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Essential Oil Blends with or without Fumaric Acid Influenced In Vitro Rumen Fermentation, Greenhouse Gas Emission, and Volatile Fatty Acids Production of a Total Mixed Ration

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Abstract: The growing interest in improving rumen fermentation and mitigating methane emissions necessitates the use of essential oil blends (EOB) and fumaric acid (FA). This study evaluated the synergistic effect of four EOB with or without FA supplementation on in vitro dry matter digestibility, greenhouse gas emission, and total volatile fatty acid production using inoculum from three rumen-cannulated Black Angus beef cows. The study was arranged in a 4 × 2 + 1 factorial design to evaluate the effects of the four EOB and two FA levels on a total mixed ration (TMR). The EOB dosage was 100 µL while FA was added at 3% of total mixed ration. The EOB × FA interaction ($p < 0.05$) influenced the dry matter, neutral detergent fiber, and hemicellulose degradabilities. All the EOB and FA (EFA) treatments decreased ($p < 0.001$) the dry matter degradability compared to the control (TMR substrate only). The EFA4 treatment reduced the neutral detergent fiber and hemicellulose degradabilities compared to the control. The ruminal pH was influenced ($p < 0.001$) by both the EOB and FA inclusion, and the EOB × FA interaction was significant. The microbial mass was higher ($p < 0.001$) in the EFA1, EFA4, and EOB4 compared to the control and the EOB3 treatments. The EFA1 and EOB1 produced less ($p < 0.001$) gas than the control by 29.1 and 32.1%, respectively. Compared with the control, the EFA1 and EOB1 treatments decreased ($p < 0.001$) methane gas by 90.8% and 86.4%, respectively, while the carbon dioxide was reduced ($p = 0.004$) by 65.7 and 57.9%, respectively. The EOB × FA interaction was significant ($p < 0.001$) for the total and individual volatile fatty acid concentrations. The inclusion of FA increased the propionate concentration by 9.5% and decreased ($p = 0.02$) the acetate concentration by 4%. In summary, the synergistic effect of the EOB and FA offers an effective way to reduce greenhouse gas emission and enhance total volatile fatty acids.

Keywords: essential oils; fumarate; ruminants; methane; batch culture

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1. Introduction

Ruminants could utilize high fibrous feed resources by relying on the rumen microbiome to extract the nutrients. Through the fermentation processes, plant lignocellulosic materials are degraded by anaerobic digestion to yield volatile fatty acids (VFA) and other methanogenic products such as hydrogen, carbon dioxide (CO_2), hydrogen sulfide (trace amount), and acetic acid [1]. Thus, ruminants have been implicated to contribute 14.5% of anthropogenic greenhouse gas (GHG) emissions toward global warming and climate change [2]. Of greater interest are methane (CH_4) and CO_2, because methanogens can reduce the CO_2 generated during anerobic digestion to CH_4 through the hydrogenotrophic methanogenesis pathway. Besides, CH_4 gas accounts for up to 12% of dietary gross energy, and its global warming potential surpasses that of CO_2 by over 28 times [1,3]. In view of

this, reducing the GHG emissions from ruminant animals without compromising their production performance is a high-priority challenge that must be addressed. Nutritional interventions for reducing the enteric CH_4 and CO_2 emission include adding dietary lipids or various feed additives to the ration [4]. Essential oils (EO) are a natural source of plant secondary metabolites and bioactive compounds, with several antimicrobial properties that hold potential to modify rumen microbiome including bacteria, protozoa, and fungi [5,6]. They influence ruminal metabolic activity, reduce methane emission, and inversely increase the molar proportion of propionate [7]. Since EO vary in their chemical structure and bioactive constituents, combining two or more single EO to form a unique EO blend (EOB), or a combination of EO with other anti-methanogenic agents, is presumed to be effective in mitigating the methane emission from ruminants [8]. The combinations of various EOB were found to modify the rumen fermentation processes in vitro [9], in vivo [10], or both [5] with promising results. Blanch et al. [5] reported that 300 mg/L of essential oil blend (Next Enhance®; NE300) containing cinnamaldehyde and garlic oils, reduced the total gas, CH_4 emission, and VFA profile and increased the propionate proportion.

Fumaric acid (FA) is a key metabolic intermediate of the propionate–succinate pathway, which is recognized to enhance ruminal propionate production by scavenging the hydrogen available for methanogens, thereby offering a potent means to reduce CH_4 emissions [4]. Previous studies [3,11] have reported a reduction in CH_4 production, total VFA, and acetate-to-propionate ratio, while propionate increased with FA supplementation. Based on the prospects of EOB and FA, it was hypothesized that combinations of EO and FA would synergistically improve feed digestion and reduce GHG emission. Furthermore, there is a paucity of available literature on the synergy of EOB containing a mixture of four or more individual EO and FA on in vitro rumen fermentation. Lin et al. [7] reported that the addition of FA and a mixture of essential oils (clove, oregano, cinnamon, and lemon) or their bioactive substances, decreased ammonia nitrogen concentration, total VFA content, acetate-to-propionate ratio, increased propionate proportion, and inhibited the growth of methanogens and protozoa. In the present study, twelve EO with different bioactive substances were mixed to form four unique EOB based on a previous study from our lab, which showed that certain EO consistently reduced GHG without a significant negative effect on nutrient digestibility [9]. The study hypothesis was that the synergistic effects of EOB with or without FA supplementation would positively influence the ruminal fermentation profile and improve the nutrient degradation. Hence, the study was conducted to investigate the effects of EOB, with or without FA, on CH_4 and CO_2 production, fermentation characteristics, gas production, and VFA concentrations.

