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A Mixture of Prebiotics, Essential Oil Blends, and Onion Peel Did Not Affect Greenhouse Gas Emissions or Nutrient Degradability, but Altered Volatile Fatty Acids Production in Dairy Cows Using Rumen Simulation Technique (RUSITEC)

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Abstract: This study evaluated the synergistic effects of prebiotics containing galacto-oligosaccharides (GOS) and/or mannan oligosaccharides (MOS), essential oil blend (EOB), and onion peel (OPE) on fermentation characteristics using the rumen simulation technique (RUSITEC) system. Three rumen-cannulated, non-lactating Holstein Friesian cows were the inoculum donors. The substrate used for the study was a total mixed ration (TMR), which consisted of corn silage, alfalfa hay, and concentrate at 6:2:2, respectively. Sixteen fermentation vessels were randomly allotted to four treatments with four replicates each over a 9-day period in a completely randomized design. The treatments assessed include: control [TMR only], GEO [TMR + GOS + EOB + OPE], MEO [TMR + MOS + EOB + OPE], and OLEO [TMR + OLG + EOB + OPE]. OLG comprises GOS and MOS in equal proportion. EOB was included at 3 µL/g, while OPE, GOS, MOS, and OLG were added at 30 mg/g TMR. Results showed that pH, gas volume, effluent volume, and ammonia-N were not affected ($p > 0.05$) by the different additives. Similarly, greenhouse gas (GHG) emissions and nutrient digestibility were not affected by the treatments. Compared to the control, total volatile fatty acids (VFA) were decreased ($p < 0.05$) by 14.8, 10.8, and 8.5% with GEO, MEO, and OLEO inclusion, respectively, while the molar proportion of acetate was increased ($p = 0.011$) by 3.3, 1.1, and 3.8% with GEO, MEO, and OLEO inclusion, respectively. MEO increased isobutyrate ($p = 0.001$) and branched chain VFA ($p = 0.013$) contents; however, GEO and OLEO inclusion reduced them. Overall, the interaction of EOB, OPE, GOS, and/or MOS did not affect nutrient digestibility or GHG emissions but reduced VFA production. Further research is recommended to assess the dose effect of the additives on GHG emissions and VFA production; and to determine the long-term effects of these interventions on the rumen microbiome and animal performance.

Keywords: fermentation; methane; nutraceutical feed additives; phytochemical; ruminants

1. Introduction

Dietary manipulation represents a promising strategy to mitigate greenhouse gas (GHG) emissions from ruminant livestock production systems. Enteric methane (CH₄) emanating from rumen fermentation accounts for a considerable portion of total GHG emissions from the livestock sector [1,2]. Feed additives such as prebiotics, plant secondary

metabolites, and agricultural by-products have gained attention as potential dietary interventions to favorably modulate rumen fermentation, ameliorate GHG emissions, and improve nutrient use in ruminants [3].

Prebiotics are non-digestible feed ingredients that selectively stimulate the growth and activity of beneficial bacteria in the gastrointestinal tract [4]. Prebiotics like mannan oligosaccharides (MOS) and galacto-oligosaccharides (GOS) are selectively fermented by beneficial gut microbes, promoting their growth and activity [4–6]. Their inclusion in ruminant diets has been shown to alter rumen fermentation patterns, volatile fatty acid (VFA) production, and CH₄ output [7,8]. Dietary MOS supplementation in beef and dairy cows maintained higher body weight during parturition, increased colostrum production, enhanced immune response to rotavirus during the dry period, and improved transfer of rotavirus antibodies to calves [5,9,10]. In sheep, MOS increased ruminal pH, total VFA concentration, and reduced ruminal ammonia [8].

Essential oils (EOs) have gained considerable attention in animal science for their potential to improve animal health and performance [11,12], and manipulate rumen microbial ecology and fermentation [12,13]. They have shown potential for mitigating GHG emissions, particularly CH₄, while positively influencing VFA production in ruminants [13,14]. Combining individual EOs into unique essential oil blends (EOBs) harnesses their bioactive substances for effective CH₄ mitigation [11,15]. Notably, Blanch et al. [16] reported that 300 mg/L of an EOB containing cinnamaldehyde and garlic oils reduced CH₄ emission, modified VFA profiles, and increased propionate proportion [16]. Additionally, previous studies from our lab [17] showed that EOB containing garlic, lemongrass, cumin, lavender, and nutmeg in a ratio of 4:2:2:1:1, respectively, reduced in vitro CH₄ and carbon dioxide (CO₂) by 86.4 and 57.9%, respectively, compared to the control diet.

Agricultural by-products such as onion peel (OPE) represent sustainable sources of bioactive compounds like flavonoids (quercetin, kaempferol), phenolic acids (gallic acid, chlorogenic acid), and organosulfur compounds with potential antioxidant and antimicrobial effects in the rumen [18,19]. Onion peel, an abundant agricultural by-product, is a rich source of bioactive compounds, and these phytochemicals could modify the rumen microbiome and beneficially influence rumen fermentation dynamics due to their antimicrobial properties [20]. Almost no information is available on the inclusion of OPE in the diets of ruminants. Therefore, the impacts of supplementing OPE on rumen fermentation, greenhouse gas emissions, and nutrient utilization in ruminants warrant further investigation.

