle.” Thus, alternative feed additives to MON have been tested to enhance animal performance. Among the currently marketed feed additives, essential oils (**EO**) and enzyme products have attracted the attention of the scientific community as potential alternatives to ionophores, especially for being considered “generally recognized as safe” and may improve animal performance (Benchaar et al., 2008; Andreazzi et al., 2018). Essential oils are aromatic compounds extracted from plants by fermentation or distillation (Chao et al., 2000) that exhibit antimicrobial properties, promote propionate production in *in vitro* studies (Chaves et al., 2008), and may improve milk and FCM production of dairy cows (Kung et al., 2008; Tassoul and Shaver, 2009).

Although enzymes for ruminants have been studied since the 1960s (as reviewed by Beauchemin and Rode, 1996), researchers have re-evaluated their potential in ruminant nutrition in recent years due to increases in feed costs, decreases in enzyme production costs, and greater availability of more active and defined enzyme preparations (Beauchemin et al., 2003). Indeed, the addition of enzyme products with amylase activity in dairy cow diets has increased diet digestibility and milk production of cows (Tricarico et al., 2005; Gencoglu et al., 2010; Andreazzi et al., 2018). Freire et al. (2017) reported greater FCM production in cross-breed dairy cows fed EO combined with amylase (**EOA**) compared with cows fed MON (22.7 and 22.0 kg/d, respectively).

Although evidence suggests that EOA can improve milk production of dairy cows, the literature still lacks data regarding the effects of EO and enzyme product combination on ruminal fermentation, digestibility, and performance of dairy cows. Thus, the objective of this study was to evaluate the effects of feed additives (MON, EO, and EOA) on nutrient intake and total

apparent digestibility, ruminal fermentation, microbial protein synthesis, serum glucose and urea concentrations, milk yield and composition, and N utilization in dairy cows. The hypothesis of this study was that EO and EOA could replace MON in diets of cows without decreasing animal performance. In addition, we expected that the dietary supplementation of EO and amylase would exert synergistic positive effects on ruminal fermentation, nutrient digestibility, and milk production of cows.

MATERIALS AND METHODS

All experimental procedures with animals were performed with the approval of the ethics committee from the School of Veterinary Medicine and Animal Sciences, University of São Paulo, São Paulo, Brazil (approval no. 9762201015). Two experiments with different designs were performed simultaneously to evaluate the effects of feed additives (MON, EO, and EOA) on ruminal fermentation, N utilization, and microbial protein synthesis (experiment 1) and to determine their effects on performance of cows (experiment 2). This study was carried out at the Dairy Cattle Research Laboratory, University of São Paulo, Pirassununga, Brazil.

Experiment 1

Animals, Design, and Treatments. Eight rumen-cannulated multiparous Holstein cows (576 ± 100 kg of BW, 146 ± 35 DIM, and 35.1 ± 4.0 kg/d of milk yield at the start of the experiment) were enrolled in a replicated 4×4 Latin square experiment design and distributed to each square according to the milk yield, DIM, and BW. Experimental periods consisted of 14 d of treatment adaptation and 7 d of sampling. Treatment sequences were randomly assigned to animals within square and included the following: no feed additives (control; **CON**); sodium MON added at 13 mg/kg of diet DM (Rumensin, Elanco, São Paulo, Brazil); a blend of EO supplemented at 44 mg/kg of DM (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); and provision of EO combined with exogenous α -amylase produced by *Bacillus licheniformis* at 330 kilo novo units/kg of diet DM (Ronozyne Rumistar, DSM Produtos Nutricionais Brasil S.A.). The enzyme product was added in an equivalent dose of 0.55 g/kg of DM, which has shown positive effects on the performance of dairy cows (Klingerman et al., 2009; Gencoglu et al., 2010). The Crina Ruminants EO is a patented blend of natural and natural-identical EO compounds that included thymol, eugenol, vanillin, and limonene on an organic carrier (McIntosh et al., 2003).

Table 1. Ingredient and chemical composition (g/kg of DM, unless otherwise stated) of TMR provided to cows in both experiments

Item	Amount
Ingredient	
Corn silage ¹	469
Ground corn	220
Soybean meal	152
Citrus pulp	68.2
Whole raw soybean	59.5
Vitamin and mineral mix ²	22.4
Limestone	3.00
Urea	2.50
Salt	2.20
Chemical composition	
DM, g/kg of fresh matter	448
OM	922
NFC ³	459
Starch	279
NDF	295
ADF	197
Indigestible ADF	77.2
Lignin	17.0
Neutral detergent insoluble protein	32.2
Acid detergent insoluble protein	22.7
CP	160
Ether extract	37.9
Total digestible nutrient 1× ⁴	753
NE _L 3×, ⁵ Mcal/kg of DM	1.73

¹Average composition (g/kg of DM, unless otherwise stated): DM, 290 g/kg of fresh matter; NDF, 484; indigestible ADF, 148; CP, 72.6; and ash, 57.1.

²Per kilogram of mix (minimum values): 88 g of Ca, 42 g of P, 18 g of S, 45 g of Mg, 20 g of K, 123 g of Na, 14 mg of Co, 500 mg of Cu, 20 mg of Cr, 1,050 mg of Fe, 28 mg of I, 1,400 mg of Mn, 18 mg of Se, 2,800 mg of Zn, 80 mg of biotin, 200,000 IU of vitamin A, 70,000 IU of vitamin D, and 1,200 IU of vitamin E.

³NFC = 1,000 – [(CP – CP from urea + urea) + ether extract + ash + NDF]; all values expressed as g/kg (Hall, 2000).

