Comparative Properties of Anise, Clove, Oregano and Peppermint Essential Oils used Individually or Combined on Nutrient Digestibility and Greenhouse Gas Emissions in Concentrate - and Fiber-Based Diets

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Summary

The current study investigated the effects of four individual essential oils namely anise, clove, oregano, and peppermint or their blends (EOB), respectively at 1:1:1:1 (EOB1), 1:2:3:4 (EOB2), 2:3:4:1 (EOB3), 3:4:1:2 (EOB4), or 4:1:2:3 (EOB5) on nutrient digestibility and greenhouse gas emissions (GHG) using high concentrate (HC) and high forage (HF) diets incubated for 24 h at 39 °C. Diet samples (500 ± 10 mg) and additives were placed into 100 mL serum bottles and rumen fluid obtained from two cannulated beef cattle. Inclusion of individual or EOB reduced ($P < 0.001$) total gas production, GHG, and *in vitro* dry matter degradability. The inclusion of oregano reduced total gas production by 95.8% and 96.7% in HC and HF diets, respectively, while EOB3 suppressed total gas production by 90.4% and 92.3% in HC and HF. When compared to the control, the oregano treatment suppressed methane (CH_4) by 99.8% and 99.9% in HC and HF, respectively while EOB3 suppressed CH_4 by 99.6% and 99.8% in HC and HF. In comparison to the control, anise reduced dry matter digestibility by 11.4% and 8.7% in HC and HF, respectively. The EOB3 treatment reduced ($P < 0.001$) fiber degradability in the HC diet, while clove and oregano reduced fiber degradability in the HF diet. Individual essential oils or EOB decreased ($P < 0.001$) the production of volatile fatty acids from both diets. It is concluded that individual essential oils or their blends may be used as feed additives to reduce GHG production from ruminants. However, the use of essential oils may be combined with other feed additives to improve nutrient degradability and ruminal fermentation.

Key words

Essential oils, greenhouse gas emissions, nutrient digestibility, ruminants

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Introduction

As the world population steadily increases so does the demand for meat and dairy products that are sourced from beef and dairy cattle. To meet this demand, there will be an increase in livestock production which will substantially increase greenhouse gas (GHG) emissions. Twine (2021) reported a rise in the contribution of the livestock industry to GHG emissions from 14.5% to 16.5%. Prominent GHGs include methane (CH_4), carbon dioxide (CO_2), and nitrous oxide originating from ruminal fermentation and management of manure, exacerbating global warming (U.S. EPA, 2024). Methane, a byproduct of enteric fermentation, results from the degradation of plant materials in the rumen, thus contributing to GHG emissions. Essential oils have been identified to offer a promising solution to address environmental concerns and combat antibiotic resistance (Ike et al., 2024; Kholif and Olafadehan, 2021).

There has been an increase in the usage of essential oils as feed additives in recent times because of their ability to enhance animal performance and decrease CH_4 production (Ike et al., 2024; Króliczewska et al., 2023). To determine how essential oils should be used, researchers are focusing on studying the mode of action to have a clearer understanding of the synergistic impact of individual and blends of essential oils on certain parameters like nutrient utilization (Kiran and Deswal, 2020). Individual or essential oil blends (EOB) contain many biologically active components that may affect rumen fermentation to affect nutrient degradability and decrease ruminal CH_4 and ammonia (NH_3) production (Foggi et al., 2024; Zhou et al., 2020). The biologically active components in essential oils confer high antibacterial ability on essential oils, making them suitable as feed additives. Blending more than one essential oil is a new strategy to utilize the synergism between different essential oils with distinct molecule configurations and biological activity due to their extraction from various plant sources. These substances serve as viable alternatives to antibiotics (Al-Suwaiegh et al., 2020; Kholif and Olafadehan, 2021). Moreover, blending essential oils can overcome possible adverse effects associated with some individual essential oils on ruminal fermentation leading to enhanced alteration of rumen fermentation as a result of a greater variety of bioactive substances (Al-Suwaiegh et al., 2020; Amin et al., 2021). The report of Ike et al. (2024) had it that an EOB at 4:2:2:1:1 for garlic, lemongrass, cumin, lavender, and nutmeg included at 200 µL/g feed, reduced neutral detergent fiber (NDF) degradability (*d*NDF) and gas and CH_4 production, but increased microbial mass (MM), undegraded DM and ruminal propionate production.