The combination of different feed additives has gained interest as a strategy to potentially achieve synergistic effects on rumen fermentation, nutrient utilization, and mitigation of environmental impacts like GHG emissions [17,21]. Patra and Yu [22] reported that combining essential oil compounds (eugenol and carvacrol) with fumarate resulted in greater reductions in CH₄ production compared to when each was used individually in an in vitro system. Similarly, Alabi et al. [17] and Ike et al. [21] reported that blends of essential oils along with fumaric acid decreased total VFA, CH₄, and hydrogen sulfide (H₂S) to a greater extent than EOB alone.

In another study, Lee [23] reported that supplementation with a synbiotic product containing a combination of a probiotic (bacteria, yeast, and molds) and MOS resulted in improved nutrient digestion and reduced GHG emissions.

Despite these promising results, it is important to note that the efficacy of the combinations may depend on factors such as the specific types and concentrations of additives, the basal diet composition, and the rumen microbial community structure. Moreover, the mechanisms underlying the potential synergistic effects are not fully elucidated and require further investigation. Therefore, the present study investigated the effects of prebiotics, essential oil blends, and onion peel supplementation on in vitro rumen fermentation characteristics, including GHG production, nutrient degradability, and VFA profile, using the rumen simulation technique (RUSITEC). The findings may provide insights into developing effective dietary strategies to mitigate GHG emissions while optimizing nutrient utilization

in ruminant production systems. We hypothesized that the biologically active compounds in the additives would work synergistically to affect ruminal fermentation and decrease GHG production.

2. Materials and Methods

2.1. Ethical Approval

The experimental protocols adhered to guidelines approved by the Institutional Animal Care and Use Committee at North Carolina A&T State University, Greensboro (protocol number LA22-0019). The dairy cattle were managed in accordance with the husbandry practices and animal welfare standards established for the university's farm facilities.

2.2. Substrate Preparation

The substrate used in the current study has been described previously [17].

2.3. Determination of Nutrient Composition

The ingredients and the total mixed ration (TMR) were analyzed using the standard procedures of AOAC [24] for DM (#930.15), ash content (#942.05), ether extract (EE; #920.39), and nitrogen (N; #954.01). Organic matter (OM) was estimated by subtracting ash content from DM and expressed in percentage. The neutral detergent fiber (NDF) was determined according to the procedure of Van Soest et al. [25], and thereafter, the acid detergent fiber (ADF; #973.18) content was analyzed using the ANKOM 200 Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY, USA). Acid detergent lignin (ADL) was determined by solubilization of cellulose with concentrated H_2SO_4 based on ANKOM Technologies analytical methods.

The nutritive values of feed ingredients and TMR were estimated using the following formulas: DM intake = $120/NDF$; DM digestibility = $88.9 - (0.779 \times ADF)$; Relative forage value = $(DM \text{ digestibility} \times DM \text{ intake}) \times 0.775$; Relative forage quality = $(DM \text{ intake} \times \text{total digestible nutrient})/1.23$; Total digestible nutrient = $104.97 - (1.302 \times ADF)$; Digestible CP = $(0.916 \times CP) - 3.09$; Gross energy = $(CP \times 0.056) + (EE \times 0.094) + (100 - CP - \text{ash} - EE) \times 0.042$; Digestible energy = $\text{gross energy} \times [70.19 - 1.364 \times (ADF - 29.83) + 0.104 \times CP + 0.149 \times EE + 0.022 \times NDF - 0.244 \times \text{ash}]/100$; Metabolizable energy = $\text{digestible energy} \times [86.38 - (9.9 \times \text{crude fiber} + 19.6 \times CP)/(100 - \text{ash})]/100$; Net energy of maintenance = $\text{metabolizable energy} \times (0.287 \times \text{metabolizable energy}/\text{gross energy} + 0.554)$; Net energy of gain = $\text{metabolizable energy} \times (0.78 \times \text{metabolizable energy}/\text{gross energy} + 0.006)$; Net energy of lactation = $\text{metabolizable energy} \times [0.6 + 0.24 \times (\text{metabolizable energy}/\text{gross energy} - 0.57)]$. Gross energy, digestible energy, metabolizable energy, net energy of maintenance, net energy of gain, and net energy of lactation were expressed in MJ/kg DM, while crude fiber, CP, EE, NDF, ADF, and ash were expressed in %. The chemical composition and nutritive values of ingredients and the total mixed ration are shown in Table 1.

Table 1. Nutrient composition of feed ingredients and total mixed ration *.

Variables	Corn Silage	Alfalfa Hay	Concentrate	TMR
Dry matter, %	38.13	83.88	90.76	68.12
Organic matter, %	96.49	90.95	83.28	93.08
Crude protein, %	6.31	16.00	20.25	10.03
Crude fat, %	4.67	3.15	8.49	4.73
Total ash, %	3.51	9.05	16.72	6.92
Neutral detergent fiber, %	59.40	49.47	74.05	61.86
Acid detergent fiber, %	14.10	9.56	15.38	12.04
Acid detergent lignin, %	14.51	18.24	10.45	13.70

Table 1. Cont.