⁴Estimated at maintenance level of intake, according to NRC (2001).

⁵Estimated at 3 times maintenance level, according to NRC (2001).

Feed additives (MON, EO, and EOA) were supplied according to the manufacturers' recommendations as dry powders added to the concentrate along with the mineral mixture during the concentrate mixing (once a week). The basal diet was formulated according to NRC (2001) recommendations and fed twice a day (0800 and 1330 h) as a TMR (Table 1). Corn silage DM content was assessed on a weekly basis for dietary adjustment purposes. Throughout the experiment, cows were housed in a barn containing individual pens (17.5 m² of area), sand bedding, feed bunks, and free access to water.

Nutrient Intake and Digestibility. Samples of each ingredient in the concentrate were collected during its preparation, and samples of corn silage and refusals from each cow were collected during the 7-d sampling periods. Composited samples of feed, refusals, and feces were dried in a forced-air oven at 55°C for 72 h and ground in a Wiley mill (Arthur H. Thomas,

Philadelphia, PA) to pass through a 1-mm screen. The contents of DM (method 930.15, AOAC International, 2000), OM (DM – ash), CP (N × 6.25; method 984.13, AOAC International, 2000), ether extract (EE; method 920.39, AOAC International, 2000), and starch (Knudsen, 1997) were evaluated in all samples. The contents of NDF, ADF, and lignin in samples were determined according to Van Soest et al. (1991), and results were expressed with residual ash. The NDF analysis was performed using α-amylase with no sodium sulfite added to the detergent (TE-149 fiber analyzer, Tecnal Equipment for Laboratory Inc., Piracicaba, Brazil). Total digestible nutrients were calculated according to NRC (2001) equations. Net energy for lactation was estimated according to Weiss et al. (1992). Nonfiber carbohydrate was calculated using the equation proposed by Hall (2000; values expressed as percentages):

$$\text{NFC} = 100 - [(\text{CP} - \text{CP from urea} + \text{urea}) + \text{NDF} + \text{EE} + \text{ash}].$$

Fecal samples (0.3 kg) were collected directly from the rectum of each cow on d 15, 16, and 17 of each experimental period, every 9 h (starting on d 15 at 0600 h), to estimate the amount of daily nutrient excretion. After collection, samples were frozen and composited (wet basis) at the end of each period to form a single sample for each animal per period. Afterward, samples were dried in a forced-air oven at 55°C for 72 h and ground in a Wiley mill (Arthur H. Thomas) to pass through a 2-mm screen. Ground samples (2-mm screen) of feed ingredients, Orts, and feces were placed in nonwoven textile bags (5 × 5 cm) following the recommendation of a maximum of 20 mg of DM/cm² (Nocek, 1988). These samples were incubated for 288 h in the rumen of 2 Holstein cows previously adapted to the basal diet of this study as described by Casali et al. (2008). After removal from the rumen, bags were washed in running tap water, dried at 55°C in a forced-air oven for 72 h, and analyzed for ADF content as described earlier. Fecal samples were evaluated for contents of DM, OM, NDF, starch, CP, and EE as mentioned before. The indigestible ADF (**iADF**) was used as an internal marker to analyze total-tract digestion, calculated using the following equations:

$$\text{digestibility of DM} = 1 - \left(\frac{\% \text{ of iADF intake}}{\% \text{ of iADF in feces}} \right),$$

$$\text{digestibility of nutrient} = 1 - \left[\left(\frac{\% \text{ of iADF intake}}{\% \text{ of iADF in feces}} \right) \times \left(\frac{\% \text{ of nutrient in feces}}{\% \text{ of nutrient intake}} \right) \right].$$

Ruminal Fermentation. Ruminal digesta was collected on d 21 of each experimental period, always by the same technician, by withdrawing equal amounts of digesta (~500 g) from multiple sites (anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral) within the rumen of cannulated cows. Collections of rumen digesta were performed before the morning feeding (time 0) and 2, 4, 6, 8, 10, 12, 14, and 16 h after the morning feeding. Immediately after each collection, samples were strained through 4 layers of cheesecloth to obtain ruminal fluid (250 mL). The ruminal fluid pH was measured using a pH meter (MB-10, Marte, Santa Rita Sapucaí, Brazil). Aliquots of 1,600 μ L of sample were mixed with 400 μ L of methanoic acid (98–100% H_2CO_2) and centrifuged at $7,000 \times g$ for 15 min at 4°C, and the supernatant of each sample was frozen for posterior VFA analysis. Also, 2-mL aliquots were mixed with 1 mL of sulfuric acid (0.5 mol/L of H_2SO_4) and frozen for analysis of ammonia N ($\text{NH}_3\text{-N}$) by the colorimetric phenol-hypochlorite method (Broderrick and Kang, 1980).

Ruminal VFA were measured using a gas chromatograph (GC-2014, Shimadzu, Tokyo, Japan) equipped with a capillary column (Stabilwax, Restek, Bellefonte, PA). The gases used were helium as the carrier gas (8.01 mL/min flow), hydrogen as the fuel gas (pressure of 60 kPa), and synthetic air as the oxidizer gas (pressure of 40 kPa). The steamer temperature was set at 220°C, the ionization detector flames were set at 250°C, and the separation column was set at 145°C for 3 min, which was then increased 10°C/min up to 200°C.