2. Materials and Methods

2.1. Study Ethical Approval

The study protocol for using essential oil blends and cannulated cows as a rumen liquid donor was approved by the Institutional Animal Care and Use Committee (LA22-0019), North Carolina A&T State University, Greensboro. The cannulated cows were maintained according to the University Farm standards.

2.2. Test Ingredients

A total of twelve individual, commercially available EO were used. Four different EOBs were formulated as follows: EOB1 [garlic, lemongrass, cumin, lavender, and nutmeg; 4:2:2:1:1]; EOB2 [anise, clove, oregano, cedarwood, and ginger; 4:2:2:1:1]; EOB3 [clove, oregano, peppermint, and eucalyptus; 3:3:2:2]; and EOB4 [clove, anise, peppermint, and oregano; 4:3:2:1]. The proportion of each essential oil used in the blends was based on a previous study from our laboratory [9]. Fumaric acid (99+%) purchased from Thermo Fisher Scientific, Branchburg, NJ, USA was used in this study. Samples of corn silage, alfalfa hay, and concentrate were obtained from the North Carolina A&T State University Farm. Samples were oven-dried (Isotemp Oven, Thermo Fisher Scientific, Allentown, PA, USA) and milled through a 1-mm screen (Cutting Mill SM100, Retsch GmbH, Haan, Germany).

A total mixed ration (TMR) formulated consisted of 60% corn silage, 20% alfalfa hay, and 20% concentrate on a dry matter (DM) basis was used as the substrate for the in vitro batch culture study. Three rumen-cannulated Black Angus beef cows (1506 ± 70 kg) were used as rumen inoculum donors. They were maintained on pasture, and fed with grass hay and a mineral mixture as supplement.

2.3. Chemical Analysis

The chemical composition of each ingredient (corn silage, alfalfa hay, and concentrate) and TMR formulated were determined using standard procedures [12]. The dry matter (DM, #930.15) was determined by oven drying at 55 °C to constant weight (Thermo Scientific Heratherm OGS100, Thermo Electron LED GmbH, Langenselbold, Germany). Nitrogen (N, #954.01) was quantified using an organic elemental analyzer (2400 CHNS, PerkinElmer, Waltham, MA, USA) and crude protein (CP) was calculated as N × 6.25. Ether extract (EE; #920.39) was determined using the ANKOM XT15 (ANKOM, Macedon, NY, USA) extractor. The ash content (CA, #942.05) was determined by combustion of samples in a muffle furnace at 550 °C (Lindberg Blue M, Thermo Fisher Scientific, Pittsburgh, PA, USA). The organic matter (OM) was determined by subtracting the weight of the ash after ignition and reported in percentage. The neutral detergent fiber (NDF) was determined [13] with modifications for using thermo-stable α-amylase and sodium sulfite; and the acid detergent fiber (ADF; #973.18) content was analyzed using automated the ANKOM fiber analyzer (F57 Fiber Filter Bags, 200 Fiber Analyzer, ANKOM Technology). To obtain ADL, the cellulose was removed from the ADF by soaking with concentrated H_2SO_4 based on the ANKOM Technologies analytical methods. The chemical composition of the ingredients and substrate are presented in Table 1 below.

Table 1. Chemical composition (% dry matter) of ingredients and total mixed ration *.

	Corn Silage	Alfalfa Hay	Concentrate	Total Mixed Ration
Dry Matter	37.0	82.8	89.6	67.8
Organic matter	96.5	91.0	83.3	93.0
Crude Protein	6.72	17.0	16.6	13.6
Crude Fat	4.63	3.26	8.62	4.89
Ash	3.52	9.03	16.7	7.03
NDF	58.9	49.7	74.4	61.9
ADF	14.4	9.35	15.2	11.7
ADL	10.4	18.2	10.4	13.7

* n = 10 replicates; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin.