Variables	Corn Silage	Alfalfa Hay	Concentrate	TMR
Dry matter intake	2.02	2.43	1.62	1.94
Dry matter digestibility	77.92	81.45	76.92	79.52
Relative forage value	122.01	153.14	96.61	119.57
Relative forage quality	142.28	182.47	111.92	140.85
Total digestible nutrients	86.61	92.52	84.95	89.29
Digestible crude protein	2.69	11.57	15.46	6.10
Gross energy, MJ/kg	4.38	4.21	4.22	4.30
Digestible energy, MJ/kg	3.82	4.08	3.75	3.94
Metabolizable energy, MJ/kg	3.14	3.35	3.08	3.23
Net energy of maintenance, MJ/kg	2.38	2.62	2.35	2.49
Net energy of gain, MJ/kg	1.77	2.10	1.77	1.92
Net energy of lactation, MJ/kg	1.99	2.19	1.96	2.08

* $n = 6$ replicates.

2.4. Ingredients and Experimental Design

The EO blend consisted of garlic, lemongrass, cumin, lavender, and nutmeg at 4:2:2:1:1, respectively. The EOB was selected based on its efficacy to reduce CH₄ production from previous studies [17,21]. The four treatments were investigated using a completely randomized design with four replicates:

- (1) Control [TMR only],
- (2) GEO [TMR + GOS + EOB + OPE],
- (3) MEO [TMR + MOS + EOB + OPE],
- (4) OLEO [TMR + OLG +EOB + OPE].

The OLG treatment consisted of GOS and MOS at 1:1 (v/v). The inclusion rate of EOB was 3 $\mu\text{L/g}$, while OPE, GOS, MOS, and OLG were added at 3 mg/g TMR.

For incubation, approximately 10 ± 0.2 g subsamples of the substrate (i.e., TMR) were weighed into pre-weighed Ankom Filter Bags (Ankom Technology Corp., Macedon, NY, USA) (70 mm $\times$ 140 mm, 150 μm pore size).

2.5. In Vitro RUSITEC Fermentation

The RUSITEC system used in the present study had two identical 8-chamber ($n = 16$ fermenters) units, and the general incubation procedure was as described in Alabi et al. [26]. The fermentation vessels had a 1000 mL capacity, with an inlet for buffer infusion and an effluent output port. The vessels were randomly divided into 4 treatment groups with 4 replicates each. Rumen inoculum was obtained from three non-lactating Holstein Friesian cows fitted with permanent ruminal cannula and fed the same TMR used as the substrate. Ruminal contents were collected from various parts of the rumen, strained through four cheesecloth layers into an insulated thermos, and immediately transported to the laboratory. The experiment lasted for 9 days, with 4 days for adaptation and 5 days for data collection.

2.6. In Vitro Fermentation

Total gas production was collected daily in Tedlar[®] gas sampling bags (Supelco[®], Bellefonte, PA, USA) connected to the effluent flasks. A DM3 gas flowmeter (Alexander Wright Ltd., London, UK) was used to estimate volume and expressed in mL/d. The GHG concentrations, including CH₄, CO₂, ammonia (NH₃), and H₂S, were estimated from the effluent flasks using a portable Biogas 5000 analyzer (Landtec, Dexter, MI, USA) as described in Alabi et al. [26].

The pH, NH₃-N, and VFA concentrations were determined as previously described in Alabi et al. [17].

2.7. Dry Matter and Fiber Fractions Digestibility

After 48 h of fermentation, the bags were removed from each RUSITEC fermenter and rinsed with cold tap water until the runoff was clear. The bags were then oven-dried at 55 °C for 72 h to estimate apparent dry matter digestibility [27,28]. Thereafter, the oven-dried residues remaining in each bag were subsequently used to determine the digestibility of the fiber fractions (NDF, ADF, and ADL), as previously described for the chemical analysis.

2.8. Statistical Analysis

Prior to analysis, all dependent variables were evaluated for normality of distribution. Data were then analyzed using the General Linear Model procedure in a one-way Analysis of Variance (ANOVA) using SAS Studio (SAS Institute Inc., Cary, NC, USA) according to the following statistical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} is the observed response variable, μ is the overall mean, T_i is the treatment effect (prebiotics, essential oil blend, and onion peel extract), and e_{ij} represents the residual error. For variables exhibiting significant effects ($p < 0.05$), means were compared and separated using Tukey's multiple comparison test.

3. Results

The pH, gas volume, volume of effluent, and $\text{NH}_3\text{-N}$ were not affected ($p > 0.05$) by GEO, MEO, and OLEO inclusion (Table 2).

Table 2. Effects of prebiotics, essential oil blends, and onion peel on pH and in vitro fermentation characteristics of the total mixed ration.