N Utilization and Microbial Protein Synthesis. Nitrogen excreted in milk was calculated using the following equation:

$$\begin{aligned} \text{milk N (g/d)} &= \text{milk CP concentration (g/kg)} \\ &\times \text{milk yield (kg/d)}/6.38. \end{aligned}$$

Nitrogen excreted in feces was calculated using the following equation:

$$\begin{aligned} \text{fecal N (g/d)} &= \text{CP in feces (g/kg)} \\ &\times \text{DM fecal excretion (kg/d)}/6.25. \end{aligned}$$

Samples of urine were collected at the same time points of feces. Urine samples were filtered, and aliquots of 10 mL were diluted into 40 mL of 0.036 *N* sulfuric acid to avoid bacterial destruction of purine derivatives (**PD**) and uric acid precipitation and then frozen. Nondiluted urine samples were also frozen for further assessment of N and creatinine concentrations. Total urine volume was estimated based on the creatinine concentration in

urine assuming a daily creatinine excretion of 24.05 mg/kg of BW (Chizzoti et al., 2008). Urine N was determined according to AOAC International (2000) method 984.13; hence, urinary N excretion was calculated by multiplying urine N by urine volume. Creatinine and uric acid concentrations of urine were determined using commercial kits (Bioclin, Belo Horizonte, Brazil) by colorimetric method, and absorbance was measured in a semiautomatic biochemistry analyzer (SBA-200, CELM, São Caetano do Sul, Brazil). Body weights were measured at the beginning of the study and at the last day of each experimental period, before the morning feeding and after milking, using an electronic livestock scale for large animals. Body condition score was measured at the same time points as BW using a 5-point system (1 = emaciated; 5 = obese) according to Wildman et al. (1982).

Microbial N synthesis was determined based on PD excretion according to Chen and Gomes (1992). Allantoin in urine and in milk were analyzed by a colorimetric method (Chen and Gomes, 1992). Ten milliliters of milk was mixed with 5 mL of trichloroacetic acid (25%); this mixture rested for 5 min and then was filtered through a paper filter (14- μ m pore size) to obtain deproteinized milk samples. The total excretion of PD (mmol/d) was calculated as the sum of allantoin and uric acid excreted in urine and milk (Orellana Boero et al., 2001). The absorbed PD (**PD_{abs}**; mmol/d) was calculated as follows:

$$\text{PD}_{\text{abs}} = (\text{PD} - 0.385 \times \text{BW}^{0.75})/0.84,$$

where 0.84 represents the recovery of **PD_{abs}** as PD, and $0.385 \times \text{BW}^{0.75}$ represents the endogenous excretion of PD (Chen and Gomes, 1992). The ruminal synthesis of N compounds (**N_{mic}**; g of N/d) was calculated based on the **PD_{abs}** using the equation of Chen and Gomes (1992):

$$\text{N}_{\text{mic}} = (70 \times \text{PD}_{\text{abs}})/(0.83 \times 0.134 \times 1,000),$$

where 70 is the N purine derivative content (mg of N/mol), 0.134 is the ratio between PD N and microbial N (Valadares et al., 1999), and 0.83 is the intestinal digestibility of microbial purines.

Milk Yield and Composition. Milk yield was electronically recorded (Alpro, DeLaval, Tumba, Sweden) during all milkings. Milk samples were collected from each cow in all milkings throughout d 17, 18, and 19 of each period and were analyzed for fat, protein, and lactose contents by infrared methodology (Lactoscan, Entelbra, São Paulo, Brazil). The 3.5% FCM was calculated according to Sklan et al. (1992):

$$3.5\% \text{ FCM} = (0.432 + 0.165 \times \text{milk fat}) \\ \times \text{milk yield (kg/d)}.$$

Statistical Analysis. Data were submitted to ANOVA and analyzed using the MIXED procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC) after verifying the normality of residuals and homogeneity of variances by PROC UNIVARIATE, according to the following model:

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_l + c\gamma_{kl} + e_{ijk},$$

where y_{ijkl} represents the observation on animal k given treatment i at period j in squared l , μ is the intercept, α_i represents the fixed effect of the i th treatment ($i = 1$ to 4), β_j represents the fixed effect of the j th period ($j = 1$ to 4), γ_l represents the fixed effect of the l th square ($l = 1$ and 2), $c\gamma_{kl}$ represents the random effect of animal within square ($k = 1$ to 8), and e_{ijk} is the random error associated with each observation.

Repeated measures over time analysis (Littell et al., 2006) using the MIXED procedure of SAS were performed for the ruminal fermentation data (0, 2, 4, 6, 8, 10, 12, and 16 h relative to the morning feed) according to the model

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_l + c\gamma_{kl} + e(a)_{ijkl} \\ + \delta_m + \alpha\delta_{im} + e(b)_{ijklm},$$

where y_{ijkl} represents the observation on animal k given treatment i at period j in square l , μ is the intercept, α_i represents the fixed effect of the i th treatment ($i = 1$ to 4), β_j represents the fixed effect of the j th period ($j = 1$ to 4), γ_l represents the fixed effect of the l th square ($l = 1$ to 2), $c\gamma_{kl}$ represents the random effect of animal within square ($k = 1$ to 8), e_{ijklm} represents the random error associated with each observation of main plot (a), δ_m represents the fixed effect of the m th time ($m = 0, 2, 4, 6, 8, 10, 12, 14$, and 16), $\alpha\delta_{im}$ represents the fixed effect of interaction between i treatment and m time, and $e(b)_{ijklm}$ represents the random error associated with each observation of subplot (b). The Akaike methodology was used to determine the covariance matrix. The degrees of freedom were calculated according to Kenward and Roger (1997). Differences among treatments were analyzed through the following orthogonal contrasts: C1 = control versus feed additives (MON + EO + EOA); C2 = comparison between ionophore treatment and treatments containing EO (MON vs. EO + EOA); and C3 = effect of α -amylase addition (EO vs. EOA). Significance level was $P \leq 0.05$, and trend was $0.05 < P \leq 0.10$.

