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Synergistic Effects of Essential Oil Blends and Fumaric Acid on Ruminal Fermentation, Volatile Fatty Acid Production and Greenhouse Gas Emissions Using the Rumen Simulation Technique (RUSITEC)

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Abstract: This study investigated the combined impact of essential oil blends (EOBs) and fumaric acid (FA) on ruminal fermentation in dairy cows using the rumen simulation technique (RUSITEC) system. Three rumen-cannulated, non-lactating Holstein Friesian cows served as inoculum donors. The substrate, a total mixed ration (TMR), comprised corn silage, alfalfa hay, and a concentrate mix in a 3:1:1 ratio. The four treatments evaluated were Control (TMR without additives), EFA1 (TMR + EOB1 + FA), EFA2 (TMR + EOB2 + FA), and EFA3 (TMR + EOB3 + FA). Sixteen fermentation chambers were randomly assigned to the treatments, each with four replicates, following a completely randomized design during a 9-day experimental period. EOBs and FA were added at 10 µL/g feed and 3% of TMR, respectively. After a 4-day adaptation, samples were collected for 5 days. Results revealed that EFA1 significantly reduced ($p = 0.0351$) CH_4 emissions by 60.2% without negatively impacting dry matter disappearance, fiber fraction digestibility, pH, or gas volume. All EFAs increased ($p < 0.001$) the propionate molar proportion and decreased ($p < 0.001$) the acetate-to-propionate ratio. EFA2 decreased ($p < 0.05$) the acetate proportion by 3.3% compared to the control. In conclusion, EFA1 is recommended as an effective nutritional intervention to mitigate CH_4 emissions and optimize ruminal fermentation in dairy cows.

Keywords: RUSITEC; additives; essential oils; fumarate; methane; fermentation; digestibility

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1. Introduction

Livestock production constitutes almost 40% of the global agricultural GDP, making a substantial contribution to the world economy [1]. The cattle industry ranked among the top three contributors, thereby playing a crucial role in global food security. Additionally, it profoundly impacts economies, rural development, social dynamics, culture, and gastronomy in many countries [2]. Dairy cows, through the help of diverse rumen microbes, can efficiently convert high-forage diets and low-quality protein feedstuffs [3,4] to generate volatile fatty acid (VFA) and microbial protein synthesis for optimum production [5]. Meanwhile, methane (CH_4) gas, a potent greenhouse gas, is produced through oxidative reduction of carbon dioxide (CO_2) and molecular hydrogen (H_2) generated during microbial

degradation of fiber and non-fiber carbohydrates [6]. The enteric CH_4 emission accounts for nearly 80 million tons per year, contributing to roughly 33% of global anthropogenic emissions [3]. Hence, there is a growing interest in modifying rumen fermentation and reducing greenhouse gas emissions from cattle production without compromising feed efficiency and animal productivity. Several feed additives, including ionophores, halogenated CH_4 analogs, probiotics, dietary lipids, unsaturated fatty acids, enzymes, and plant secondary compounds, have been explored to lower methane energy losses associated with GHG emissions in cattle production [3,5,7,8]. The use of essential oils (EOs) and fumaric acid (FA) has gained considerable attention because of their potential to positively influence ruminal fermentation and nutrient utilization and mitigate CH_4 emissions in ruminant animals [5,9–13].

Essential oils (EOs) are volatile or semi-volatile, naturally occurring secondary metabolites extractable from various plant parts by solvent extraction, cold pressing, or steam distillation [14,15]. The derived EO imparts organoleptic, antimicrobial, anti-inflammatory, and antioxidant effects due to specific aldehydes or phenols [16,17]. The concentration of phytoactive components depends on factors such as plant parts, cultivars, growing conditions, and extraction methods [18]. Variations in bioactive constituents among EOs significantly influence their effectiveness as antimicrobial agents and modifiers of rumen fermentation [3]. In previous studies, Patra and Yu [5] observed a significant reduction in CH_4 production with increasing doses of all five essential oils (clove, eucalyptus, garlic, origanum, and peppermint) investigated. Meanwhile, Benetel et al. [19] found that white thyme and oregano showed significantly higher potential in reducing CH_4 production among ten individual essential oils investigated. The use of essential oil blends (EOBs) is reported to be more advantageous than single EOs [20]. An EOB typically contains a mixture of individual EOs with diverse bioactive compounds [21]. The combination of different EOs can exert synergistic, additive, or antagonistic effects, altering the rumen microbiome and providing a comprehensive approach to addressing multiple pathways in CH_4 production [17,22]. However, the effects of EOBs depend on factors like diet, substrate, bioactive compounds, incubation time, and inclusion level [21,23].