The study assumed that essential oil would influence the activity of ruminal microbes and fermentation kinetics to reduce GHG production. Thus, the focus of this study is to observe the impacts of individual anise, clove, oregano, and peppermint or their blends on improving nutrient retention and reducing greenhouse gas emissions of diets containing concentrate and forage using the *in vitro* batch culture technique over a 24-h incubation period.

Materials and Methods

Study Location and Animals

The study was conducted in the Department of Animal Sciences, North Carolina A&T State University. Two cannulated beef cows from North Carolina A&T Beef Unit were used for this study. The cows were in a good state of health, disease-free, and maintained according to the IACUC-approved protocol LA22-0019. The beef cattle were fed free-choice grass hay and had access to a mixed grass pasture.

Feed Sample Processing and Chemical Analysis

Samples of two diets based on corn silage [i.e., considered as high forage (HF) or concentrates (i.e., considered as the high concentrate (HC)] were obtained from NC A&T Farm (Table 1). Samples were processed for analysis as previously reported (Alabi et al., 2024; Ike et al., 2024). Dry matter (DM; #930.15), nitrogen (N; #954.01), and ether extract (EE; #920.39) were analyzed according to AOAC (2019). The NDF and acid detergent fiber (ADF) were analyzed using Ankom 200 Fiber Analyzer (Ankom, Macedon, USA) according to Van Soest et al. (1991) and AOAC (2019) (method 973.18), respectively using heat stable alpha-amylase and sodium sulfite for the NDF.

Table 1. Chemical composition (% dry matter) of the substrates (i.e., diets) used in ruminal *in vitro* incubation

Parameters	High Forage	High Concentrate
Dry matter, fresh weight	96.2	95.8
Crude protein	6.72	16.6
Ether extract	5.95	5.60
Neutral detergent fiber	44.7	32.0
Acid detergent fiber	21.3	13.7
Acid detergent lignin	1.67	2.86

Ingredients and Treatments

Before the start of the study, clean 100 mL serum bottles were arranged and labeled using a permanent marker. After labeling the required number of bottles, Ankom F57 (Ankom Technology Corp., Macedon, USA) filter bags were labeled using a black Lumocolor permanent special pencil, soaked in acetone for 5 minutes, and placed under the fume hood to dry for 20 minutes, then oven-dried at 55 °C for 4-h. The bags were taken out of the oven after 48 h, and placed in the desiccator for about 15 minutes. Thereafter, they were individually removed from the desiccator using a tweezer and weighed using a digital scale. The substrates (0.5 ± 0.05 g each of HC and HF) were put in the filter bags and then sealed using a heat sealer. Immediately after sealing the bags, they were placed into their designated 100 mL serum bottle.

Four essential oils (i.e., anise, clove, oregano, and peppermint) were used individually or blended into five EOBs with different proportions. EOB1 was formulated from the previously mentioned individual essential oils respectively, using a 1:1:1:1 ratio, a 1:2:3:4 ratio for EOB2, a 2:3:4:1 ratio for EOB3, a 3:4:1:2 ratio for EOB4, and a 4:1:2:3 ratio for EOB5. The EOBs were mixed together before starting the incubation, and then pipetted into the serum bottles using a micropipette.