Experiment 2

A randomized complete block design experiment was carried out to compare the effects of feed additives (MON, EO, EOA) for longer periods of supplementation on productive performance of dairy cows. In this experiment, 30 multiparous Holstein cows (574 ± 68 kg of BW, 152 ± 54 DIM, and 30.9 ± 4.1 kg/d of milk yield at the start of the experiment) were blocked according to DIM, milk yield, and BW. Cows within block were randomly distributed to MON, EO, or EOA as described in experiment 1. The experiment lasted 10 wk, where the first week was used for sampling to covariate analyses (no feed additives provided) and the following 9 wk were used to evaluate treatment effects. Housing and feeding conditions were the same as those described in experiment 1.

Sampling. Refusals of each animal were collected daily 1 wk before the beginning of feed additive supply (for covariate adjustment purposes) and every 3 wk to determine nutrient intake of the animals. Fecal samples were collected every 3 wk on Friday, Saturday, and Sunday (at 9 h-intervals, starting at 0600 h on Friday and finishing at 2100 h on Sunday). Milk yield was recorded in all milkings, and milk was sampled every week on Sunday, Monday, and Tuesday. Methods of chemical analyses were the same as those described in experiment 1. Blood samples were collected 4 h after the morning feeding every 3 wk (on Thursday) by venipuncture from the coccygeal vein into sterile evacuated containers without clotting activator. After clotting, samples were centrifuged for 15 min at $2,000 \times g$ at 4°C , and the supernatant serum was transferred to centrifuge tubes, which were frozen. Blood serum was analyzed for glucose and urea concentrations using colorimetric commercial kits (Bioclin), and absorbance was measured using a semiautomatic biochemistry analyzer (SBA-200, CELM).

Statistical Analysis. Data were submitted to ANOVA using the MIXED procedure of SAS as repeated measures (Littell et al., 2006) according to the model

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \omega_{ij} + B_1(X_{il} - \bar{X}) + \gamma_l + \alpha_i \\ + \gamma_{il} + e_{ijkl},$$

where y_{ijkl} represents the observation on animal k given treatment i at period l in block j , μ is the intercept, α_i represents the fixed effect of the i th treatment ($i = 1$ to 3), β_j represents the random effect of the j th block ($j = 1$ to 10), ω_{ij} is the random error for treatment evaluation (treatment and block interaction effect), B_1 is a

Table 2. Nutrient intake and total apparent digestibility in mid-lactation cows fed a blend of essential oils and amylase (experiment 1: Latin square design)

Item	Treatment ¹				SEM	P-value ²			
	CON	MON	EO	EOA		Treatment	C1	C2	C3
Intake, kg/d									
DM	27.6	26.5	27.4	26.9	0.701	0.592	0.368	0.404	0.550
OM	25.3	24.3	25.1	24.6	0.643	0.573	0.362	0.397	0.523
NDF	7.91	7.54	7.85	7.77	0.211	0.551	0.410	0.258	0.762
Starch	7.82	7.52	7.73	7.62	0.196	0.599	0.321	0.439	0.621
CP	4.49	4.30	4.46	4.35	0.111	0.523	0.329	0.406	0.450
Indigestible ADF	2.05	1.91	2.01	2.02	0.058	0.466	0.368	0.199	0.857
Ether extract	1.08	1.03	1.06	1.04	0.027	0.529	0.262	0.401	0.654
Intake, kg/100 kg of BW									
DM	4.73	4.56	4.69	4.58	0.113	0.630	0.349	0.570	0.485
NDF	1.36	1.30	1.34	1.32	0.035	0.660	0.361	0.462	0.675
Digestibility coefficient									
DM	0.659	0.693	0.688	0.679	0.0076	0.236	0.064	0.517	0.584
OM	0.678	0.708	0.709	0.698	0.0087	0.492	0.155	0.796	0.633
NDF	0.447	0.515	0.477	0.448	0.0133	0.169	0.230	0.085	0.392
Starch	0.933	0.938	0.943	0.946	0.0029	0.364	0.143	0.366	0.681
CP	0.634	0.671	0.675	0.667	0.0088	0.273	0.058	0.978	0.728
Ether extract	0.800	0.851	0.822	0.836	0.0120	0.489	0.201	0.455	0.680

¹CON = no feed additive (control); MON = sodium monensin added at 13 mg/kg (Rumensin, Elanco, São Paulo, Brazil); EO = blend of essential oils supplemented at 44 mg/kg (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); EOA = provision of essential oils and amylase (330 kilo novo units/kg; Ronozyme Rumistar, DSM Produtos Nutricionais Brasil S.A.).