2.4. In Vitro Batch Culture Incubation

The in vitro batch culture assay followed the standard procedures from our lab [9]. The study was arranged as a 4 × 2 + 1 factorial design to evaluate the effects of the four EOB and two FA levels (with or without). The nine treatments were designated as follows: EOB1 with FA (EFA1); EOB2 with FA (EFA2); EOB3 with FA (EFA3); EOB4 with FA (EFA4); EOB1 without FA (EOB1); EOB2 without FA (EOB2); EOB3 without FA (EOB3); EOB4 without FA (EOB4); and control (TMR substrate only). The EOB dosage was 100 µL while the FA was added at 3% of the TMR. The ruminal contents were obtained from the various rumen regions of three ruminally cannulated Black Angus beef cows. It was strained through four layers of cheesecloth into an insulated thermos and transported immediately to the Ruminant Nutrition Laboratory. The buffer was prepared according to McDougall's recipe and maintained in a water bath at 39 °C until dispensed into 100 mL serum bottles. Samples (500 ± 50 mg) of the substrate were weighed into ANKOM filter bags (ANKOM Technology Corp., Macedon, NY, USA) and placed in the serum bottle for dry matter and fiber degradation, while some samples were weighed directly into the serum bottles for estimation of the microbial mass and the efficiency of microbial production. Six bottles with no substrate were also included as blank. The rumen fluid was mixed with the buffer

solution in a proportion of 1:3 (vol/vol) at 39 °C under continuous flushing with CO_2. Sixty milliliters of buffered medium were added anaerobically to each serum bottle. The bottles were sealed immediately with a 14 mm butyl rubber stopper plus aluminum crimp cap and incubated at 39 °C for 24 h on an orbital shaker at a speed of 125 rpm. The incubation was repeated on a different date to make two runs, with nine replicates per run.

2.5. Sampling and Analyses of Gas Production and Digestibility

After 6 and 24 h of incubation, all the culture serum bottles were measured by inserting a 22 mm gauge needle attached to a manometer (VWR International, Randor, PA, USA) to determine the gas pressure, which was later used to estimate the total gas production. The concentrations of methane, ammonia, carbon dioxide, and hydrogen sulfide gases were determined using a portable gas analyzer (Biogas 5000, Landtec, Dexter, MI, USA) [9]. Thereafter, samples of the ruminal liquid contained in each bottle were collected to determine the pH values (Fisherbrand™ FE150 pH benchtop meter, Fisher Scientific, Waltham, MA, USA). Then, the ANKOM bags were removed from the bottles, rinsed, and dried in an oven [55 °C; 48 h] to determine the apparent DM degradability. The NDF, ADF, and ADL content of the residues in the fiber bags were also determined. The in vitro apparent degradable dry matter (IVADDM) was derived from the microbial mass data, and the in vitro true degradable dry matter (IVTDDM) was estimated after 24 h of incubation.

2.6. Estimation of Microbial Mass

The microbial mass was determined according to the protocol described [9,14]. The contents from the serum bottles (n = 3 per treatment per run) were transferred into pre-weighed centrifuge tubes (Thermo Fisher Scientific, Rochester, NY, USA) after they had been incubated for 24 h. As a correction factor, blanks (bottles without substrate during incubation) were also included in the process. The samples were centrifuged at 15,000× g for 15 min at 4 °C. Afterward, the supernatant was decanted, and the centrifuged substrate was kept in a freezer for 24 h. The frozen samples were freeze dried for 72 h using a freeze dryer (L-200, BÜCHI Lyovapor, New Castle, DE, USA). The tubes were then reweighed, and the microbial mass was calculated as: Feed (DM) incubated − [pellet (DM) − blank pellet (DM)]/Feed (DM) incubated.

2.7. Ammonia Nitrogen Determination

The ammonia nitrogen (NH_3-N) contents in the ruminal liquid were determined by the Kjeldahl method. After 24 h of incubation, 25 mL of ruminal liquid from each bottle was collected into 5 mL diluted H_2SO_4 (72%) and stored in −20 °C prior to analysis. The samples were thawed, added to 50 mL of NaOH (32%), and distilled into a 25 mL boric acid indicator using a BÜCHI Distillation Unit (K-355, BÜCHI Lyovapor, New Castle, DE, USA). The distillate was then titrated against diluted HCl (0.1N) until it changed back to its original color.

2.8. Volatile Fatty Acid Analysis

After 24 h of incubation, samples of the ruminal liquid (15 mL) were collected from six bottles per treatment, preserved with 3 mL of 25% (wt/wt) metaphosphoric solution, and immediately frozen at −20 °C for the VFA determination. The VFA profile in the ruminal liquid was quantified by using gas chromatography (Agilent 7890B GC system/5977B GC-MSD/7693 autosampler, Agilent Technologies, Santa Clara, CA, USA) with a capillary column (Zebron ZB-FFP, Phenomenex Inc., Torrance, CA, USA) as previously described [14]. A metaphosphoric acid and crotonic acid (trans-2-butenoic acid) mixture was used as the internal standard, while acetic, propionic, butyric, isobutyric, valeric, and isovaleric acid were used as quantitative external standards [13,15].

2.9. Statistical Analysis

Data generated on nutrient degradability, greenhouse gas production, fermentation parameters, and volatile fatty acids concentration were analyzed using the General Linear Model in a 4 × 2 + 1 factorial arrangement (SAS 9.4 version; SAS Institute Inc., Cary, NC, USA). The treatments were 4 EOB with or without fumaric acid. The main effects of EOB and FA treatments and their interactions were examined. Significant means were separated at $p \leq 0.05$ using the Duncan multiple range test.