Batch Culture, Degradability and Measurement of Gases

This study utilized a 2 × 10 factorial design with 10 treatments and 2 diets. First, 100 µl of each essential oil and their blends were carefully pipetted onto the substrate bags. Preparation, dispensing of artificial saliva and rumen fluid, and initiation of fermentation have been extensively described (Alabi et al., 2024; Ike et al., 2024). Artificial saliva was prepared according to McDougall's buffer recipe containing the following components per liter: 9.83 g $NaHCO_3$, 3.69 g Na_2HPO_4, 0.60 g KCl, 0.47 g NaCl, 0.30 g $(NH_4)_2SO_4$, 0.061 g $MgC_{12}.6H_2O$, 0.0293 g $CaC_{12}.2H_2O$. The buffer was maintained at 39 °C in a water bath. It was then mixed with ruminal fluid in a 3:1 (v/v) ratio, and the pH was measured using a bench top pH meter (model B10P, VWR International, Randor, PA, USA). Thereafter, 60 mL of the artificial saliva and ruminal liquor were dispensed into the serum bottles containing the substrate (Anele et al., 2014). The serum bottles were sealed with butyl rubbers stoppers, crimped with aluminum seals and incubated in an orbital shakers set to 39 °C and 125 rpm for 24 h. All treatments were evaluated in two separate runs, each consisting of 4 replicates. Additionally, during each incubation run, 4 bottles containing only the buffered inoculum (blanks) were included to establish baseline fermentation gas production.

The fermentation was stopped after 24 h by placing the bottles on ice for 5 min. Gas production was measured at the end of incubation by piercing the rubber stopper with a 22 mm gauge needle fitted to a digital gas pressure manometer (VWR International, USA). Gas production was calculated using a regression equation derived from the relationship between gas volume and pressure: $V = 4.974 \times p + 0.171$ (n = 500; $R^2 = 0.98$; where: V is a gas volume (mL); p is the measured pressure (psi). Blanks were included to account for gas production from the buffered inoculum, and corrected gas pressure values were used to estimate the gas production (Mauricio et al., 1999).

Following gas measurements, a table-top gas analyzer (Biogas 5000, Landtec, Dexter, MI, USA) was used to measure CH_4, CO_2, NH_2, and H_2S concentrations. An aliquot of the gas from each bottle was introduced into the analyzer with a 22 G × 1 ½ (0.7 mm × 40 mm) gauge needle attached to the end of the inlet tygon tube. The analyzer unit was purged between sampling to eliminate any residual gas from the previous sample. The analyzer was calibrated according to the manufacturer's instructions.

Nutrient Degradability and Microbial Mass Measurement

After measuring the gas pressure, liquid content was transferred into centrifuge tubes and centrifuged for 15 minutes at 10,000 rpm, and filter bags were removed from all the bottles and rinsed thoroughly under a continuous flow of cold water until the water was clear. The filter bags were oven-dried at 55 °C for 48 h for apparent DM degradability (*d*DM). *In vitro* apparent degradable DM (IVADDM) and *in vitro* true degradable DM (IVTDDM) were calculated following the methodology outlined by Anele et al. (2014). The oven-dried residues remaining in each bag were subsequently used to determine the digestibility of fiber fractions (NDF, ADF, and ADL) following the ANKOM Fiber Analyzer procedures (ANKOM Technology, Macedon, NY, USA), as previously outlined in the chemical analysis of the diets. Degradabilities of NDF (*d*NDF), ADF (*d*ADF), and ADL (*d*ADL) were determined by subtracting the dried residue weight from the initial weight of the dried substrate.

Determination of MM was done following the procedure of Blümmel and Lebzien (2001). An equal number of bottles with the same treatments, but without filter bags, were incubated for 24 h to estimate pellet weight for both the treatments and the blanks. The contents of the bottles were transferred into pre-weighed centrifuge tubes (Thermo Fisher Scientific, Rochester, NY, USA) and centrifuged at 20,000× g for 15 minutes at 4 °C. Blanks were processed similarly to correct for buffered ruminal inoculum residues. Following centrifugation, the supernatant was discarded, and the pellets were frozen for 24 h. The frozen pellets were then placed in a freeze dryer (L-200, BUCHI Lyovapor, New Castle, DE, USA) for approximately 72 h. After freeze-drying, the samples were weighed to determine pellet weight, and MM was calculated as described by Blümmel and Lebzien (2001).