Fumaric acid (FA) is a natural organic acid that serves as a hydrogen sink, thereby redirecting hydrogen from methanogenesis toward VFA production [24]. By competing for hydrogen with methanogenic archaea, FA reduces CH_4 emissions, resulting in increased VFA production, particularly propionate. As a hydrogen acceptor, fumarate acts as a propionate precursor in the rumen, undergoing reduction and decarboxylation reactions to form succinate and propionate, respectively [25]. Baraz et al. [26] reported that the addition of hydrogen acceptors to ruminal fermentation can effectively reduce methanogenesis by limiting the availability of hydrogen, a key substrate for methane formation. Previous studies have reported significant reductions in CH_4 production and the acetate-to-propionate ratio, as well as a significant increase in propionate concentration with FA supplementation [6,8,25]. Meanwhile, diet-dependent effects (high-forage versus low-forage diets) could also influence the divergent outcomes following FA supplementation [6,7]. Previously, an in vitro fermentation study revealed that the synergy of EOBs without or with FA reduced CH_4 and CO_2 gases, increased propionate concentration, and decreased the acetate-to-propionate ratio in black Angus beef cows [27]. This study hypothesized that a combination of EOB and FA may be effective in mitigating methane emissions from dairy cows without suppressing nutrient digestibility. Moreover, most of the available information reported in the literature are in vitro batch culture studies; there is a paucity of information on the combined effects of EOB and FA from rumen simulation technique (RUSITEC) fermenters. Therefore, the objective of this study was to investigate the synergistic effects of EOB and FA on fermentation characteristics, GHG emission, and VFA production of total mixed ration for dairy cows using the RUSITEC system.

2. Materials and Methods

2.1. Study Ethical Approval

The study protocol involving the use of essential oil blends and cannulated cows was approved by the Institutional Animal Care and Use Committee (LA22-0019), North Carolina A&T State University, Greensboro. The dairy cows were maintained according to the University Farm standards.

2.2. Substrate Preparation

Samples of corn silage, alfalfa hay and concentrate (ground corn grain, corn gluten, soybean meal, Soyplus, soybean hulls, and vitamin–mineral premix) obtained from the Dairy Unit, NC A&T State University Farm were dried at 55 °C for 72 h (Isotemp Oven, Thermo Fisher Scientific, Allentown, PA, USA) and then ground through a 1 mm screen (Cutting Mill SM100, Retsch GmbH, Haan, North Rhine-Westphalia, Germany). A total mixed ration (TMR) was formulated with corn silage, alfalfa hay, and concentrate at a ratio of 3:1:1 on a dry matter (DM) basis. The chemical composition of the ingredients and TMR was carried out using the standard procedures [27]. The chemical composition of ingredients and TMR are presented in Table 1. Approximately 10 ± 0.2 g of substrate was weighed into pre-weighed nylon bags (70 mm × 140 mm; pore size = 150 µm; Ankom filter bags (Ankom Technology Corp., Macedon, NY, USA)) for incubation.

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

Chemical Composition	Corn Silage	Alfalfa Hay	Concentrate	Total Mixed Ration
Dry Matter	38.1	83.9	90.8	68.1
Organic matter	96.5	90.9	83.3	93.1
Crude Protein	6.31	16.0	20.3	13.2
Crude Fat	4.67	3.15	8.49	4.73
Ash	3.51	9.05	16.7	6.92
Neutral detergent fiber	59.4	49.5	74.1	61.9
Acid detergent fiber	14.1	9.6	15.4	12.0
Acid detergent lignin	14.5	18.2	10.5	13.7

* n = 8 replicates.

2.3. Test Ingredients and Study Design

Eleven commercially available EOs were used to formulate three EOBs 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], and EOB3 [Clove, Anise, Peppermint, and Oregano; 4:3:2:1]. The proportion of each essential oil used in the blends was based on an extensive review of previous studies in the literature and our laboratory [20,27]. Fumaric acid (99+%) was procured from Thermo Fisher Scientific, Branchburg, NJ, USA. Four treatments evaluated in a completely randomized design were Control (TMR without additives), EFA1 (TMR + EOB1 + FA), EFA2 (TMR + EOB2 + FA), and EFA3 (TMR + EOB3 + FA). Based on the preliminary in vitro batch culture study in our laboratory, a lower inclusion level of EOB (10 µL/g) caused no adverse effect on gas production and dry matter digestibility (unpublished data). The inclusion dosage of EOBs was 10 µL/g feed, resulting in a total of 100 µL per 10 g of feed incubated while FA was added at 3% of TMR.

2.4. RUSITEC Fermentation

The RUSITEC system consists of two identical, 8-chamber fermenters (n = 16) equipped with 1000 mL fermentation vessels. They were randomized into 4 groups with 4 replicates per group. Each vessel had an inlet for the infusion of buffer and an effluent output port. At the start of the experiment, each fermentation vessel was filled with 700 mL of rumen fluid and 200 mL of artificial saliva prepared according to McDougall's buffer recipe [$NaHCO_3$: 9.83 g/L, Na_2HPO_4: 3.69 g/L, KCl: 0.60 g/L, NaCl: 0.47 g/L,

$(NH_4)_2SO_4$: 0.30 g/L, $MgCl_2.6H_2O$: 0.061 g/L, $CaCl_2.2H_2O$: 0.0293 g/L]. The initial pH of the rumen fluid, artificial saliva, and the mixture of rumen fluid and artificial saliva were 6.38, 8.57, and 7.06, respectively. Three non-lactating Holstein Friesian cows fitted with permanent ruminal cannula were the donors of the inoculum used. The cows were fed the same corn silage, alfalfa hay, and concentrate used to formulate the TMR/substrate used in the RUSITEC fermenters.

Ruminal contents were obtained from various rumen regions of the cannulated cows. After straining the rumen contents through four layers of cheesecloth into an insulated thermos, it was transported immediately to the Ruminant Nutrition Laboratory. On the first day of incubation, rumen solids (≈50 g of wet weight) were placed inside each vessel and were removed after 24 h of incubation. Sample bags containing the substrate were added to the vessel and allowed to ferment for 48 h. Fermenters were then submerged in a 39 °C water bath. The flow through fermenters was maintained by continuous infusion of artificial saliva at a rate of 21 rpm (Watson-Marlow Pump 205U, Watson-Marlow Ltd., Cornwall, England). The experiment lasted for 9 d (i.e., 4 d of adaptation and 5 d of data collection).