Data were analyzed using the model below:

$$Yijk = \mu + EOBi + FAj + (EOB \times FA)ij + eijk$$

where Yijk is the dependent variable, μ is the overall mean, EOBi is the essential oil blend effect, FAk is the fumaric acid effect, (EOB × FA)ij is the interaction effect between the essential oil blend and fumaric acid, and eijk is the residual error.

3. Results

3.1. Dry Matter and Fiber Fractions Degradability

The interaction effects of the EOB and FA significantly ($p < 0.01$) influenced dry matter degradability (DMD), NDF degradability (NDFD), and hemicellulose degradability (HEMD; Table 2). All the EOB, either with or without FA, decreased ($p < 0.001$) DMD compared to the control. Significantly lower ($p < 0.05$) DMD, NDFD, and HEMD values were observed in the EFA4 treatment. The inclusion of FA reduced ($p = 0.028$) DMD by 6.2% while NDFD, ADFD, ADLD, HEMD, and cellulose degradability were not affected.

Table 2. Effects of essential oil blends with or without fumaric acid on dry matter and fiber fractions degradability of the total mixed ration.

Treatments	DMD (%)	NDFD (%)	ADFD (%)	ADLD (%)	HEMD (%)	CELD (%)
Control	52.99 ^{a}	70.72 ^{a}	56.03	19.78	14.70 ^{a}	36.25
EFA1	39.47 ^{bc}	67.72 ^{ab}	55.17	17.74	12.61 ^{ab}	37.37
EFA2	40.35 ^{bc}	67.37 ^{ab}	54.40	17.80	12.97 ^{ab}	36.60
EFA3	38.22 ^{bc}	68.19 ^{ab}	55.94	19.26	12.25 ^{ab}	36.68
EFA4	36.80 ^{c}	66.23 ^{b}	56.30	17.97	9.94 ^{b}	38.33
EOB1	38.89 ^{bc}	68.46 ^{ab}	56.96	19.09	11.50 ^{ab}	37.87
EOB2	42.57 ^{bc}	69.53 ^{ab}	55.71	19.29	13.82 ^{ab}	36.41
EOB3	44.03 ^{b}	70.66 ^{a}	56.06	17.46	14.60 ^{a}	38.60
EOB4	39.51 ^{bc}	66.62 ^{ab}	55.38	20.37	11.24 ^{ab}	35.01
SEM	0.769	0.357	0.203	0.380	0.352	0.401
p value						
EOB	0.1370	0.0353	0.3582	0.9066	0.0152	0.7103
FA	0.0280	0.0543	0.1845	0.3142	0.2561	0.7603
EOB × FA	<0.001	0.0085	0.1539	0.6575	0.0116	0.5303

DMD, Dry matter degradability; NDFD, Neutral detergent fiber degradability; ADFD, Acid detergent fiber degradability; ADL, Acid detergent lignin degradability; HEMD, Hemicellulose degradability; CELD, Cellulose degradability; SEM, Standard error of means; $^{a–c}$ Means with different superscripts within the same column differ, $p < 0.05$.

3.2. In Vitro Digestibility and Fermentation Parameters

The interaction of the EOB and FA influenced ($p < 0.001$) the pH, undegraded DM, IVADDM, IVTDDM, partitioning factor (PF), and microbial mass (Table 3). The pH, which ranged from 6.61 to 6.69, was influenced ($p < 0.001$) by both the EOB and FA inclusion. Undegraded DM values were significantly ($p < 0.001$) higher in the treatments tested than in the control but were similar among the EOB and EFA treatments. Treatments EFA1, EFA4, and EOB4 decreased the IVADDM ($p < 0.001$) by almost 33% compared to control. All the EOB decreased ($p < 0.001$) the IVTDDM compared to the control. The EOB1 and EOB4 treatments had higher ($p < 0.001$) PF compared with EFA4. Higher ($p < 0.001$)

microbial mass was noted for the EFA1, EFA4, and EOB4 compared to the control and EOB3 treatments. The NH_3-N concentration was significantly influenced ($p < 0.001$) by the FA addition and the EOB × FA interaction. All the EOBs and EFAs decreased ($p < 0.001$) NH_3-N compared to the control. Meanwhile, EFA2 resulted in a 26% decrease in the NH_3-N content in relation to the control. Also, FA inclusion reduced ($p < 0.001$) the ruminal NH_3-N content by nearly by 31%.

Table 3. Effects of essential oil blends with or without fumaric acid on in vitro digestibility and fermentation parameters of the total mixed ration.