Treatments ¹	pH	Gas Volume (mL)	Volume of Effluent (mL)	Ammonia Nitrogen
Control	7.25	1648	438	433
GEO	7.30	1418	476	472
MEO	7.26	1487	441	503
OLEO	7.22	1537	479	445
SEM	0.047	128.6	28.6	77.3
<i>p</i> -value	0.634	0.640	0.620	0.923

EOB is essential oil blend, GOS is galacto-oligosaccharides, OPE is onion peel, MOS is mannan oligosaccharides, SEM is the standard error of the means, and TMR is total mixed ration. ¹ Control is the TMR without additives; GEO is the control diet supplemented with GOS, EOB, and OPE; MEO is the control diet supplemented with MOS, EOB, and OPE; and OLEO is the control diet supplemented with OLG, EOB, and OPE. OLG contains GOS and MOS in equal proportion. The inclusion rate of EOB was 3 $\mu\text{L/g}$, while OPE, GOS, MOS, and OLG were added at 3 mg/g TMR.

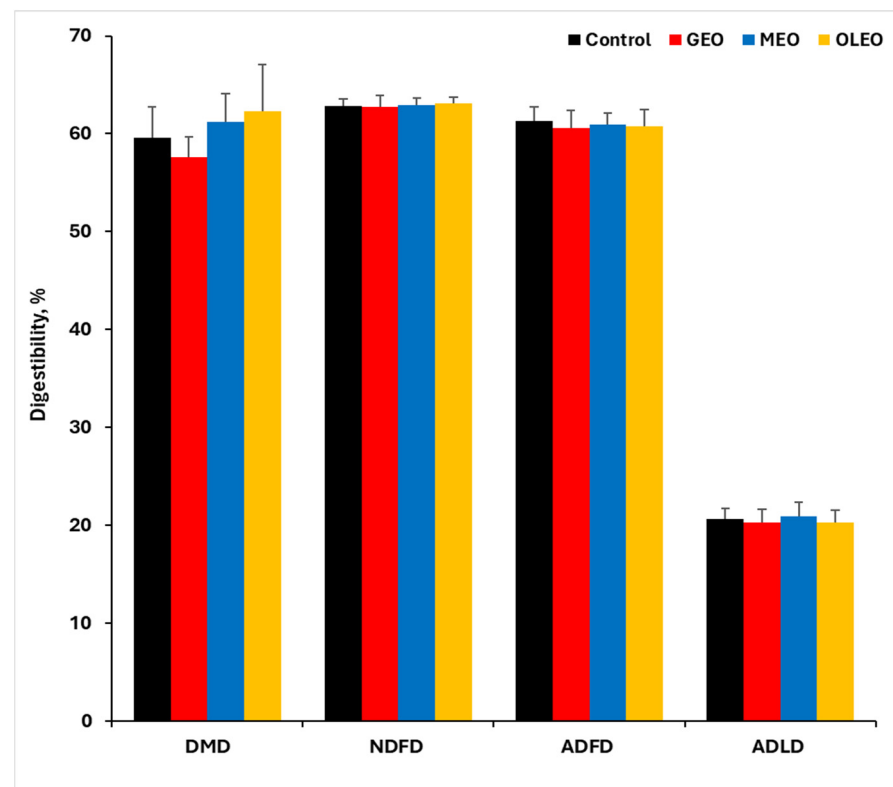
The addition of GEO, MEO, and OLEO produced no significant effect on CH_4 , CO_2 , NH_3 , and H_2S production ($p > 0.05$) compared to the control group (Table 3). Figure 1 shows the effect of the additives on DM and fiber fraction digestibility. The results showed that DM digestibility (DMD), NDF digestibility (NDFD), ADF digestibility (ADFD), and ADL digestibility (ADLD) were not affected ($p > 0.05$) by the additives compared to the control diet.

The inclusion of GEO, MEO, and OLEO decreased ($p < 0.05$) total VFA by 14.8, 10.8, and 8.5%, respectively, while the molar proportion of acetate was increased ($p = 0.011$) by 3.3, 1.1, and 3.8% with GEO, MEO, and OLEO inclusion, respectively (Table 4). MEO increased ($p < 0.001$) isobutyrate contents by 3%, but the same VFA was decreased by 6.1% and 4.4% in the GEO and OLEO treatments, respectively. The branched chain VFA (BCVFA) increased ($p = 0.013$) by 2.9% with MEO administration, whereas GEO and OLEO reduced BCVFA by 9.7 and 6.0%, respectively. Higher ($p = 0.050$) acetate/propionate ratios (APR) of 6.4, 3.7, and 10.8% were observed in GEO, MEO, and OLEO treatments, respectively.

Table 3. Effects of prebiotics, essential oil blends, and onion peel on greenhouse gas production in the total mixed ration.