2.5. Sampling for Total Gas, Greenhouse Gases, Ammonia Nitrogen (NH_3-N), and VFA Determinations

During the 5-day sampling period, the total gas produced was collected daily in a Tedlar® gas sampling bag (Supelco®, Bellefonte, PA, USA) connected to the effluent flasks. The gas pressure readings were taken daily (Gas Flowmeter DM3, Alexander Wright Ltd., London, UK), and gas production was expressed in mL/d. Concentrations of greenhouse gases produced, such as methane, carbon dioxide, ammonia, and hydrogen sulfide, were estimated from the effluent flasks using a portable gas analyzer (Biogas 5000, Landtec, Dexter, MI, USA) equipped with internal electrochemical and dual wavelength infrared cells with a reference channel. Following calibration as per the manufacturer's instructions, gas readings were obtained by connecting the analyzer to the gas collection opening of each effluent flask. To ensure accuracy, the unit was purged between each sampling to remove any residual gas from the previous measurement.

The volume of effluent was measured daily at the time of feed bag exchange with a graduated cylinder, and pH values were determined immediately (Fisherbrand™ FE150 pH benchtop meter, Fisher Scientific, Waltham, MA, USA). Thereafter, 25 mL of liquid effluent was collected into a 50 mL Eppendorf tube containing 5 mL diluted H_2SO_4 (72%) for NH_3-N analysis [27]. Then, 15 mL of liquid effluent was collected, preserved with 3 mL of 25% (wt/wt) metaphosphoric solution, and immediately frozen at −20 °C until required for VFA determination. The VFA concentrations were quantified using gas chromatography (Agilent 7890B GC system with a Flame Ionization Detector, 7693 autosampler, Agilent Technologies, Santa Clara, CA, USA) and a capillary column (Zebron ZB-FFP, Phenomenex Inc., Torrance, CA, USA) following standard procedures [28]. Overall, 20 replicate samples were collected for each treatment.

2.6. Dry Matter, Organic Matter, and Fiber Digestibility

Following the same sampling period described above, sample bags were removed from each fermenter after 48 h of fermentation, washed with cold water until the water was clear, and oven-dried at 55 °C for 72 h. The residue weight was used to estimate dry matter disappearance (DMD) [29]. A known quantity of the residue from each sample was ashed at 550 °C for 3 h in crucibles and weighed to determine organic matter digestibility (OMD) [30,31]. The residues in each bag were used for fiber (NDF, ADF, and ADL) analysis using the Ankom Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) described in [27]. Hemicellulose content was determined by subtracting ADF from NDF, while the cellulose content was estimated by subtracting ADL from ADF [28].

2.7. Analysis

Data obtained were analyzed using the General Linear Model in a one-way ANOVA (SAS 9.4 version; SAS Institute Inc., Cary, NC, USA). Means of significant variables were separated at $p \leq 0.05$ using the Duncan multiple range test. The statistical model used was Yij = μ + Ti + eij, where Yij is the dependent variable, μ is the overall mean, Ti is the essential oil blend and fumaric acid effect, and eij is the residual error.

3. Results

The synergistic effects of the essential oil blends and fumaric acid (EFA) on pH, volume of effluent, gas volume, in vitro DMD, OMD, and NH_3-N are presented in Table 2. The pH, volume of effluent, and gas volume were not affected ($p > 0.05$) by EFA inclusion. Similarly, DMD, OMD and NH_3-N were not significantly influenced by the inclusion of EFA in the TMR.

Table 2. Effects of essential oil blends and fumaric acid on pH, volume of effluent, in vitro digestibility, and ammonia nitrogen of the total mixed ration.

Treatments	pH	Volume of Effluent (mL)	Gas Volume (mL)	NH_3-N (mg/dL)	DMD (%)	OMD (%)
Control	7.27	466	1810	5.04	56.5	87.8
EFA1	6.89	444	1556	4.73	55.8	85.7
EFA2	7.27	522	1466	4.71	54.5	87.1
EFA3	7.19	464	1591	4.80	52.2	87.6
SEM	0.094	13.2	69.1	0.192	1.03	0.71
p value	0.429	0.180	0.333	0.930	0.473	0.762

DMD, dry matter disappearance; OMD, organic matter digestibility; NH_3-N, ammonia nitrogen; SEM, standard error of means.

The effects of essential oil blends and fumaric acid on NDF digestibility (NDFD), ADF digestibility (ADFD), ADL digestibility (ADLD), hemicellulose digestibility (HEMD) and cellulose digestibility (CELD) are presented in Table 3. Fiber fraction digestibility was not suppressed ($p > 0.05$) by EFA inclusion in the control diets.

Table 3. Effects of essential oil blends and fumaric acid on fiber fraction degradability of the total mixed ration.