Treatments	pH	Undegraded DM	IVADDM	IVTDDM	PF	Mmass (g/kg DM)	NH_3-N (mg/dL)
Control	6.64 ^{b}	0.159 ^{b}	0.592 ^{a}	0.693 ^{a}	2.57 ^{ab}	0.065 ^{cd}	16.14 ^{a}
EFA1	6.61 ^{d}	0.184 ^{a}	0.391 ^{d}	0.636 ^{b}	2.74 ^{ab}	0.124 ^{a}	12.21 ^{ef}
EFA2	6.64 ^{b}	0.182 ^{a}	0.450 ^{bcd}	0.644 ^{b}	2.62 ^{ab}	0.099 ^{abc}	11.88 ^{f}
EFA3	6.62 ^{cd}	0.189 ^{a}	0.523 ^{ab}	0.628 ^{b}	2.76 ^{ab}	0.054 ^{d}	12.61 ^{def}
EFA4	6.64 ^{bc}	0.186 ^{a}	0.393 ^{d}	0.631 ^{b}	2.29 ^{b}	0.120 ^{a}	13.73 ^{bcd}
EOB1	6.64 ^{bc}	0.181 ^{a}	0.423 ^{cd}	0.644 ^{b}	2.99 ^{a}	0.113 ^{ab}	14.01 ^{bc}
EOB2	6.69 ^{a}	0.183 ^{a}	0.494 ^{bc}	0.640 ^{b}	2.85 ^{ab}	0.074 ^{bcd}	14.06 ^{bc}
EOB3	6.62 ^{bcd}	0.178 ^{a}	0.498 ^{bc}	0.647 ^{b}	2.65 ^{ab}	0.076 ^{bcd}	14.85 ^{b}
EOB4	6.69 ^{a}	0.183 ^{a}	0.399 ^{d}	0.636 ^{b}	2.96 ^{a}	0.120 ^{a}	13.39 ^{cde}
SEM	0.004	0.0014	0.0106	0.0029	0.034	0.0045	0.217
p value							
EOB	<0.001	0.8969	<0.001	0.3819	0.3646	<0.001	0.5107
FA	0.0001	0.0901	0.4328	0.0601	0.0363	0.7162	<0.001
EOB × FA	<0.001	<0.001	<0.001	<0.001	0.0305	<0.001	<0.001

IVADDM, In vitro apparent degradable dry matter; IVTDDM, In vitro true degradable dry matter; PF, Partitioning factor; Mmass, Microbial mass; SEM, Standard error of means; $^{a–f}$ Means with different superscripts within the same column differ, $p < 0.05$.

3.3. Gas Production and GHG Emissions

The effects of the EOBs and FA on the total gas production, methane, carbon dioxide, ammonia (NH_3), and hydrogen sulfide (H_2S) are presented in Table 4. The interaction of the EOB and FA produced a significant ($p < 0.001$) effect on total gas production, CH_4, CO_2, NH_3, and H_2S concentrations. The EFA1 and EOB1 treatments produced less ($p < 0.001$) gas than the control by 29.1% and 32.1%, respectively. The EOB with or without FA significantly ($p < 0.001$) reduced the CH_4 and CO_2 gases. Compared with control, the EFA1 and EOB1 treatments decreased ($p < 0.001$) the CH_4 gas by nearly 90.8% and 86.4%, respectively, while the CO_2 was reduced ($p = 0.004$) by approximately 65.7% and 57.9%, respectively. Both the EFA4 and EOB4 treatments had lower ($p < 0.001$) NH_3 gas compared with the other treatments, whereas the EOB4 produced the least ($p < 0.001$) H_2S gas. Among the EOB group, the EOB3 had the highest ($p < 0.001$) gas volume, while the EOB1 had the least gas volume, both at the 6 and 24 h post incubations. The inclusion of the FA e increased ($p < 0.001$) the gas production only at 6 h. EOB1 significantly ($p < 0.001$) reduced thCH_4 by 79–85% and CO_2 by 36–53% compared to other blends. However, the EOB4 had lower NH_3 (60–68%) and H_2S (79–92%) concentrations when compared to the other EO blends.

3.4. Volatile Fatty Acids Production

The effects of the EOB and FA on the total and molar proportions of the volatile fatty acid production are shown in Table 5. The interactions between the EOB and FA were significant ($p < 0.001$) for the total volatile fatty acid (TVFA), acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and acetate-to-propionate ratio (APR). The EFA1 increased ($p < 0.001$) the butyrate concentration by 80.7% compared to the control. The isobutyrate concentration was reduced ($p < 0.001$) in all the EOB with or without FA when compared with the control. The EFA1 increased ($p < 0.001$) the valerate concentration compared to the rest of treatments. EFA4 increased the isovalerate concentration, whereas the EFA1 had the lowest value. The EOB2 and EOB4 increased ($p < 0.001$) the APR by 38.6 and 28.6%, respectively, compared to the control, while the EFA1 and EOB1 reduced it by

18.9 and 17.1%. Also, FA inclusion decreased (p = 0.02) acetate concentration by 4% and increased propionate concentration by 9.5%.

Table 4. Effects of essential oil blends with or without fumaric acid on total gas volume and greenhouse gases production of the total mixed ration.