Treatments ¹	Methane (mg/g DM)	Carbon Dioxide (mg/g DM)	Ammonia (mmol/g DM)	Hydrogen Sulfide (mg/g DM)
Control	64.1	297	433	3574
GEO	51.3	234	472	5852
MEO	61.3	289	503	6658
OLEO	63.6	300	445	4397
SEM	10.06	39.8	77.3	1103.1
<i>p</i> -value	0.786	0.617	0.923	0.198

EOB is essential oil blend, GOS is galacto-oligosaccharides, OPE is onion peel, MOS is mannan oligosaccharides, SEM is the standard error of the means, and TMR is total mixed ration. ¹ Control is the TMR without additives; GEO is the control diet supplemented with GOS, EOB, and OPE; MEO is the control diet supplemented with MOS, EOB, and OPE; and OLEO is the control diet supplemented with OLG, EOB, and OPE. OLG contains GOS and MOS in equal proportion. The inclusion rate of EOB was 3 µL/g, while OPE, GOS, MOS, and OLG were added at 3 mg/g TMR.

**Figure 1.** Effect of GEO, MEO, and OLEO on dry matter and fiber fraction digestibility.

The correlation analysis revealed several significant relationships between fermentation characteristics (Table 5). Notable correlations include an inverse correlation ($p < 0.001$) between pH and CH₄ ($r = -0.71$), CO₂ ($r = -0.72$), NH₃ ($r = -0.61$), and H₂S ($r = -0.52$). Also, pH was inversely correlated with gas volume ($r = -0.58$; $p < 0.001$), total VFA ($r = -0.57$; $p < 0.001$), and acetate ($r = -0.33$; $p < 0.01$), but linearly correlated ($p < 0.001$) with butyrate ($r = 0.42$), iso-butyrate ($r = 0.44$), valerate ($r = 0.52$), isovalerate ($r = 0.59$), and BCVFA ($r = 0.52$). Meanwhile, gas volume and effluent volume exhibited a linear correlation ($p < 0.01$) with CH₄, CO₂, NH₃, and H₂S. Additionally, total VFA had positive correlations ($p < 0.05$) with CH₄, CO₂, NH₃, and H₂S. Acetate had a weak linear relationship ($p < 0.05$) with CH₄ ($r = 0.27$) and CO₂ ($r = 0.28$). Propionate had a weak linear correlation ($p < 0.05$) with DMD ($r = 0.28$), CH₄ ($r = 0.26$), and NH₃ ($r = 0.29$). Butyrate demonstrated inverse correlations with DMD ($r = -0.34$; $p < 0.01$), CH₄ ($r = -0.40$; $p < 0.001$), CO₂ ($r = -0.40$;

$p < 0.001$), and NH_3 ($r = -0.32$; $p < 0.01$). ADLD had a linear relationship ($p < 0.01$) with iso-butyrate ($r = 0.38$) and BCVFA ($r = 0.36$).

Table 4. Effects of prebiotics, essential oil blends, and onion peel on total (mmol/g DM) and molar proportion (%) of VFA production (mM, except APR) of the total mixed ration.

Treatments ¹	VFA	C ₂	C ₃	C ₄	Iso-C ₄	C ₅	Iso-C ₅	BCVFA	C ₂ :C ₃
Control	62.1 ^a	53.7 ^b	27.3	14.5	0.482 ^{ab}	3.85	0.181	0.6615 ^{ab}	1.99 ^b
GEO	52.9 ^b	55.5 ^{ab}	26.4	14.1	0.427 ^c	3.43	0.170	0.5975 ^b	2.11 ^{ab}
MEO	55.4 ^{ab}	54.3 ^{ab}	26.5	14.5	0.496 ^a	4.07	0.186	0.6805 ^a	2.06 ^{ab}
OLEO	56.8 ^{ab}	55.7 ^a	25.7	14.1	0.449 ^{bc}	3.84	0.173	0.6215 ^{ab}	2.20 ^a
SEM	2.36	0.48	0.52	0.32	0.0122	0.253	0.0078	0.01925	0.056
<i>p</i> -value	0.045	0.011	0.215	0.676	0.001	0.339	0.435	0.013	0.050

BCVFA is branched chain VFA, C₂ is acetate, C₂:C₃ is acetate/propionate ratio, C₃ is propionate, C₄ is butyrate, C₄ is iso-butyrate, EOB is essential oil blend, GOS is galacto-oligosaccharides, Iso-C₅ is iso-valerate, MOS is mannan oligosaccharides, OPE is onion peel, SEM is the standard error of the means, TMR is total mixed ration, and VFA is the total volatile fatty acids. ¹ Control is the TMR without additives; GEO is the control diet supplemented with GOS, EOB, and OPE; MEO is the control diet supplemented with MOS, EOB, and OPE; and OLEO is the control diet supplemented with OLG, EOB, and OPE. OLG contains GOS and MOS in equal proportion. The inclusion rate of EOB was 3 $\mu\text{L/g}$, while OPE, GOS, MOS, and OLG were added at 3 mg/g TMR. Means with different superscripts within the same column differ, $p < 0.05$.

Table 5. Pearson correlation coefficients between fermentation characteristics, greenhouse gases, and volatile fatty acid production.