Treatments	NDFD (%)	ADFD (%)	ADLD (%)	HEMD (%)	CELD (%)
Control	65.6	58.5	26.1	7.06	32.4
EFA1	65.3	56.9	26.0	8.37	30.9
EFA2	65.1	56.8	23.8	8.37	32.9
EFA3	64.8	56.2	23.7	8.60	32.5
SEM	0.24	0.36	0.72	0.339	0.47
p value	0.674	0.108	0.488	0.367	0.460

NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility; ADL, acid detergent lignin digestibility; HEMD, hemicellulose digestibility; CELD, cellulose digestibility; SEM, standard error of means.

The effects of EOBs and FA on the total and molar proportion of VFA are presented in Table 4. The molar proportion of propionate was increased ($p < 0.001$), while total VFA, butyrate concentration, and acetate: propionate ratio were decreased ($p < 0.05$) with EFA inclusion. The EFA2 treatment decreased the acetate proportion by 3.3% compared to the control. Higher ($p = 0.013$) isovalerate content by nearly 27% was observed in EFA2 compared to EFA1.

Table 4. Effects of essential oil blends and fumaric acid on total and molar proportion of VFA production (mM, except APR) of the total mixed ration.

Treatments	TVFA	Acetate	Propionate	Butyrate	Iso-Butyrate	Valerate	Iso-Valerate	APR
Control	58.6 ^{a}	0.576 ^{a}	0.278 ^{b}	0.120 ^{a}	0.0056	0.0169	0.0037 ^{ab}	2.08 ^{a}
EFA1	52.1 ^{b}	0.573 ^{a}	0.302 ^{a}	0.100 ^{b}	0.0050	0.0174	0.0030 ^{b}	1.91 ^{b}
EFA2	51.2 ^{b}	0.557 ^{b}	0.310 ^{a}	0.103 ^{b}	0.0053	0.0208	0.0038 ^{a}	1.81 ^{b}
EFA3	51.8 ^{b}	0.568 ^{ab}	0.301 ^{a}	0.105 ^{b}	0.0050	0.0190	0.0033 ^{ab}	1.90 ^{b}
SEM	1.04	0.0026	0.0025	0.0021	0.00017	0.00115	0.00014	0.021
p value	0.039	0.045	<0.001	0.001	0.624	0.179	0.013	<0.001

TVFA, total volatile fatty acids; APR, acetate propionate ratio; SEM, standard error of means; ^{a,b} means with different superscripts within the same column differ, $p < 0.05$.

The effects of EOBs and FA on CH_4, CO_2, ammonia (NH_3), and hydrogen sulfide (H_2S) are presented in Table 5. CH_4 gas emission was reduced ($p = 0.0351$) by about 60.2% in EFA1 compared to the control group. However, CO_2, NH_3, and H_2S production were not significantly ($p > 0.05$) affected by the treatments.

Table 5. Effects of essential oil blends and fumaric acid on greenhouse gas production of the total mixed ration.

Treatments	Methane (mg/g DM)	Carbon Dioxide (mg/g DM)	Ammonia (mmol/g DM)	Hydrogen Sulfide (mg/g DM)
Control	48.3 ^{a}	271	737	7653
EFA1	19.2 ^{b}	230	738	6722
EFA2	37.4 ^{ab}	226	582	5916
EFA3	38.4 ^{ab}	253	600	4026
SEM	3.67	18.2	62.3	748.7
p value	0.035	0.799	0.714	0.391

SEM, standard error of means; ^{a,b} means with different superscripts within the same column differ, $p < 0.05$.

The correlation between fermentation characteristics is presented in Table 6. There was a positive linear correlation between pH and volume of effluent (r = 0.251; $p < 0.05$). Positive correlations ($p < 0.05$) existed between gas volume and cellulose digestibility. Positive correlations also existed between organic matter digestibility and NH_3-N (r = 0.457; $p < 0.01$), NDF digestibility (r = 0.326; $p < 0.05$), and hemicellulose digestibility (r = 0.452; $p < 0.01$). There was a linear positive relationship between NH_3-N and NDF digestibility (r = 0.318; $p < 0.05$) and ADL digestibility (r = 0.286; $p < 0.05$), but an inverse relationship (r = −0.364; $p < 0.01$) with cellulose digestibility. There were positive correlations between NDF digestibility and ADF digestibility (r = 0.401; $p < 0.01$), ADL digestibility (r = 0.486; $p < 0.01$), and hemicellulose digestibility (r = 0.273; $p < 0.05$) but an inverse relationship (r = −0.448; $p < 0.01$) with cellulose digestibility. ADF digestibility was linearly correlated with ADL digestibility (r = 0.840; $p < 0.01$) but inversely correlated with hemicellulose digestibility (r = −0.772; $p < 0.01$) and cellulose digestibility (r = −0.541; $p < 0.01$). ADL digestibility exhibited an inverse relationship with hemicellulose digestibility (r = −0.545; $p < 0.01$) and cellulose digestibility (r = −0.910; $p < 0.01$). There was a linear relationship between hemicellulose digestibility and cellulose digestibility (r = 0.257; $p < 0.05$).

Table 6. Pearson correlation coefficients between fermentation characteristics.