Treatments	Gas Volume (mL/g DM)	Methane (mg/g DM)	Carbon Dioxide (mg/g DM)	Ammonia (mmol/g DM)	Hydrogen Sulphide (mg/g DM)
Control	181 ^{a}	7.69 ^{a}	37.3 ^{a}	61.7 ^{ab}	213 ^{bc}
EFA1	128 ^{d}	0.71 ^{d}	12.9 ^{b}	75.5 ^{a}	511 ^{a}
EFA2	158 ^{abc}	5.18 ^{ab}	27.2 ^{ab}	73.1 ^{a}	202 ^{bc}
EFA3	160 ^{ab}	5.36 ^{ab}	29.5 ^{ab}	58.3 ^{ab}	179 ^{bc}
EFA4	147 ^{bcd}	4.42 ^{abc}	24.7 ^{ab}	26.6 ^{b}	43.5 ^{bc}
EOB1	123 ^{d}	1.05 ^{cd}	15.7 ^{b}	90.1 ^{a}	505 ^{a}
EOB2	148 ^{bcd}	5.10 ^{ab}	25.3 ^{ab}	80.2 ^{a}	217 ^{b}
EOB3	158 ^{abc}	6.29 ^{ab}	31.0 ^{ab}	72.1 ^{a}	217 ^{b}
EOB4	133 ^{cd}	3.95 ^{bcd}	20.3 ^{ab}	25.7 ^{b}	38.3 ^{c}
SEM	2.3	0.379	1.62	3.86	24.85
p value					
EOB	<0.001	<0.001	0.003	<0.001	<0.001
FA	0.077	0.808	0.883	0.316	0.857
EOB × FA	<0.001	<0.001	0.004	<0.001	<0.001

SEM, Standard error of means; $^{a–d}$ Means with different superscripts within the same column differ, $p < 0.05$.

Table 5. Effects of essential oil blends with or without fumaric acid on total and molar proportion of VFA production of the total mixed ration.

Treatments	TVFA (mM)	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	APR
Control	77.90 ^{a}	0.678 ^{bc}	0.201 ^{bc}	0.104 ^{e}	0.0049 ^{a}	0.011 ^{b}	0.0021 ^{ab}	3.39 ^{d}
EFA1	59.49 ^{d}	0.579 ^{f}	0.211 ^{ab}	0.188 ^{a}	0.0031 ^{c}	0.017 ^{a}	0.0014 ^{e}	2.75 ^{e}
EFA2	62.28 ^{cd}	0.657 ^{cd}	0.188 ^{cd}	0.138 ^{c}	0.0041 ^{b}	0.011 ^{b}	0.0019 ^{bc}	3.50 ^{cd}
EFA3	72.52 ^{ab}	0.666 ^{bcd}	0.198 ^{bc}	0.120 ^{d}	0.0039 ^{b}	0.010 ^{b}	0.0018 ^{bc}	3.36 ^{d}
EFA4	59.83 ^{cd}	0.652 ^{d}	0.166 ^{ef}	0.166 ^{b}	0.0039 ^{b}	0.011 ^{b}	0.0022 ^{a}	3.94 ^{b}
EOB1	59.46 ^{d}	0.606 ^{e}	0.216 ^{a}	0.161 ^{b}	0.0037 ^{b}	0.011 ^{b}	0.0017 ^{d}	2.81 ^{e}
EOB2	69.44 ^{abc}	0.708 ^{a}	0.151 ^{f}	0.126 ^{cd}	0.0038 ^{b}	0.009 ^{b}	0.0017 ^{cd}	4.70 ^{a}
EOB3	66.94 ^{bcd}	0.683 ^{b}	0.177 ^{de}	0.123 ^{d}	0.0041 ^{b}	0.010 ^{b}	0.0019 ^{bcd}	3.86 ^{bc}
EOB4	58.75 ^{d}	0.662 ^{bcd}	0.152 ^{f}	0.169 ^{b}	0.0039 ^{b}	0.011 ^{b}	0.0020 ^{bc}	4.36 ^{a}
SEM	1.101	0.0054	0.0033	0.0038	0.00007	0.0004	0.00004	0.089
p value								
EOB	<0.001	<0.001	<0.001	<0.001	<0.001	0.0019	<0.001	<0.001
FA	0.9547	0.0210	0.0199	0.2520	0.1967	0.0580	0.7181	0.0047
EOB × FA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

TVFA, Total volatile fatty acids; APR, Acetate Propionate Ratio; SEM, Standard error of means; $^{a–f}$ Means with different superscripts within the same column differ, $p < 0.05$.

4. Discussion

4.1. Dry Matter and Fiber Fractions Degradability

The decrease in the DMD as a result of EOB (with or without FA) inclusion would adversely affect the actual amounts of various nutrients accessible to the animals. Also, the decrease in the NDFD and HEMD would be correlated with the lower TDN and, consequently, the dietary energy content. Some authors reported that the EOs and EOBs had negative effects on DM and fiber digestibility [16,17], which has been attributed to the phenolic and non-phenolic compounds that intrude and disintegrate into the cell membrane of the rumen fibrolytic bacteria and protozoa, inhibiting their metabolic activities. Metwally et al. [10] reported significantly lower in situ rumen dry matter degradability of a TMR with the EO blend (Crina® Ruminant) but the affected individual ingredients (grass silage,

maize silage, soybean meal, rapeseed meal, and wheat grain) differently. The absence of an effect on the ADFD and ADLD with the inclusion of EOBs with or without FA in this current study agrees with a previous report by [9], who observed no effect of EOBs on the ADFD and ADLD of high-forage and high-concentrate diets.