Variables	pH	DMD	NDFD	ADFD	ADLD	CH ₄	CO ₂	NH ₃	H ₂ S
pH	1.00	−0.16	0.05	0.06	0.03	−0.71 ***	−0.72 ***	−0.61 ***	−0.52 ***
Gas volume	−0.58 ***	0.02	0.17	0.15	0.00	0.91 ***	0.93 ***	0.79 ***	0.61 ***
Effluent volume	−0.56	0.01	−0.10	0.01	−0.13	0.42 ***	0.37 **	0.41 ***	0.31 **
NH ₃ -N	0.01	0.07	−0.21	0.00	0.18	0.09	0.07	0.13	0.11
TVFA	−0.57 ***	0.09	−0.11	−0.23	0.22	0.45 ***	0.47 ***	0.34 **	0.23 *
Acetate	−0.33 **	0.00	0.02	−0.03	−0.18	0.27 *	0.28 *	0.21	0.17
Propionate	−0.21	0.28 *	−0.11	−0.03	0.13	0.26 *	0.21	0.29 *	0.19
Butyrate	0.42 ***	−0.34 **	0.09	−0.04	0.07	−0.40 ***	−0.40 ***	−0.32 **	−0.19
Iso-butyrate	0.44 ***	0.05	0.08	−0.07	0.38 **	−0.10	−0.14	0.02	−0.06
Valerate	0.52 ***	−0.15	0.08	0.15	−0.15	−0.52 ***	−0.47 ***	−0.54 ***	−0.36 **
Isovalerate	0.59 ***	−0.12	−0.03	−0.18	0.28	−0.30 **	−0.33 **	−0.17	−0.17
BCVFA	0.52 ***	−0.02	0.05	−0.11	0.36 *	−0.18	−0.22	−0.05	−0.10
APR	0.03	−0.25 *	0.08	0.00	−0.14	−0.06	−0.02	−0.11	−0.06

ADFD is acid detergent fiber digestibility, ADLD is acid detergent lignin digestibility, and BCVFA is branched chain volatile fatty acids. C₂ is acetate, C₂:C₃ is the acetate/propionate ratio, C₃ is propionate, C₄ is butyrate, C₄ is iso-butyrate, DMD is dry matter digestibility, EOB is essential oil blend, GOS is galacto-oligosaccharides, Iso-C₅ is iso-valerate, MOS is mannan oligosaccharides, NDFD is neutral detergent fiber digestibility, NH₃-N is ammonia-N, OPE is onion peel, SEM is the standard error of the means, TMR is the total mixed ration, and VFA is the total volatile fatty acids. * Correlation is significant at $p < 0.05$ level; ** $p < 0.01$ level; *** $p < 0.001$ level.

4. Discussion

In the present study, the combination of essential oils and prebiotics like MOS or GOS, or plant extracts such as OPE, was explored as a strategy to potentially enhance their individual effects on rumen fermentation, nutrient utilization, and greenhouse gas mitigation. The mechanisms behind the potential synergistic effects of essential oils and prebiotics/plant extracts are not fully understood but may involve complementary modes of action. Combinations leveraging complementary modes of action show promise for improving ruminant production efficiency while mitigating environmental impacts [17,21]. Essential oils can directly inhibit certain rumen microbial groups, while prebiotics and plant compounds selectively promote the growth of beneficial microbes [13,22,29]. Additionally, prebiotics may mitigate the potential negative impacts of essential oils on fiber-degrading bacteria [5,30]. Onion peel, rich in bioactive compounds such as flavonoids and phenolics, has shown antimicrobial and antioxidant properties [19]. The potential health benefits of OPE, including its antimicrobial effects, could contribute to improved rumen health and fermentation efficiency [18]. Contrary to expectations, no synergistic effect was observed

with the combinations in the present study. However, it is important to note that the efficacy of these combinations can be influenced by many factors, such as the specific types and concentrations of essential oils, prebiotics, and plant extracts used, administration dosages, diet composition, rumen microbial community structure, and experimental conditions [31].

In the present study, pH values ranged from 7.22 to 7.30 across the treatments, indicating minimal effects on ruminal fermentation since it is associated with gas production, DMD, and VFA concentration [32]. Abnormal ruminal pH, resulting from dysfunctional ruminal acid metabolism, can adversely impact the microbial community structure and lead to life-threatening metabolic disorders, such as acidosis [32].

In agreement with the current results, Khorrami et al. [33] reported that ruminal pH was not altered with the inclusion of thyme and cinnamon in the diet of Holstein steers. Similarly, another meta-analysis including 34 *in vivo* experiments confirmed that essential oil had no effect on ruminal pH, regardless of the inclusion level [34]. Ruminal fluid pH is a crucial factor influencing microbial fermentation activity and metabolism in the rumen [35].

Gas production is an indicator of the availability of fermentable carbohydrates for enteric fermentation in ruminants and can be influenced by several factors, including diet composition, rumen microbiome composition, and metabolic activity, as well as the types and dosages of feed additives used [15,36]. Monitoring gas production can provide insights into the dynamics of ruminal fermentation and the potential impacts of dietary interventions or feed additives. The lack of significant changes in gas production and effluent volume, in the present study, suggests that these interventions are safe for use in ruminant diets without causing adverse effects on rumen fermentation [26]. This observation is consistent with a previous study from our lab in which EOB and fumaric acid inclusion in the diets of dairy cows had no adverse effects on gas production [26].