Variables	pH	Vol. Effl.	Gas Vol.	DMD	OMD	NH_3-N	NDFD	ADFD	ADLD	HEMD	CELD
pH	1.000										
Vol. Effl.	0.251 *	1.000									
Gas Vol.	0.043	0.154	1.000								
DMD	−0.166	−0.041	−0.028	1.000							
OMD	0.069	0.207	0.044	−0.228	1.000						
NH_3-N	0.077	0.002	−0.162	−0.100	0.457 **	1.000					
NDFD	0.114	0.168	−0.061	−0.072	0.326 *	0.318 *	1.000				
ADFD	0.128	0.141	−0.135	0.026	−0.213	0.012	0.401 **	1.000			
ADLD	0.064	0.048	−0.229	−0.004	−0.036	0.286 *	0.486 **	0.840 **	1.000		
HEMD	−0.055	−0.032	0.100	−0.077	0.452 **	0.242	0.273 *	−0.772 **	−0.545 **	1.000	
CELD	−0.002	0.033	0.252 *	0.026	−0.112	−0.364 **	−0.448 **	−0.541 **	−0.910 **	0.257 *	1.000

Vol. Effl., volume of effluent; Gas Vol., gas volume; DMD, dry matter disappearance; OMD, organic matter digestibility; NH_3-N, ammonia nitrogen; NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility; ADLD, acid detergent lignin digestibility; HEMD, hemicellulose digestibility; CELD, cellulose digestibility; * correlation is significant at $p < 0.05$ level; ** $p < 0.01$ level.

The correlation between volatile fatty acid production and greenhouse gas emissions is presented in Table 7. An inverse correlation between TFVA and iso-butyrate (r = −0.317; $p < 0.01$), NH_3 (r = −0.304; $p < 0.05$), and H_2S (r = −0.330; $p < 0.01$) was noted. An inverse relationship ($p < 0.01$) was also noted for acetate and propionate (r = −0.558), butyrate (r = −0.311), valerate (r = −0.819), and iso-valerate (r = −0.751). Contrarily, acetate exhibited a linear relationship with iso-butyrate (r = 0.343), APR (r = 0.782), NH_3 (r = 0.254) and H_2S (r = 0.275). Propionate had a linear relationship with valerate (r = 0.381; $p < 0.01$) but a strong inverse relationship ($p < 0.01$) with butyrate (r = −0.600), iso-butyrate (r = −0.591), and APR (r = −0.949). Butyrate demonstrated linear correlations with iso-butyrate (r = 0.357; $p < 0.01$), valerate (r = 0.242; $p < 0.05$), iso-valerate (r = 0.574; $p < 0.01$), and APR (r = 0.332; $p < 0.01$). Iso-butyrate had an inverse relationship ($p < 0.01$) with valerate (r = −0.437) but a linear correlation with APR (r = 0.584). Moreover, valerate exhibited a linear correlation with iso-valerate (r = 0.746; $p < 0.01$) but an inverse relationship with APR (r = −0.589; $p < 0.01$), CH_4 (r = −0.243; $p < 0.05$), NH_3 (r = −0.352; $p < 0.01$), and H_2S (r = −0.335; $p < 0.01$). Also, iso-valerate had an inverse relationship with APR (r = −0.320; $p < 0.01$). There were strong linear relationships ($p < 0.01$) between CH_4 and CO_2 (r = 0.901) and NH_3 (r = 0.625) and H_2S (r = 0.743). A positive linear correlation existed ($p < 0.01$) between CO_2 and NH_3 (r = 0.736) and H_2S (r = 0.826). NH_3 also demonstrated a linear connection with H_2S (r = 0.861; $p < 0.01$).

Table 7. Pearson correlation coefficients between volatile fatty acids production and greenhouse gas emissions.

Variables	TVFA	Acetate	Propionate	Butyrate	Iso-Butyrate	Valerate	Iso-Valerate	APR	CH_4	CO_2	NH_3	H_2S
TVFA	1.000											
Acetate	−0.088	1.000										
Propionate	0.022	−0.558 **	1.000									
Butyrate	0.127	−0.311 **	−0.600 **	1.000								
Iso-Butyrate	−0.317 **	0.343 **	−0.591 **	0.357 **	1.000							
Valerate	−0.042	−0.819 **	0.381 **	0.242 *	−0.437 **	1.000						
Iso-Valerate	0.029	−0.751 **	0.074	0.574 **	−0.096	0.746 **	1.000					
APR	−0.050	0.782 **	−0.949 **	0.332 **	0.584 **	−0.589 **	−0.320 **	1.000				
CH_4	−0.120	0.177	−0.116	0.003	0.021	−0.243 *	−0.233	0.168	1.000			
CO_2	−0.222	0.167	−0.140	0.022	0.072	−0.197	−0.198	0.185	0.901 **	1.000		
NH_3	−0.304 *	0.254 *	−0.133	−0.057	0.230	−0.352 **	−0.227	0.208	0.625 **	0.736 **	1.000	
H_2S	−0.330 **	0.275 *	−0.164	−0.050	0.198	−0.335 **	−0.216	0.245 *	0.743 **	0.826 **	0.861 **	1.000

TVFA, total volatile fatty acids; APR, acetate propionate ratio; CH_4, methane; CO_2, carbon dioxide; NH_3, ammonia; H_2S, hydrogen sulfide; * correlation is significant at $p < 0.05$ level; ** $p < 0.01$ level.