Determination of Volatile Fatty Acid

The preserved rumen fluid samples were thawed and centrifuged at 10,000× g for 15 minutes at 4 °C. The volatile fatty acids (VFA) were determined following the protocol of Ruiz-Moreno et al. (2015) with modifications as described previously (Alabi et al., 2024; Ike et al., 2024) using gas chromatography with flame ionization detection (FID). A metaphosphoric–crotonic acid mixture served as the internal standard. The sample injection volume was 1 µL, with a split ratio of 1:12. The injector port was maintained at a constant temperature of 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min, ensuring efficient transport of the sample through the GC column. A temperature gradient was applied in the oven for optimal separation of analytes. Initially, the oven was set to 120 °C for 0.8 minutes, followed by an 8 °C·min^{-1} increase until reaching 140 °C, which was held for 1.8 minutes. The detector temperature was maintained at 280 °C. The FID operation was supported by controlled flows of hydrogen and air at rates of 30 mL·min^{-1} and 400 mL·min^{-1}, respectively. Additionally, nitrogen was used as a make-up gas at a flow rate of 25 mL·min^{-1}, ensuring a stable baseline and consistent detector performance. An internal standard mixture of metaphosphoric acid and crotonic acid (trans-2-butenoic acid) was employed, while acetate (C_2), propionate (C_3), butyrate (C_4), isobutyrate (iso-C_4), valerate (C_5), and isovalerate (iso-C_5) served as quantitative external standards (Ruiz-Moreno et al., 2015).

Statistical Analysis

Data analysis was done using the MIXED procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC) using a 2 × 10 factorial design. The statistical model used was: $Y_{ijk} = \mu + A_i + D_j + AD_{ij} + \Sigma_{ijk}$; where Y_{ijk} is the observation, μ is the mean, A_i is the treatment, D_j is the

diet, AD_{ij} is the interaction between treatment and diet, and Σ_{ijk} represents error. The probability of difference (PDIFF) of the least squares means statement in the MIXED procedure of SAS was used. The means having $P < 0.05$ represent significant statistical differences.

Results

Total Gas and Greenhouse Gases Production

The total gas and GHG produced were significantly ($P < 0.001$) influenced by diet×additive interactions (Table 2). Diet type affected ($P < 0.001$) gas, CO_2, and H_2S production, while additive type affected ($P < 0.001$) total gas and individual GHG. Individual and EOB inclusions reduced ($P < 0.001$) gas production in both diets in comparison to the control. A similar pattern was noted for individual GHG. Individual oregano had the lowest production of gas and individual GHG in both diets. Oregano and EOB3 were the most effective treatments to reduce gas and GHG.

Nutrient Disappearance

Significant ($P < 0.001$) diet × additive and diet effects were observed for undegraded DM, MM, IVADDM, IVTDDM, and *d*DM (Table 3). Additionally, undegraded DM, IVTDDM and *d*DM were affected by the additive ($P < 0.05$). Within each diet, administration of individual and EOB reduced ($P < 0.001$) the undegraded DM, IVADDM, IVTDDM, and *d*DM, and increased MM. The highest amounts of undegraded DM were observed in the HF diet. Oregano, clove, and EOBs administered to the HC diet had the highest MM values.

As shown in Table 4, significant ($P < 0.001$) diet × additive and diet effects were observed for *d*NDF, *d*ADF, and *d*ADL, while additives affected only *d*NDF. The addition of EOB3 to the HC diet gave the lowest ($P < 0.001$) values for *d*NDF, *d*ADF, and *d*ADL, while clove and oregano reduced *d*NDF in the HF diet. The lowest *d*ADF and *d*ADL values were observed in EOB5 in the HF diet. The addition of peppermint to the HF diet resulted in the highest ($P < 0.001$) values for *d*NDF and *d*ADF in contrast to the remaining treatments.

Volatile Fatty Acids

Significant ($P < 0.01$) diet × additive interactions between the total and each VFA (Table 5) were noted. The proportions of C_2, C_3, C_4, and C_2:C_3 were affected by diet type, while the concentration of total VFA, and proportions of each VFA (except C_2) were influenced by the additives. The administration of individual essential oils or EOB reduced ($P < 0.001$) the production of total VFA in the diets. Oregano and clove in the HC diet, and all individual essential oils or EOB in the HF diet recorded the lowest total VFA production. However, the administration of peppermint in the HC diet had the highest ($P = 0.002$) C_2, while EOB2 in the HF diet had the lowest proportion. Peppermint and EOB4 had the highest C_3 proportion in the HF diet compared to anise administration in both diets which had the lowest C_3 proportion. The highest C_2:C_3 ratio was observed with the administration of anise in the HC diet, while the lowest ratio was observed with the administration of EOB2 in the HF diet. The lowest proportions of iso-C_4 and C_5 were observed in the control treatment of both diets.