GHG emissions from livestock production, accounting for around 15% of global emissions, were a substantial contributor to climate change in the Anthropocene era [37]. Noteworthy are the CH₄ emissions, which has a higher (28 times) global warming potential than CO₂ [2]. In ruminants, enteric CH₄ production leads to dietary gross energy loss of 2–15%, which negatively impacts both the environment and the productivity and profitability of ruminant farming operations [17,38]. In the present study, the addition of GEO, MEO, and OLEO had no significant effect on CH₄, CO₂, NH₃, and H₂S production. This contradicts a previous study from our lab in which a combination of EOB containing garlic, lemongrass, cumin, lavender, and nutmeg and fumaric acid significantly reduced CH₄ gas emissions by 60% [26]. This could be attributed to the low dosage of EOB (3 µL/g) used in this present study, which is much lower (3 µL/g in the present experiment vs. 10 µL/g in the previous one) than that used in the previous study [26]. Previous studies have shown that the effects of EOs on GHG mitigation are largely dose-dependent, with higher dosages exerting a greater GHG suppression effect [15,16].

Several studies have investigated the potential synergistic effects of combining essential oils, fumarate, prebiotics, and other feed additives on rumen fermentation and GHG mitigation [21,39,40]. Yatoo et al. [41] reported a 14% reduction in CH₄ production in EOB treated groups compared to the control group. Similarly, Patra et al. [42] observed a 23.5% reduction in CH₄ production in sheep by combining *Terminalia chebula*, which is rich in tannin, and *Allium sativum*, which contains essential oils. Baraz et al. [39] found that combining thymol essential oil with disodium fumarate resulted in greater reductions in CH₄ production compared to either compound alone in an *in vitro* system. Building on this, Alabi et al. [17] reported that the combination of EOB and fumaric acid decreased CH₄ and CO₂ emissions in an *in vitro* fermentation of beef diet. Furthermore, the addition of fumarate to the active components of the EOB, which include eugenol, carvacrol, citral, and cinnamaldehyde, further decreased CH₄ and increased total VFA in comparison with the sole EOB. Rossi et al. [31] evaluated a blend of essential oils, bioflavonoids, and tannins in dairy cows and reported a tendency for lower CH₄ emissions. This may be due to the antimicrobial effects of the additives to modulate rumen fermentation. Similarly, Coşkun-

tuna et al. [38] reported that adding lavender meal and lavender essential oil to the diets of dairy cows reduced CH₄ yield.

Dry matter and fiber digestibility were not affected by GEO, MEO, and OLEO inclusion compared to the control diets. In agreement, Yatoo et al. [41] reported similar digestibility for DM, NDF, and ADF in growing male buffaloes fed diets containing EOB (equal proportion of ajwain oil, garlic oil, and cinnamon leaf oil). In addition, Khorrami et al. [33] reported that supplementation of thymol and cinnamon oil at 500 mg/kg DM had no effect on DM, NDF, or ADF digestibility in Holstein steers. However, inclusion of MOS at 1.6% in the diet of Hu rams for 7 weeks increased DM, NDF, and ADF digestibility [5]. Additionally, Patra et al. [42] observed an improvement in the DM, OM, NDF, ADF, and cellulose digestibilities in sheep fed diets containing a mixture of *T. chebula*, which is rich in tannin, and *A. sativum* essential oil. Liu et al. [43] reported that the administration of essential oils and prebiotics to a starter feed at 44 mg/calf/day improved calf growth, ruminal development, gut health, nutrient digestibility, and immunity.

VFA is one of the crucial products of microbial fermentation in the rumen, supplying metabolites for energy and other metabolic processes, and accounts for 50–75% of the total energy requirements [44,45]. Results of VFA production are paralleled with those of the rumen pH since VFA production typically reduces ruminal pH for a few hours after feeding, followed by an increase as the VFAs are absorbed. Monitoring VFA profiles provides insights into ruminal fermentation dynamics and energy availability for the animal. In the present study, GEO, MEO, and OLEO inclusion decreased total VFA by 14.8, 10.8, and 8.5%, respectively. This could result from the complex interaction of the additives with microbial activity during ruminal digestion and the metabolic pathways that are responsible for VFA production. Previously, a meta-analysis study showed that dietary supplementation with EOs reduced rumen total VFA concentration, indicating that essential oils reduce energy availability in beef cattle [29], as high essential oil administration reduces nutrient degradability and energy availability [21,26]. Meanwhile, feeding a TMR containing 50:50 forage concentrate supplemented with EOB, which consisted of thyme, clove, and cinnamon, to fistulated sheep produced no significant effect on total VFA concentration, the molar proportion of individual VFA, or the acetate/propionate ratio [46]. Increasing VFA concentration involves creating conditions that are unfavorable for methanogens, as they are VFA utilizers, and the strategies to inhibit methanogen populations include adjusting pH or using chemical inhibitors such as 3NOP or 2-bromoethanesulfonate [44].