The correlation between fermentation characteristics, volatile fatty acid production, and greenhouse gas emissions is presented in Table 8. There was a strong linear correlation ($p < 0.05$) between gas volume and CH_4 (r = 0.720), CO_2 (r = 0.804), NH_3 (r = 0.706), and H_2S (r = 0.666). An inverse relationship ($p < 0.01$) existed between OMD and propionate (r = −0.345) and valerate (r = −0.390), but it had a linear relationship with acetate (r = 0.290), iso-butyrate (r = 0.489), APR (r = 0.363), NH_3 (r = 0.411), and H_2S (r = 0.365). ADF

digestibility was linearly correlated with TVFA (r = 0.383; $p < 0.01$) but inversely correlated with iso-butyrate (r = −0.486; $p < 0.01$), CO_2 (r = −0.256; $p < 0.05$), and NH_3 (r = −0.312; $p < 0.01$). ADL digestibility was linearly correlated with TVFA (r = 0.292; $p < 0.05$) but inversely correlated with iso-butyrate (r = −0.353; $p < 0.01$), CH_4 (r = −0.319; $p < 0.01$), CO_2 (r = −0.316; $p < 0.01$), and NH_3 (r = −0.309; $p < 0.05$). Hemicellulose digestibility exhibited an inverse relationship with TVFA (r = −0.423; $p < 0.05$), propionate (r = −0.283; $p < 0.05$), and valerate (r = −0.389; $p < 0.01$), while it had a linear relationship with acetate (r = 0.313; $p < 0.01$), iso-butyrate (r = 0.578; $p < 0.01$), APR (r = 0.329; $p < 0.01$), NH_3 (r = 0.377; $p < 0.01$), and H_2S (r = 0.290; $p < 0.05$). Cellulose digestibility showed a linear correlation with CH_4 (r = 0.323; $p < 0.01$), CO_2 (r = 0.294; $p < 0.05$), and NH_3 (r = 0.241; $p < 0.05$).

Table 8. Pearson correlation coefficients between fermentation characteristics, volatile fatty acid production, and greenhouse gas emissions.

Variables	Vol. Effl.	Gas Vol.	DMD	OMD	NH_3-N	NDFD	ADFD	ADLD	HEMD	CELD
TVFA	−0.197	−0.042	0.154	−0.311	−0.248	−0.031	0.383 **	0.292 *	−0.423 **	−0.161
Acetate	−0.193	0.045	−0.116	0.290 *	0.288 *	0.177	−0.181	−0.068	0.313 **	−0.032
Propionate	0.148	−0.124	0.145	−0.345 **	−0.356 *	−0.148	0.171	0.141	−0.283 *	−0.088
Butyrate	0.026	0.109	−0.028	0.096	0.077	−0.026	−0.034	−0.112	0.018	0.147
Iso-Butyrate	0.063	0.002	−0.218 *	0.489 **	0.645 **	0.098	−0.486 **	−0.353 **	0.578 **	0.177
Valerate	0.108	−0.065	0.066	−0.390 **	−0.349 *	−0.100	0.304 *	0.194	−0.389 **	−0.068
Iso-Valerate	0.096	−0.064	0.022	0.036	0.164	0.040	0.212	0.189	−0.196	−0.131
APR	−0.183	0.128	−0.157	0.363 **	0.369 **	0.173	−0.199	−0.135	0.329 **	0.057
CH_4	0.113	0.720 **	−0.072	0.173	−0.113	−0.122	−0.225	−0.319 **	0.151	0.323 **
CO_2	0.115	0.804 **	−0.106	0.133	−0.137	−0.062	−0.256 *	−0.316 **	0.226	0.294 *
NH_3	0.200	0.704 **	−0.176	0.411 **	−0.035	0.072	−0.312 **	−0.309 *	0.377 **	0.241 *
H_2S	0.271 *	0.666 **	−0.154	0.365 **	0.021	0.164	−0.168	−0.169	0.290 *	0.134

Vol. Effl., volume of effluent; Gas Vol., gas volume; DMD, dry matter disappearance; OMD, organic matter digestibility; NH_3-N, ammonia nitrogen; NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility; ADLD, acid detergent lignin digestibility; HEMD, hemicellulose digestibility; CELD, cellulose digestibility; TVFA, total volatile fatty acids; APR, acetate propionate ratio; CH_4, methane; CO_2, carbon dioxide; NH_3, ammonia; H_2S, hydrogen sulfide; * correlation is significant at $p < 0.05$ level; ** $p < 0.01$ level.

4. Discussion

The need to use efficient and sustainable strategies to address methane emissions in ruminants without compromising feed digestibility is crucial. This study explored the combined effects of EOBs and FA on fermentation and greenhouse gas (GHG) emissions by adopting a synergistic approach to enhance methane reduction. Microbial fermentation activity depends significantly on ruminal pH due to the pH sensitivity of cellulolytic bacteria in the rumen. The pH of the fermentation media (rumen fluid and artificial saliva) was 7.06 at the commencement of the study. The final pH, which ranged from 6.89 to 7.27, did not differ from the initial pH, and this could be attributed to the continuous supply of artificial buffer in the RUSITEC system. In support, a meta-analysis showed that supplementation of Agolin® Ruminants (coriander seed oil, eugenol, geranyl acetate, geraniol, and fumaric acid) produced no significant variation in the rumen pH of Holstein dairy cows [32]. On the contrary, several in vitro batch culture studies on individual or blends of essential oils, with or without fumaric acid, reported significant differences in ruminal pH [5,27,31,33]. Essential oils (EOs) have been reported to elevate rumen pH, either numerically [9,23] or significantly [15] when compared to control diets [31]. This effect positions EOs as a viable strategy to mitigate ruminal acidosis in ruminants [34]. An in vivo study involving lactating Shame goats fed a Berseem hay and corn grain-based diet reported that the administration of 2 mL/goat/d of anise oil, thyme oil, or clove oil increased ruminal pH [15].