²Orthogonal contrasts: C1 = CON vs. feed additives (MON + EO + EOA); C2 = MON vs. EO + EOA; C3 = EO vs. EOA.

fixed effect regression coefficient, X_{il} is the covariate measurement of the k th cow within the j th block and i th treatment; $\bar{X}$ is the overall mean of the covariate measurements, γ_l is the fixed effect of the l th week ($j = 1$ to 3), $\alpha \times \gamma_{il}$ is an interaction effect, and e_{ijkl} is the random residual error. The degrees of freedom were calculated according to Kenward and Roger (1997). The covariance structure for each parameter was determined based on the smallest Akaike information criterion values. Data of DM and nutrient intake, milk yield and composition, BW, and BCS from the first week of the experiment were used as covariates because they reduced Akaike criterion values, and covariate adjustment was not performed for digestibility and serum metabolites concentration data. Differences among treatments were analyzed using orthogonal contrasts: C1 = comparison between ionophore treatment and treatments containing EO (MON vs. EO + EOA), and C2 = effect of α -amylase addition (EO vs. EOA). Significance level was $P \leq 0.05$, and a trend was $0.05 < P \leq 0.10$.

RESULTS

Experiment 1: Latin Square Design

In general, feed additives (MON, EO, and EOA) had no effect on feed intake and tended to increase digestibility of DM ($P = 0.064$) and CP ($P = 0.058$; Table

2). Cows fed MON and those fed treatments containing EO (EO and EOA) showed similar ($P \geq 0.199$) nutrient intake. However, there was a trend for MON to increase ($P = 0.085$) NDF digestibility in cows compared with cows fed EO and EOA. The combination of EO with α -amylase (EOA) did not affect ($P \geq 0.392$) nutrient intake or digestibility in cows in relation to EO.

Feed additives decreased ($P = 0.026$) ruminal $\text{NH}_3\text{-N}$ concentration and tended to increase ($P = 0.080$) total VFA concentration in rumen fluid (Table 3). Furthermore, treatments containing EO decreased ($P = 0.017$) ruminal pH, increased $\text{NH}_3\text{-N}$ concentration ($P = 0.004$), and tended to increase ($P = 0.076$) total VFA ruminal concentration in relation to MON. The EOA treatment tended to decrease ($P = 0.065$) ruminal $\text{NH}_3\text{-N}$ concentration without affecting ($P = 0.519$) VFA concentration in the rumen compared with EO treatment. Regarding the specific ruminal VFA concentration, feed additives increased ($P \leq 0.046$) the concentrations of acetate, butyrate, and branched-chain fatty acids in the rumen fluid. In addition, cows fed treatments containing EO had greater ($P = 0.041$) ruminal butyrate concentration compared with those cows fed MON. Cows fed EO treatments tended to exhibit greater ($P \leq 0.010$) concentrations of acetate, propionate, and branched-chain fatty acids in ruminal fluid compared with those fed MON.

Dietary additives improved ($P = 0.016$) N transfer into the milk and tended to decrease ($P = 0.057$) the

Table 3. Ruminal fermentation in mid-lactation cows fed a blend of essential oils and amylase (experiment 1: Latin square design)

Item	Treatment ¹					P-value ²					
	CON	MON	EO	EOA	SEM	Treatment	Time	Treatment × time	C1	C2	C3
pH	6.03	6.08	5.96	5.96	0.023	0.096	<0.001	0.388	0.492	0.017	0.992
NH ₃ -N, mg/dL	18.7	15.9	18.6	17.2	0.45	0.002	<0.001	0.892	0.026	0.004	0.065
Acetate, mol %	60.3	61.0	60.2	60.9	0.42	0.690	0.689	0.572	0.690	0.689	0.572
Propionate, mol %	22.6	21.8	22.8	22.2	0.480	0.886	<0.001	0.591	0.794	0.539	0.670
Butyrate, mol %	12.2	12.3	12.2	12.1	0.242	0.993	<0.001	0.979	0.972	0.811	0.877
BFCA, ³ mol %	4.94	4.92	4.77	4.74	0.086	0.790	0.439	0.472	0.527	0.439	0.897
Total VFA, mM	146	150	161	167	3.27	0.095	<0.001	0.933	0.080	0.076	0.519
Acetate, mM	88.9	91.9	97.5	102	1.79	0.047	<0.001	0.967	0.046	0.069	0.359
Propionate, mM	32.7	32.7	36.8	37.1	1.15	0.240	<0.001	0.974	0.226	0.099	0.928
Butyrate, mM	17.7	18.2	19.4	20.0	0.365	0.027	<0.001	0.480	0.027	0.041	0.471
BFCA, mM	6.99	7.16	7.43	7.67	0.100	0.019	<0.001	0.602	0.021	0.051	0.288
C2:C3 ratio	2.78	2.85	2.73	2.77	0.078	0.950	<0.001	0.754	0.971	0.589	0.835

¹CON = no feed additive (control); MON = sodium monensin added at 13 mg/kg (Rumensin, Elanco, São Paulo, Brazil); EO = blend of essential oils supplemented at 44 mg/kg (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); EOA = provision of essential oils and amylase (330 kilo novo units/kg; Ronozyme Rumistar, DSM Produtos Nutricionais Brasil S.A.).

²Time points = 0, 2, 4, 6, 8, 10, 12, 14, and 16 h after feeding. Orthogonal contrasts: C1 = CON vs. feed additives (MON + EO + EOA); C2 = MON vs. EO + EOA; C3 = EO vs. EOA.

³Branched-chain fatty acids.

fecal N (Table 4). Cows fed treatments containing EO tended to show higher ($P = 0.073$) N balance and lower ($P = 0.077$) N in urine compared with cows fed MON. Treatments had no effect ($P \geq 0.410$) on microbial protein synthesis of cows.

Neither tendencies nor treatment effects were observed on milk yield and composition, but feed additives improved ($P = 0.040$) the efficiency of milk production and tended to reduce ($P = 0.067$) the BW of cows (Table 5). In addition, cows fed EO and EOA had the same efficiency of milk yield as cows fed MON.