The acetate proportion was increased by 3.3, 1.1, and 3.8% with GEO, MEO, and OLEO inclusion, respectively. The increase in acetate production observed in the present study suggests a shift towards acetogenic pathways, which could enhance milk fat synthesis in dairy cows, as acetate is a precursor for lipogenesis. According to Urrutia and Harvatiné [47], acetic acid can be converted into acetyl coenzyme A and directly enter the tricarboxylic acid cycle to participate in fat synthesis, thereby increasing milk fat by increasing *de novo* fatty acid synthesis. However, an increase in acetate content could result in a potential increase in CH₄ production, as acetate is a primary precursor for methanogenesis [48]. This is because acetate formation releases H⁺ that can be utilized by methanogens to reduce CO₂ to CH₄ [49]. The absence of significant variation in CH₄ production could be due to the complex interactions between the feed additives used in the present study and the rumen microbiota. In a previous study, Rossi et al. [31] used a coated blend of essential oils from cloves (*Syzygium aromaticum*), coriander seed (*Coriandrum sativum*), and geranium (*Pelargonium cucullatum*), condensed tannins from chestnuts (*Castanea sativa*), and bioflavonoids from olives (*Olea europea*) and reported higher propionate and lower acetate proportions in the ruminal fluid of donor cows after *in vitro* incubation. Vargas et al. [50] investigated two diets (high forage and concentrate) and two pH levels with a RUSITEC system and reported that diet had no significant influence on acetate and CH₄ production, while decreasing rumen pH from 6.8 to 6.4 reduced feed digestibility and daily outputs of fermentation end-products (gas, VFA, acetate, and NH₃). Additionally, Yi et al. [51] observed that adding capsaicin and *Yucca schidigera* extract to beef cattle diets favorably

altered rumen VFA profiles with a decrease in acetate and isobutyrate proportions without affecting the propionate proportion.

Both isobutyrate and BCFVFA levels increased with MEO administration; however, these decreased with GEO and OLEO inclusion. The increased isobutyrate levels with the MEO treatment may indicate enhanced microbial protein synthesis, as isobutyrate is involved in amino acid synthesis by the rumen microbes. The lower isobutyrate proportion observed with the GEO treatment is consistent with a previous report that prebiotics modulate BCFVFA production [22]. BCFVFAs, such as isobutyrate and isovalerate, are derived from the deamination of branched-chain amino acids. They serve as the primary carbon sources for microbial growth in the rumen [52]. A reduction in BCFVFAs may indicate altered proteolytic activity in the rumen [53]. A decrease in BCFVFAs could limit the availability of carbon skeletons necessary for microbial protein synthesis, as BCFVFAs are the main carbon chain sources supporting microbial growth [54]. The varying VFA profiles observed with the feed additives affirm that the efficacy of these additives in modifying VFA production is dependent on several factors, such as the composition and inclusion levels of the additives, duration of administration, diet or substrate used, the composition of the rumen microbiome, and the overall feeding or experimental conditions employed [44].

Although EOB, OPE, and oligosaccharides had limited or weak effects on most of the parameters in the present study, they still offer potential benefits in ruminant feeding. More studies, mainly with different levels of administration, different treatment periods, different diets, and different animal species, should be considered. The dose of EOB (3 $\mu\text{L/g}$ of feed) used in the present study can be considered to be very low compared to the amount reported by Ike et al. [21] (200 $\mu\text{L/g}$ of feed), Alabi et al. [17], and Brice et al. [11] (100 $\mu\text{L/g}$ of feed), as well as Alabi et al. [26] (10 $\mu\text{L/g}$ of feed). The duration for which the additives were applied may be considered another reason for the weak effects of the additives. Ruminal microbes always develop adaptation mechanisms to recover their original composition after a period of exposure to a particular treatment, which is known as additive–microbes interaction [13,55]. In the present study, the period of treatment was 9 days, compared to 24 h in other studies [11,17,21], which could allow the microbes to adapt to the additives. It should be emphasized that any adaptation to the additives in the RUSITEC system will differ from live animals, which always absorb the fermentation byproducts into other parts of the digestive system or excrete them out.

Additionally, more research is needed to fully understand the long-term effects, combination, and optimal dosage of the evaluated additives in ruminal microbiomes and metabolomics for better understanding the mechanisms by which such additives affect or do not affect ruminal fermentation and feed digestion.

5. Conclusions

Overall, the results suggest that while the additives did not significantly impact most of the fermentation characteristics, they exhibited selective effects on VFA production and profile. These findings contribute to the growing body of knowledge on the potential use of prebiotics, essential oils, and onion peel as rumen modifiers, with implications for improving ruminant productivity, nutrient utilization, and environmental sustainability. However, more research is still needed at different levels of administration to optimize effective combinations and inclusion levels that would minimize greenhouse gas emissions, elucidate mechanisms of action, and assess their practical applications across various production systems. Furthermore, the economic feasibility and practical implementation of these blends of additives in animal production systems should be considered.

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