The final pH levels recorded in the present study surpassed those observed in lactating Shame goats (6.08 to 6.29) [15], lactating Holstein cows (6.26 to 6.41) [9], and dairy cows offered Agolin® Ruminants (6.46 to 6.78) [32]. This discrepancy may be attributed to the inherent ability of live animals to regulate saliva production through rumination time, in contrast to the continuous supply of artificial buffer in the RUSITEC system. Additionally,

rumen pH could be greatly influenced by the dosage of essential oils used, rate of VFA production, absorption of fermentation acids, and passage rate to the lower GI tract [15,34]. Castillejos et al. [35] reported a higher pH of 7.38 with a higher dose (5000 mg/L) of eugenol, thymol, guaiacol, limonene, and vanillin supplementation in a 24 h in vitro batch culture of a diet containing 60% alfalfa hay and 40% concentrate (corn grain, barley grain, soybean meal, and vitamin–mineral premix), but no effects were observed at lower doses. Additionally, the inclusion of higher levels of citral (0.05 and 0.1%) and sandalwood (3 and 5%) essential oils increased in vitro batch culture pH levels compared to pH values at lower doses [33]. Benchaar et al. [9] observed that pH tended to increase when 750 mg/d of a mixture of essential oils (thymol, eugenol, vanillin, guaiacol, and limonene) was supplemented in alfalfa silage or corn silage-based diets for Holstein dairy cows. However, the diets' effects and the diets by essential oil interactions were not significantly affected. Additionally, discrepancies in incubated substrate and incubation media volume between in vitro batch culture and the RUSITEC systems resulted in varying dosage per unit of substrate-to-inoculum media. Consequently, in in vitro batch culture, higher concentrations of EOBs or feed additives are frequently employed [31]. This probably accounts for notable differences in treatment effects observed between in vitro and in vivo scenarios, posing challenges in translating doses from one system, particularly from in vitro batch culture to RUSITEC, in vivo trials, and/or real-time farm situations.

Gas production serves as an indicator of the availability of degradable carbohydrates, especially cellulose, for enteric fermentation [36]. The non-significant effect of EFA on gas volume indicates that microbial fermentation was not impaired. This contradicts previous reports where a decline in gas production is typically concerned with the inclusion of EOB and/or FA in in vitro ruminal fermentation; however, the extent of this decline varies based on the dosage of inclusion and diet composition [19,20,31,37]. For instance, the synergy of thyme essential oil (300 µL/L) and disodium fumarate in a diet containing alfalfa hay and a concentrate reduced gas production by 6.5% [26]. Additionally, gas production decreased by 13.6 to 17.1% with 200 mg/L of essential oil active components, with or without fumarate [22]. Parra et al. [33] reported a 16% decrease in cumulative gas production with the highest dose of sandalwood (5% culture media) compared to the control. Given the similarity of the RUSITEC system to ruminal fermentation in live animals, the inclusion of either EFA1, EFA2, or EFA3 at the investigated levels could be incorporated into the diets of dairy cows without causing adverse effects on digestibility and gas production.

Ruminal NH_3-N concentration is a key indicator of converting dietary nitrogen into microbial N for optimal protein synthesis. Dietary protein degradability could be influenced by solubility, susceptibility to microbial protease, and residence time, thereby dictating the release pattern of peptides, amino acids, and NH_3 [38]. In the present study, all EFA treatments exerted similar effects on ruminal NH_3-N concentration. This implies that proteolysis, peptidolysis, deamination process, and metabolic activities of proteolytic bacteria, as well as hyper-ammonia-producing bacterial growth, were not impaired. Consistent with this observation, a previous study reported that supplementation of diet with a mixture of essential oils had no effect on the ruminal fluid concentration of NH_3-N [9]. Contrarily, the synergy of thyme essential oil and disodium fumarate significantly altered NH_3-N production [26], while a monosodium fumarate and essential oils combination reduced ammonia nitrogen in the rumen of Hu sheep [39].

Dry matter digestibility plays a crucial role in determining the nutritive value of feed and the bioavailability of inherent nutrients and provides valuable information for sustaining and enhancing the production performance of animals consuming the diets [30]. In the present study, EOB and FA inclusion did not suppress DMD and OMD. This implies that dairy cows would be able to adequately utilize the various nutrients in the feeds. This is an encouraging result in the use of EOB in ruminant feeding because previous studies involving essential oils (alone or as a blend, with or without FA) often observed a decrease in DMD compared with the control [5,20,27,31,35,40–42]. The present study demonstrated

that a combination of essential oil blends and fumaric acid can be supplemented in dairy cows' diets without compromising feed digestibility. Dose-dependent suppression of in vitro digestibility has been reported in which higher dosages of EOs or EOBs reduced DM digestibility [41–43]. The addition of *Tagetes minuta* and *Lippia turbinata* essential oils (EOs) at a dose of 300 µL/L resulted in a significant reduction in organic matter digestibility across breeding, rearing, fattening, and dairy diets [31]. Additionally, various combinations of three individual essential oils administered at a dosage of 0.8 mL/L in a total mixed ration consisting of corn silage, alfalfa hay, grass hay, and a concentrate mixture led to a decrease in in vitro dry matter digestibility [42]. In contrast to 24 h in vitro batch culture studies where essential oil blends were incorporated at a dosage of 100 µL/500 mg substrate (equivalent to 200 µL/g) [20,27], our current investigation introduced EOBs at a reduced dosage of 10 µL/g feed for a 48 h RUSITEC fermentation. This represents a substantial 20-fold reduction in the quantity used compared to those previous studies. This result infers that the synergy of EOBs at a lower dosage is sufficient to confer economical, nutritional, and environmental health benefits.