4.2. In Vitro Digestibility and Fermentation Parameters

Ruminal fluid pH is a critical index to evaluate microbial fermentation activity, because most ruminal cellulolytic bacteria are pH sensitive [18]. In this study, the rumen pH (6.61–6.69) was influenced by both the EOB inclusion and the EOB × FA interaction. The reduced pH was linked to a decrease in the gas production, DM disappearance, and total VFA concentration [19]. Expectedly, pH was reduced by 0.03 unit with the FA inclusion without compromising the TVFA concentration. This contradicts the assumption that a reduction in the rumen pH could result in VFA accumulation, thereby limiting microbial fermentation [18]. The undegraded DM and IVTDDM values were significantly lower than the control but are similar among the EOB and EFA treatments. The chemical structures of the essential oils, the synergy of various bioactive substances in the EOB, the inclusion dosage, and the diet type are key factors responsible for the variations in in vitro apparent and true dry matter digestibility. The lack of effect on IVTDDM among the EOB groups agrees with the findings of [20] who reported that anise, clove, ginger, and oregano administered individually at varying doses had no effect on IVTDDM.

The PF points out the relationship between the feed degradability, the gas production, and the efficiency of microbial biomass synthesis [9,20]. In this study, the EOB1 and EOB4 had higher PF compared with the EFA4 treatment, despite no variation in undegraded DM and IVTDDM values among all the treated groups. This implies that lower amounts of degraded DM and OM were needed to generate 1 mL of gas, and that more volatile fatty acids and microbial biomass would be generated per unit of degraded substrate during the fermentation process. The microbial mass is a crucial outcome of ruminal fermentation and plays a vital role in the microbial protein synthesis. The higher microbial mass observed in the EFA1, though similar to the EFA4 and EOB4, could be attributed to the lower gas produced and the inhibition of methanogens [19].

4.3. Gas Production and GHG Emissions

Methane is linked to dietary energy loss, a decrease in the production efficiency of ruminant animals, and an environmental hazard. It is formed by reducing CO_2 with hydrogen, which is produced by different ruminal microbes during feed digestion [17]. The effectiveness of EOB as rumen microbiome modifier to improve feed efficiency and reduce methane emission has been reported [8,9]. In this study, the EFA1 and EOB1 reduced the total gas production gas by 29.1% and 32.1%, the CH_4 gas by approximately 90.8% and 86.4%, and the CO_2 gas by nearly 65.7% and 57.9%, respectively. This study corroborates the fact that the EOB vary in their abilities to impact ruminal fermentation and inhibit GHG emission, particularly CH_4 production [9]. Combining multiple EOs with different bioactive compounds can have synergistic effects on rumen fermentation and methane reduction. Each EO may target specific microbial populations or metabolic pathways in the rumen, contributing to a more comprehensive methane-mitigation strategy. In addition, the effectiveness of specific EO combinations may vary depending on factors such as the type and proportion of the EOs used, the diet composition, and the animal species [5,7,9]. In this study, a higher proportion of garlic (40%) in EOB1 could be responsible for the most potent effect in reducing the CH_4 and CO_2. Patra and Yu [8] noted that garlic oil has significant amounts of organosulfur compounds, including alliin and allicin, diallyl sulphide, diallyl disulphide (DADS), and allyl mercaptan, with which it exerts anti-methanogenic effect more on methanogenic archaea than on rumen bacterial population. Reduced CH_4 emissions could be attributed to a decrease in the ruminal protozoa population, thereby resulting in better nutrient utilization efficiency. A higher protozoa population increases ruminal protein degradation and reduces dietary protein and energy utilization efficiency. Molho-

Ortiz et al. [19] reported that the inclusion of garlic, cinnamon, eucalyptus, and rosemary essential oils decreased methane production compared to their aqueous extracts in a basal diet. Moreover, the addition of FA to EOB1 further enhanced the antimicrobial activity of this unique blend to inhibit methanogenesis. Also, fumaric acid is a key intermediate in the succinate–propionate pathway and propionic acid precursor, which absorbs the H_2 sink to reduce CH_4 from ruminal fermentation [7,11]. This suggests that the FA could have diverted the H_2 toward the propionate pathway. In support, Baraz et al. [21] reported that the combination of disodium fumarate and thyme essential oil decreased gas and methane production in vitro compared with their single use. Bayaru et al. [11] reported that fumaric acid supplementation reduced methane production by 23.0% in Holstein steers. The observed reduction in total gas produced during enteric fermentation and GHG emission could be attributed to the reduction in the rumen microbial population, particularly hyperammonia-producing bacteria, by the EOB and FA. In agreement, Lin et al. [7] reported a significant decrease in microbial populations of protozoa, methanogens, *Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens* in Hu sheep fed a combination of monosodium fumarate and EOB containing eugenol, carvacrol, citral and cinnamaldehyde. Furthermore, the efficacy of EOB and FA on GHG reduction is diet dependent and is more pronounced on low quality than high quality diets. Li et al. [3] reported that fumaric acid supplementation exerted a greater CH_4-decreasing effect on Xinong Saanen dairy goats fed low forage: concentrate particle size diet (31.72%) compared with high forage: the concentrate particle size diet (17.9%). Bayaru et al. [11] also noted that fumaric acid reduced carbon dioxide production by 20.5%.