Experiment 2: Randomized Complete Block Design

The EOA treatment tended to increase ($P \leq 0.066$) DMI and OM intake in comparison with EO (Table 6).

No treatment × time effect was detected on parameters evaluated. Although milk yield was similar among treatments, milk content of lactose and protein were lesser ($P \leq 0.039$) in cows fed diets containing EO or EOA than those fed MON (Table 7). Compared with EO alone, EOA increased ($P \leq 0.032$) milk protein yield and content and milk lactose content. No treatment effects were observed on blood metabolites.

DISCUSSION

In general, feed additives (MON, EO, and EOA) promoted slight changes in total-tract nutrient digestibility (tended to increase DM digestibility and CP digestibility) without altering DMI of cows. However, the most interesting findings of this study were observed in

Table 4. Nitrogen utilization and microbial protein synthesis in mid-lactation cows fed a blend of essential oils and amylase (experiment 1: Latin square design)

Item	Treatment ¹					P-value ²				
	CON	MON	EO	EOA	SEM	Treatment	C1	C2	C3	
N intake, g/d	718	688	714	696	17.8	0.524	0.329	0.406	0.450	
N balance, g/d	184	187	217	200	11.8	0.083	0.120	0.073	0.229	
Fecal N, g/g of N intake	0.366	0.329	0.326	0.333	0.0088	0.274	0.057	0.980	0.734	
Milk N, g/g of N intake	0.231	0.252	0.236	0.250	0.0071	0.013	0.016	0.141	0.046	
Urinary N, g/g of N intake	0.150	0.155	0.134	0.137	0.0050	0.263	0.463	0.077	0.861	
Microbial protein synthesis, kg/d	2.08	2.17	2.01	2.06	110	0.853	0.994	0.410	0.796	

¹CON = no feed additive (control); MON = sodium monensin added at 13 mg/kg (Rumensin, Elanco, São Paulo, Brazil); EO = blend of essential oils supplemented at 44 mg/kg (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); EOA = provision of essential oils and amylase (330 kilo novo units/kg; Ronozyme Rumistar, DSM Produtos Nutricionais Brasil S.A.).

²Orthogonal contrasts: C1 = CON vs. feed additives (MON + EO + EOA); C2 = MON vs. EO + EOA; C3 = EO vs. EOA.

Table 5. Milk yield and composition, BW, and BCS of mid-lactation cows fed a blend of essential oils and amylase (experiment 1: Latin square design)

Item	Treatment ¹				SEM	P-value ²			
	CON	MON	EO	EOA		Treatment	C1	C2	C3
Yield, kg/d									
Milk	34.6	35.9	35.0	35.3	0.74	0.300	0.156	0.220	0.703
FCM ³	32.8	33.9	32.4	34.0	0.78	0.513	0.548	0.533	0.221
Lactose	1.56	1.63	1.57	1.61	0.034	0.389	0.294	0.288	0.376
Fat	1.10	1.08	1.07	1.16	0.039	0.665	0.894	0.647	0.258
Protein	1.04	1.09	1.05	1.07	0.023	0.312	0.251	0.233	0.355
Milk composition, g/kg									
Lactose	45.3	45.4	45.5	45.6	0.28	0.693	0.438	0.408	0.727
Fat	32.3	31.6	31.2	32.3	0.85	0.807	0.591	0.880	0.429
Protein	30.2	30.3	30.4	30.4	0.18	0.639	0.262	0.713	0.627
Efficiency ⁴	1.28	1.38	1.31	1.36	0.040	0.074	0.040	0.242	0.194
BW, kg	602	600	596	598	16.8	0.520	0.067	0.921	0.157
BCS	2.53	2.53	2.50	2.50	0.009	0.371	0.317	0.161	0.231

¹CON = no feed additive (control); MON = sodium monensin added at 13 mg/kg (Rumensin, Elanco, São Paulo, Brazil); EO = blend of essential oils supplemented at 44 mg/kg (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); EOA = provision of essential oils and amylase (330 kilo novo units/kg; Ronozyme Rumistar, DSM Produtos Nutricionais Brasil S.A.).

²Orthogonal contrasts: C1 = CON vs. feed additives (MON + EO + EOA); C2 = MON vs. EO + EOA; C3 = EO vs. EOA.

³3.5% FCM = $(0.432 + 0.165 \times \% \text{ fat in milk}) \times \text{milk yield (kg/d)}$ according to Sklan et al. (1992).

⁴Efficiency = milk yield (kg/d)/DMI (kg/d).

ruminal fermentation wherein feed additives increased acetate, butyrate, and branched-chain fatty acid concentrations. In addition, feed additives containing EO (EO + EOA) tended to increase or increased total VFA, acetate, propionate, butyrate, and branched-chain fatty acid concentrations in the rumen of cows. Because of these effects, treatments containing EO improved the feed efficiency and N utilization of cows.