Discussion

Gas and Greenhouse Gases Production

The significant diet × additive interactions in the gas and GHG production indicate the importance of selecting the individual essential oils or blends based on the targeted diet. There have been reports of different responses between the additives in previous studies (Alabi et al., 2023; Brice et al., 2022; Ike et al., 2024) that each specific essential oil would have unique performance characteristics due to its chemical structure. Incorporating the individual essential oils or their blends into both diets resulted in an overall decrease in total gas production. The suppression ranking is as follows: anise<peppermint<clove<oregano, while the order for the blends is as follows: EOB5<EOB4<EOB2<EOB1<EOB3. Oregano suppressed the total gas production the most by 95.8% (HC) and 96.7% (HF). The EOB3, which consists of 40% of oregano, suppressed total gas by 90.4% and 92.3% in the HC and HF diets, respectively. Different responses to individual essential oils confirm the assumptions that the activity of essential oils depends on different molecular structures and biologically active constituents (Ike et al., 2024; Kholif and Olafadehan, 2021). The observed reduction in total gas produced could be a result of the associated decrease in the IVDMD, especially with the additives that had oregano (Dijkstra et al., 2005). The suppression of total gas at different levels indicated the toxic nature of the EOs and their blends on the activities of bacteria and protozoa (Perczak et al., 2019).

It has been reported by previous studies that the reduction of greenhouse gases with the addition or inclusion of essential oils or EOB (Alabi et al., 2024; Ike et al., 2024; Zhou et al., 2020) was as a result of the direct inhibition of the activities of methanogenic archaea family or by some methanogenic pathways being indirectly suppressed (Knapp et al., 2014). According to Zhou et al. (2020), the administration of oregano significantly decreased the relative abundance of the archaea community's phylum *Euryarchaeota*. The reports were also corroborated by Zhou et al. (2019) who noted a decrease in ruminal protozoa abundance with the administration of oregano essential oil in the feed for sheep. The reduction pattern for CH_4 in this study was similar to that observed for the total gas produced. The EOs and EOBs did not differ significantly in their reduction of CH_4 in both diets except for anise, oregano suppressed CH_4 the most by 99.8 and 99.9% for HC and HF, respectively, and EOB3 (40% oregano inclusion) which suppressed CH_4 by 99.6 (HC) and 99.8% (HF) from the control. Macheboeuf et al. (2008) noted a 98% reduction in the production of CH_4 by using 5 mM of oregano or cinnamon essential oils. The suppression of CH_4 greatly by oregano and EOB3 agrees with previous reports(Benetel et al., 2022; Brice et al., 2022; Zhou et al., 2020). This clearly shows that oregano and its inclusion in blends can help inhibit CH_4 production as CH_4 results in energy loss which ultimately will affect production efficiency in livestock, thus minimizing the adverse effects of CH_4 on the environment. According to Brice et al. (2022), the mixtures of different blends have differential impact on feed fermentation as well as CH_4 and the other GHGs. The suppression effect of oregano and EOB3 on CO_2, NH_3 and H_2S concentration in both diets could be because of the presence of oregano in all the blends as noted in the results of Zhou et al. (2020) and Brice et al. (2022).