The inclusion of the various EOB and FA treatments exerted similar effects on the breakdown of different fiber fractions. The levels of degradation were higher for NDFD (64.8 to 65.6%) and ADFD (56.2 to 58.5%) compared to ADLD (23.7 to 26.1%) and CELD (30.9 to 32.9%) with somewhat reduced degradability, while the range of 7.08 to 8.60% for HEMD was extremely low. The reduction in HEMD values in the current study is lower than values from a previous study [27] from our lab and could be attributed to a reduction in the population [32] of cellulolytic bacteria and other generalist, non-cellulolytic microbes via extracellular enzymes [43] that are responsible for hemicellulose degradation. The strong negative correlation observed between ADLD and the digestibility of cellulose and hemicellulose supports the idea that increased digestion of cellulose and hemicellulose leads to a decrease in the non-digestible fraction of feed, which is primarily composed of lignin.

Microbial activity in the rumen of ruminants breaks down carbohydrates and converts them to VFAs, CO_2, CH_4, NH_3, and microbial cells [44,45]. VFAs are the principal source (70%) of metabolizable energy for ruminants, and they can significantly impact the fat and protein contents of meat and milk. Acetic (C_2), propionic (C_3), isobutyric, butyric (C_4), isovaleric, valeric (C_5), and caproic (C_6) acids are the most common VFAs [46]. Factors such as substrate composition, operational conditions, and microbial population in the anaerobic digestion system have been reported [47] to be responsible for the production of different ratios of VFAs. The control treatment exerted the greatest effect on the production of TVFA, acetate, and butyrate compared to others. This is an indication that the control group will generate more energy for microbial functions in the rumen and other bodily uses by the animals. Higher butyrate concentration by the control indicates that more energy will be provided for rumen epithelial cells and colonocytes, which are important mediators of water, mineral, and nutrient absorption [48]. Conversely, EFA (1-3) had an increased production of propionate compared to the control. Propionate in the rumen of ruminants is the main precursor required for gluconeogenesis in the liver, which supplies 32% to 73% of glucose demands [49], which essentially reverses glycolysis [50]. Therefore, all EFAs will allow the formation of glucose from non-hexose precursors for improved performance. The present result is contrary to a previous study by Benetel et al. [19], who reported that different essential oils (star anise, citronella, clove bud, globulus, staigeriana, ginger, Ho wood, melaleuca, oregano, and white thyme) did not have any effect on acetic, propionic, and butyric acids at 24 h of in vitro incubation. Differences in the inclusion levels of the EOs, the composition of blends, and the substrates could be responsible for these variations.

Increasing interest in the abatement of methane and other greenhouse gas emissions from ruminants has necessitated the use of phytogenics and their extracts to alleviate rumen methanogenesis and other gases being produced as products of fermentation [5,22,45]. Results show that EFA1 exerted a greater effect on methane compared to the control. The

effect of essential oils on methane gas production after 24 h of in vitro incubation by [19] is consistent with the present study. Compared with the control, the lowest value for methane was observed for EFA1, which reduced methane by 60.25%. This value was lower than the 90.77% reported by [27] for the same parameter. The discrepancy could be attributed to the fermentation methods (RUSITEC vs. in vitro batch culture) and the dosage of EOB used. Expectedly, the strong inverse relationship between propionate and the acetate:propionate ratio is a confirmation that an increase in propionate concentration will lower the acetate:propionate ratio, thereby increasing the energy efficiency in ruminants [20,21,36] since lower APR indicates less methane energy loss. The strong linear correlation between CH_4 and CO_2 is consistent with our expectation, as CO_2 reduction is a primary contributor to CH_4 emission in ruminants through the hydrogenotrophic methanogenesis pathway [4,6]. Consequently, an escalation in the production of CO_2 and H_2 during ruminal fermentation is likely to lead to a significant release of CH_4 unless measures are taken to divert H_2 towards propionate formation [25,51]. The linear correlation observed between CH_4 and NH_3 suggests that changes in methane gas levels correspond closely to variations in ammonia gas production. This finding aligns with the findings outlined in Table 5, where reductions in methane gas within the treatment group generally resulted in proportional decreases in ammonia gas, except for EFA1. The inverse relationship exhibited by both ADFD and ADLD on CH_4, CO_2, and NH_3 suggests that strategies that increase ADF and ADL digestibility would invariably minimize greenhouse gas emissions.

5. Conclusions

The present results revealed that all EFA treatments did not suppress gas production and feed digestibility. Furthermore, the synergy of EOB1 (10 μL/g) and FA (3%) is recommended as a suitable nutritional intervention to mitigate CH_4 emission. The findings suggest that a lower dosage of EOB is still effective in providing economical, nutritional, and environmental health benefits. This indicates its practical suitability for adoption by farmers seeking a balanced approach to enhancing feed quality and promoting environmental well-being. Future investigations should explore the reconfiguration of the proportions of examined EOBs to enhance both nutrient digestibility and VFA production.

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