In agreement with the current study, other authors reported no effects on DMI and total apparent digestibility when supplementing MON (Ferreira de Jesus et al., 2016; Vendramini et al., 2016), EO (Benchaar et al., 2006, 2007; Vendramini et al., 2016), or products containing amylase (Nozière et al., 2014; Takiya et al., 2017) to lactating dairy cows. On the other hand, exogenous amylase has increased feed efficiency in dairy

Table 6. Nutrient intake and total apparent digestibility in mid-lactation cows fed a blend of essential oils and amylase (experiment 2: randomized complete block design)

Item	Treatment ¹				P-value ²				
	MON	EO	EOA	SEM	Treatment	Time	Treatment × time	C1	C2
Intake, kg/d									
DM	22.8	22.4	23.6	0.31	0.157	<0.001	0.139	0.716	0.062
OM	20.9	20.6	21.7	0.28	0.164	<0.001	0.122	0.698	0.066
Starch	9.71	9.85	10.0	0.147	0.625	0.746	0.794	0.732	0.604
NDF	6.41	6.42	6.60	0.093	0.558	<0.001	0.607	0.573	0.370
CP	3.74	3.78	3.86	0.060	0.658	0.868	0.642	0.496	0.567
Indigestible ADF	1.69	1.68	1.71	0.026	0.862	0.009	0.498	0.814	0.633
Ether extract	0.90	0.91	0.93	0.014	0.637	0.605	0.667	0.444	0.605
Digestibility coefficient									
DM	0.680	0.689	0.690	0.0045	0.581	0.005	0.512	0.302	0.968
OM	0.700	0.711	0.710	0.0049	0.579	0.060	0.361	0.301	0.971
Starch	0.843	0.858	0.856	0.0057	0.480	0.026	0.141	0.235	0.864
NDF	0.466	0.473	0.481	0.0084	0.703	0.030	0.760	0.475	0.666
CP	0.679	0.680	0.680	0.0063	0.999	0.009	0.607	0.974	0.992
Ether extract	0.831	0.839	0.844	0.0066	0.743	0.057	0.968	0.476	0.782

¹CON = no feed additive (control); MON = sodium monensin added at 13 mg/kg (Rumensin, Elanco, São Paulo, Brazil); EO = blend of essential oils supplemented at 44 mg/kg (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); EOA = provision of essential oils and amylase (330 kilo novo units/kg; Ronozyme Rumistar, DSM Produtos Nutricionais Brasil S.A.).

²Time: weeks. Orthogonal contrasts: C1 = MON vs. EO + EOA; C2, = EO vs. EOA.

cows, but differences were attributed to decreased DMI while maintaining milk production (Gencoglu et al., 2010; Ferraretto et al., 2011). Essential oils either tended to improve or improved the feed efficiency with no effects on DMI in early-lactating cows (Tassoul and Shaver, 2009; Drong et al., 2016). Thus, it is reasonable that either EO or EOA would increase feed efficiency of dairy cows. Positive responses to dietary additives, such as enzymes and EO, are more prone to be observed in situations in which fiber digestibility is impaired or when energy is the first limiting nutrient to milk production, as occur during early lactation (Beauchemin et al., 2003).

Feed additives decreased $\text{NH}_3\text{-N}$ concentration in the rumen, especially due to the lowest value of ruminal $\text{NH}_3\text{-N}$ concentration in cows treated with MON. Earlier studies indicated that MON decreases AA deamination and consequently reduces $\text{NH}_3\text{-N}$ accumulation in the rumen (Yang and Russell, 1993). In addition, authors demonstrated that MON inhibits hyperammonia-producing bacteria (Ruiz et al., 2001). The blend of EO supplied to cows in the current experiment also has been associated with decreases in ruminal deamination. McIntosh et al. (2003) reported that pure cultures of hyperammonia-producing bacteria (i.e., *Clostridium sticklandii* and *Peptostreptococcus anaerobius*) are very sensitive to EO. Giannenas et al. (2011) also found a decrease in hyperammonia-producing bacteria counts in dairy ewes fed EO. However, there was a tendency in the EOA group to accumulate less $\text{NH}_3\text{-N}$ in the ru-

men compared with cows fed EO. The explanation for the latter outcome is not clear because products with α -amylase activity have not affected ruminal $\text{NH}_3\text{-N}$ concentration (DeFrain et al., 2005; Tricarico et al., 2005; Nozière et al., 2014; Takiya et al., 2017). Rather than a decrease in deamination, EOA treatment could increase $\text{NH}_3\text{-N}$ utilization by fibrolytic bacteria or increase the substrate available for microbial protein synthesis, hence turning it more efficient. Tricarico et al. (2008) added α -amylase to a culture medium rich in starch and observed rapid growth of *Butyrivibrio fibrisolvens* D1, which have high fibrolytic activity.

Indeed, dietary additives affected ruminal fermentation by increasing all VFA concentration in the rumen (with the exception of propionate), suggesting an increase in ruminal digestibility of nutrients. To the best of our knowledge, no in vivo study has evaluated the effects of EO on ruminal digestibility of nutrients in dairy cows. Cows fed treatments containing EO had the lowest ruminal pH values, which is associated with the highest values of ruminal total VFA concentrations. The effects of EO on total VFA concentration in ruminal fluid have been inconsistent in in vitro and in vivo studies (Castillejos et al., 2005; Benchaar et al., 2007; Vendramini et al., 2016). Castillejos et al. (2005) observed an increase in the total VFA production using a continuous culture with a substrate containing forage:concentrate ratio of 60:40 (alfalfa silage as forage source) and EO. Benchaar et al. (2007) found higher values of total VFA production when providing EO

Table 7. Milk yield and composition and blood metabolites in mid-lactation cows fed a blend of essential oils and amylase (experiment 2: randomized complete block design)