Table 2. Effects of individual essential oils and their blends on total gas production, and greenhouse gases produced from high forage and high concentrate diets at 24 h of incubation

Diet	Additive[1]	Gas (mL·g^{-1} DM)	CH_4 (mg·g^{-1} DM)	CO_2 (mg·g^{-1} DM)	H_2S (mmol·g^{-1} DM)	NH_3 (mmol·g^{-1} DM)
HC	Control	109.8^{a}	5.46^{a}	32.0^{a}	630.5^{a}	161.7^{a}
	Anise	72.72^{b}	1.86^{b}	19.7^{b}	254.2^{b}	69.85^{c}
	Clove	20.01^{f}	0.11^{d}	3.53^{f}	32.11^{d}	3.28^{d}
	Oregano	4.62^{j}	0.01^{d}	0.73^{h}	1.62^{d}	0.30^{d}
	Peppermint	52.22^{d}	0.23^{d}	13.5^{d}	50.56^{d}	2.31^{d}
	EOB1	13.01^{ghi}	0.04^{d}	2.65^{fgh}	11.64^{d}	0.81^{d}
	EOB2	13.57^{ghi}	0.04^{d}	2.56^{fgh}	2.95^{d}	0.78^{d}
	EOB3	10.50^{hi}	0.02^{d}	1.92^{fgh}	1.24^{d}	0.38^{d}
	EOB4	18.15^{fg}	0.05^{d}	3.69^{f}	1.60^{d}	0.85^{d}
	EOB5	28.22^{e}	0.11^{d}	6.51^{e}	8.02^{d}	6.02^{d}
HF	Control	109.0^{a}	5.21^{a}	31.3^{a}	283.0^{b}	106.7^{b}
	Anise	65.70^{c}	1.12^{c}	16.2^{c}	144.5^{c}	53.23^{d}
	Clove	10.66^{hi}	0.03^{d}	1.83^{fgh}	11.89^{d}	1.05^{d}
	Oregano	3.58^{j}	0.003^{d}	0.60^{h}	0.89^{d}	0.23^{d}
	Peppermint	49.30^{d}	0.16^{d}	12.6^{d}	30.50^{d}	1.39^{d}
	EOB1	14.55^{gh}	0.03^{d}	2.98^{fg}	6.49^{d}	0.45^{d}
	EOB2	11.11^{hi}	0.02^{d}	2.21^{fgh}	1.65^{d}	0.31^{d}
	EOB3	8.43^{ij}	0.01^{d}	1.27^{gh}	0.76^{d}	0.34^{d}
	EOB4	14.37^{gh}	0.03^{d}	2.79^{fgh}	1.01^{d}	0.32^{d}
	EOB5	20.57^{f}	0.05^{d}	3.58^{f}	1.56^{d}	0.40^{d}
SEM		2.97	0.15	0.88	15.06	5.08
P value						
Diet		<0.001	0.059	0.002	<0.001	0.106
Additive		<0.001	<0.001	<0.001	<0.001	<0.001
Diet × Additive		<0.001	<0.001	<0.001	<0.001	<0.001

Note: Means in the same column with different superscripts differ, $P < 0.05$. *P* value is the observed significance level of the *F*-test for diet × additive interactions; SEM = standard error of the mean. HC is a high concentrate diet, HF is high forage, CH_4 is methane, CO_2 is carbon dioxide, NH_3 is ammonia, H_2S is hydrogen sulfide.

[1] The substrates were administered with no essential oils (control) or with individual anise, clove, oregano, and peppermint or their blends (EOB), respectively at 1:1:1:1 (EOB1), 1:2:3:4 (EOB2), 2:3:4:1 (EOB3), 3:4:1:2 (EOB4), or 4:1:2:3 (EOB5) at 100 µL·g^{-1} DM.

Table 3. Effects of individual essential oils and their blends on some fermentation parameters and *in vitro* DM disappearance of high forage and high concentrate diets at 24 h of incubation