Ammonia production is related to feed protein digestion and deamination of amino acids by mostly proteolytic bacteria and a group of hyper-ammonia-producing bacteria [17]. In the current study, the EFA4 and EOB4 significantly reduced NH_3 gas compared with other treatments, whereas the EOB4 produced the least H_2S gas concentrations (79–92% lower) when compared to the other EOBs. These observations confirm that different EOBs could exert varying inhibitory effects on Gram-positive rumen bacteria, including ammonia hyper-producing bacteria, and protozoa. The reduction in ammonia concentration with EOB without or with FA inclusion is consistent with some previous reports [8,9,22]. In addition, Lin et al. [23] reported that a monosodium fumarate and essential oils combination reduced ammonia nitrogen in the rumen of Hu sheep.

Gas production depicts the accessibility of degradable carbohydrates, particularly cellulose, for enteric fermentation, and is positively correlated to VFA production [19]. In the present study, EFA1 and EOB1 reduced the total gas production (29.1% and 32.1%, respectively) and the total VFA by approximately 23.7% compared to the control. Our results are consistent with the study by [9], who reported that EOB reduced total gas production when compared with the control, and that variation exists in cumulative gas production among the EOBs groups.

4.4. Volatile Fatty Acid Production

Since VFA are the primary source of metabolizable energy for ruminants, strategies to increase their production from the diet would be beneficial to the animal. Phytochemicals including natural essential oils have been reported to alter the rumen microbiota, thus changing the end-products of ruminal fermentation such as volatile fatty acids [6]. In this study, the higher propionate molar proportion obtained in the EOB1 without or with FA inclusion (EFA1) suggests that such EOB could enhance the energy availability in beef cattle. This further implies that the EOB1 modifies the rumen microbiome by promoting the relative abundance of bacteria that are positively correlated with propionate concentration. Meanwhile, the inclusion of FA further increased propionate concentration by 9.5% and decreased the acetate contents by 4%. In the rumen, fumarate works as an alternative hydrogen acceptor and a metabolic precursor of propionate and succinate through decarboxylation and reduction reactions, respectively [11,22]. Additionally, Baraz et al. [21] reported that the simultaneous use of disodium fumarate and thyme essential oil

decreased gas production and molar proportions of acetate and butyrate, while propionate was increased. Lin et al. [23] observed that 200 mg/L of essential oil active components with or without fumarate reduced the gas production by 13.60 to 17.10%. Blanch et al. [5] observed a decrease in the total VFA production when testing a commercial EO blend (Next Enhance® 300) containing cinnamaldehyde and garlic oils at 400 mg/L. In addition, it has also been reported that a blend of disodium fumarate and thyme essential oil caused a significant increase in the molar proportion of propionate, and a decrease in acetate, butyrate, and acetate-to-propionate ratio [21]. Busquet et al. [24] demonstrated that garlic oil addition at 300 mg/L increased the proportion of propionate and butyrate and reduced acetate proportion. Bayaru et al. [11] reported a higher propionic acid, a decrease in butyric acid and isovaleric acid, and no significant change in acetic acid following FA supplementation alone. The EFA1 treatment increased the butyrate concentration by 80.7% compared to untreated TMR, suggesting better gut health. Increased butyrate concentration might also indicate that the predominant butyrate-utilizing bacteria might have been inhibited by the synergy of the EOB1 and fumarate. Furthermore, the accumulation of hydrogen gas when methane gas is suppressed could inhibit the growth of butyrate-utilizing bacteria [8]. Higher propionate and butyrate concentrations could also enhance the rumen structure and functions, thus contributing to nutrient absorption, gut wellness, and better health benefits to the cattle. Remling et al. [4] noted that propionate and butyrate also stimulate the growth of rumen papillae, thereby increasing the absorption surface for ruminants. Branched-chain VFA (BCVFA), such as isobutyrate, isovalerate, valerate, and 2-methylbutyric acid, are by-products of amino acid deamination in the rumen which are utilized by ruminal microbes as a source of carbon skeleton to synthesize branched-chain amino acids [18]. Higher valerate and isovalerate concentration in EFA1 and EFA4, respectively, indicates that such treatments would enhance cellulolytic bacteria population and fiber digestibility in the rumen.

5. Conclusions

The present results show that a synergy of EOBs and FA offers an effective way to reduce methane and carbon dioxide production. Both EFA1 and EOB1 had the greatest effect in reducing methane and carbon dioxide gases. The inclusion of EFA1 and EOB1 increased propionate concentration and decreased the acetate-to-propionate ratio. The varied effects of EOB with or without FA on nutrient digestibility, fermentation characteristics, microbial mass, and total VFA production implied modification of the rumen microbiome. Therefore, future studies to improve dry matter digestibility using a unique recombination of EOBs with FA or other additives would be required. Additionally, future studies will consider the inclusion levels of the EOB and FA to reduce their effect on dry matter digestibility.

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