Item	Treatment ¹				P-value ²				
	MON	EO	EOA	SEM	Treatment	Time	Treatment × time	C1	C2
Yield, kg/d									
Milk	31.5	31.1	31.9	0.71	0.734	<0.001	0.812	0.985	0.438
FCM ³	32.0	30.8	31.9	0.75	0.477	<0.001	0.862	0.498	0.293
Lactose	1.48	1.41	1.48	0.03	0.126	<0.001	0.971	0.321	0.067
Fat	1.11	1.07	1.11	0.032	0.617	<0.001	0.612	0.604	0.400
Protein	0.99	0.93	0.99	0.019	0.063	<0.001	0.880	0.273	0.032
Milk composition, g/kg									
Lactose	46.4	45.7	46.2	0.11	<0.001	0.007	0.613	<0.001	<0.001
Fat	35.7	34.5	34.9	0.88	0.653	0.001	0.262	0.392	0.757
Protein	31.0	30.3	31.0	0.16	0.003	<0.001	0.405	0.039	0.003
Efficiency ⁴	1.40	1.43	1.35	0.02	0.190	<0.001	0.017	0.804	0.077
Blood glucose, mg/dL	41.2	41.1	41.7	0.64	0.941	<0.001	0.341	0.915	0.743
Blood urea, mg/dL	30.2	31.9	29.3	0.71	0.941	0.148	0.827	0.833	0.226

¹CON = no feed additive (control); MON = sodium monensin added at 13 mg/kg (Rumensin, Elanco, São Paulo, Brazil); EO = blend of essential oils supplemented at 44 mg/kg (Crina Ruminants, DSM Produtos Nutricionais Brasil S.A., São Paulo, Brazil); EOA = provision of essential oils and amylase (330 kilo novo units/kg; Ronozyme Rumistar, DSM Produtos Nutricionais Brasil S.A.).

²Time: weeks. Orthogonal contrasts: C1 = MON vs. EO + EOA; C2, = EO vs. EOA.

³3.5% FCM = $(0.432 + 0.165 \times \% \text{ fat in milk}) \times \text{milk yield (kg/d)}$, according to Sklan et al. (1992).

⁴Efficiency = milk yield (kg/d)/DMI (kg/d).

to cows fed alfalfa silage as a forage source, but they reported a slight decrease in VFA production when providing EO to cows fed corn silage as a dietary forage source. One possible reason for trends toward a greater ruminal concentration of total VFA and butyrate in cows fed EO (besides the increase of ruminal digestibility of nutrients) is that branched-chain fatty acids can stimulate cellulolytic bacteria activity (Buttery and Foulds, 1985), which could favor acetate and butyrate fermentation pathways. Combining amylase with EO seems to not have any beneficial effect on ruminal fermentation because similar values of VFA concentration and VFA molar proportions were found in cows fed EO or EOA. Few studies assessed the ruminal fermentation of cows fed amylase derived from *B. licheniformis*, but amylase has increased the propionate molar proportion in first-lactation cows fed high-starch diets (Nozière et al., 2014), contrasting with what was observed in the current study.

Feed additives tended to decrease the fecal N excretion and improved N transfer into milk. These outcomes can be explained by the lower $\text{NH}_3\text{-N}$ accumulation in the rumen and the trend toward a greater CP digestibility in cows fed feed additives. Low efficiencies of dietary N utilization for milk protein synthesis are partially related to $\text{NH}_3\text{-N}$ losses in the rumen (Tamminga, 1992). In the current experiment, feed additives increased the concentration of several VFA, which indicates a greater energy availability for $\text{NH}_3\text{-N}$ uptake by the rumen bacteria. Because rumen microbes largely depend on $\text{NH}_3\text{-N}$ to synthesize protein (Hristov and Broderick, 1996), enhancing microbial protein synthesis would promote N transfer into milk.

In experiment 1, treatments had no effect on milk yield and composition of dairy cows, but feed additives increased the efficiency of milk production. Feed additives tended to increase DM digestibility while presenting similar DMI in relation to CON, which partially explains the increased efficiency of milk yield in cows. In addition, the greater concentrations of acetate and butyrate in the rumen suggest an increase of DM degradation in the rumen. Interestingly, EO and EOA showed similar outcomes for milk production efficiency compared with MON in both experiments. The positive effects of MON on milk production efficiency in dairy cows is supported by Duffield et al. (2008), who performed a meta-analysis from 77 trials and reported an increase of 2.5% on milk production efficiency (energy basis) of cows fed MON. The milk production outcomes when feeding EO are inconsistent. For instance, Freire et al. (2017) reported an increase in FCM and ECM production of cross-breed cows supplemented with EOA. Contrasting with the current experiment,

Khiaosa-ard and Zebeli (2013) reported no effect of EO supplementation to dairy cows on milk production efficiency after analyzing data from 15 experiments with different blends of EO or a single extract. In experiment 2, treatments containing EO decreased milk lactose and protein content in relation to MON, whereas EOA improved the milk lactose and protein content in relation to EO. The reasons for differences in milk lactose content are not clear because ruminal propionate and blood glucose concentrations were not affected by treatments. The differences in milk protein content are likely related to ruminal $\text{NH}_3\text{-N}$ concentration outcomes.

CONCLUSIONS

This study demonstrated the potential effects of feed additives (MON, EO, and EOA) on rumen fermentation, whereas the combination of EO and α -amylase increased ruminal concentration of acetate, butyrate, and branched-chain fatty acids while decreasing ruminal concentration of $\text{NH}_3\text{-N}$. Further, EOA improved N transfer into milk compared with EO alone but had small effects on milk production and composition of cows. Milk production efficiency was improved by feed additives, and the second experiment demonstrated no differences in milk production efficiency among them. Either EO or EOA can replace MON without decreasing animal performance. There is no evidence that EO along with amylase has a positive synergistic effect on milk yield, but EOA may increase milk protein content and production.

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