Diet	Additive[1]	Undegraded (g·g^{-1} DM)	MM (g·kg^{-1} DM)	IVADDM (g/g DM)	IVTDDM (g·g^{-1} DM)	*d*DM (%)
HC	Control	0.119^{f}	0.081^{fg}	0.596^{a}	0.761^{a}	51.1^{a}
	Anise	0.138^{e}	0.117^{e}	0.487^{c}	0.722^{b}	45.3^{bc}
	Clove	0.141^{de}	0.214^{ab}	0.274^{efg}	0.711^{cd}	40.0^{de}
	Oregano	0.148^{c}	0.216^{a}	0.268^{fg}	0.703^{d}	30.6^{ghi}
	Peppermint	0.142^{cde}	0.105^{ef}	0.507^{bc}	0.715^{bc}	42.9^{bcd}
	EOB1	0.143^{cde}	0.212^{ab}	0.285^{ef}	0.712^{cd}	36.7^{ef}
	EOB2	0.142^{cde}	0.202^{abc}	0.303^{ef}	0.712^{cd}	34.3^{fgh}
	EOB3	0.146^{cd}	0.206^{abc}	0.294^{ef}	0.707^{cd}	33.7^{fgh}
	EOB4	0.145^{cd}	0.187^{abcd}	0.333^{e}	0.709^{cd}	38.7^{def}
	EOB5	0.142^{cde}	0.159^{d}	0.390^{d}	0.712^{cd}	38.7^{def}
HF	Control	0.168^{b}	0.052^{h}	0.556^{ab}	0.661^{e}	47.9^{ab}
	Anise	0.179^{a}	0.086^{fg}	0.460^{c}	0.635^{f}	43.8^{bcd}
	Clove	0.180^{a}	0.202^{abc}	0.214^{g}	0.630^{f}	26.4^{ij}
	Oregano	0.183^{a}	0.181^{cd}	0.256^{fg}	0.626^{f}	24.1^{j}
	Peppermint	0.180^{a}	0.065^{gh}	0.499^{c}	0.632^{f}	42.2^{cd}
	EOB1	0.184^{a}	0.183^{bcd}	0.252^{fg}	0.626^{f}	36.0^{efg}
	EOB2	0.182^{a}	0.165^{d}	0.289^{ef}	0.627^{f}	31.2^{ghi}
	EOB3	0.184^{a}	0.181^{cd}	0.259^{fg}	0.626^{f}	29.5^{hi}
	EOB4	0.181^{a}	0.187^{abcd}	0.248^{fg}	0.631^{f}	35.7^{efg}
	EOB5	0.182^{a}	0.168^{d}	0.287^{ef}	0.630^{f}	35.6^{efg}
SEM		0.002	0.005	0.01	0.004	0.72
P value						
Diet		<0.001	<0.001	<0.001	<0.001	<0.001
Additive		0.015	0.158	0.178	0.045	0.012
Diet × Additive		<0.001	<0.001	<0.001	<0.001	<0.001

Note: Means in the same column with different superscripts differ, $P < 0.05$. *P* value is the observed significance level of the *F*-test for diet×additive interactions; SEM = standard error of the mean. HC is high concentrate diet, HF is high forage, MM is microbial mass, IVDMD is *in vitro* dry matter degradability, IVADDM is *in vitro* apparent degradable dry matter, IVTDDM is *in vitro* true degradable dry matter; *d*DM is dry matter degradability.

[1] The substrates were administered with no essential oils (control) or with individual anise, clove, oregano, and peppermint or their blends (EOB), respectively at 1:1:1:1 (EOB1), 1:2:3:4 (EOB2), 2:3:4:1 (EOB3), 3:4:1:2 (EOB4), or 4:1:2:3 (EOB5) at 100 µL·g^{-1} DM.

Table 4. Effects of individual essential oils and their blends on fiber digestibility of high forage and high concentrate diets at 24 h of incubation

Diet	Additive[1]	*d*NDF (%)	*d*ADF (%)	*d*ADL (%)
HC	Control	47.3^{def}	42.1^{abc}	11.2^{ab}
	Anise	47.3^{def}	36.9^{abc}	8.64^{bcd}
	Clove	43.2^{fg}	31.2^{cdef}	8.96^{bc}
	Oregano	36.1^{hi}	33.5^{bcdef}	7.02^{cdef}
	Peppermint	38.7^{gh}	32.7^{cdef}	8.49^{bcd}
	EOB1	37.9^{gh}	25.1^{ef}	8.37^{bcd}
	EOB2	37.8^{gh}	27.0^{def}	8.19^{bcd}
	EOB3	30.3^{i}	22.0^{f}	12.6^{a}
	EOB4	38.4^{gh}	24.4^{ef}	7.10^{cdef}
	EOB5	41.3^{fgh}	29.0^{def}	7.65^{bcde}
HF	Control	60.2^{ab}	46.2^{abc}	3.85^{efg}
	Anise	60.4^{ab}	46.5^{abc}	3.62^{fg}
	Clove	43.3^{fg}	40.4^{abc}	3.44^{fg}
	Oregano	44.8^{efg}	47.2^{ab}	4.20^{efg}
	Peppermint	62.7^{a}	49.5^{a}	4.85^{defg}
	EOB1	54.4^{bc}	47.2^{ab}	4.15^{efg}
	EOB2	46.7^{def}	39.7^{abc}	2.75^{g}
	EOB3	51.1^{cde}	50.9^{a}	4.79^{defg}
	EOB4	52.1^{cd}	46.3^{abc}	3.30^{fg}
	EOB5	47.1^{def}	29.7^{def}	1.97^{g}
SEM		0.90	2.23	0.36
P value				
Diet		<0.001	<0.001	<0.001
Additive		<0.001	0.167	0.055
Diet × Additive		<0.001	<0.001	<0.001

Note: Means in the same column with different superscripts differ, $P < 0.05$. *P* value is the observed significance level of the *F*-test for diet×additive interactions; SEM = standard error of the mean. HC is high concentrate diet, HF is high forage diet, *d*NDF is neutral detergent fiber degradability; *d*ADF is acid detergent fiber degradability, *d*ADL is acid detergent lignin degradability.

[1] The substrates were administered with no essential oils (control) or with individual anise, clove, oregano, and peppermint or their blends (EOB), respectively at 1:1:1:1 (EOB1), 1:2:3:4 (EOB2), 2:3:4:1 (EOB3), 3:4:1:2 (EOB4), or 4:1:2:3 (EOB5) at 100 μL·g^{-1} DM.

Table 5. Effects of individual essential oils and their blends (EOB) on total (mmol/g DM) and molar proportion of volatile fatty acids produced from high forage (HF) and high concentrate (HC) diets at 24 h of incubation

Diet	Additive[1]	Total	C_2	C_3	C_4	Iso-C_4	C_5	Iso-C_5	C_2:C_3
HC	Control	88.3^{a}	0.624^{bcd}	0.207^{ab}	0.133^{b}	0.008^{d}	0.021^{f}	0.007^{abcd}	3.02^{cde}
	Anise	73.9^{cd}	0.626^{abcd}	0.182^{c}	0.148^{a}	0.009^{c}	0.027^{ef}	0.009^{ab}	3.45^{a}
	Clove	62.4^{ef}	0.623^{bcd}	0.202^{ab}	0.114^{cd}	0.011^{ab}	0.046^{abc}	0.005^{cdefg}	3.08^{cde}
	Oregano	59.6^{f}	0.630^{abcd}	0.210^{ab}	0.116^{cd}	0.011^{ab}	0.028^{def}	0.005^{cdefg}	3.01^{cde}
	Peppermint	72.1^{d}	0.648^{a}	0.195^{b}	0.100^{f}	0.009^{c}	0.041^{bcde}	0.007^{abc}	3.32^{abc}
	EOB1	62.4^{ef}	0.634^{abcd}	0.195^{b}	0.110^{de}	0.011^{a}	0.046^{abc}	0.004^{fg}	3.27^{abcd}
	EOB2	61.6^{ef}	0.626^{abcd}	0.206^{ab}	0.115^{cd}	0.011^{ab}	0.037^{cdef}	0.005^{cdefg}	3.04^{cde}
	EOB3	62.8^{ef}	0.635^{abc}	0.203^{ab}	0.112^{cd}	0.010^{b}	0.037^{cdef}	0.004^{fg}	3.14^{bcde}
	EOB4	63.0^{ef}	0.639^{ab}	0.198^{ab}	0.111^{cd}	0.011^{ab}	0.037^{cdef}	0.004^{defg}	3.27^{abcd}
	EOB5	63.9^{ef}	0.630^{abcd}	0.200^{ab}	0.111^{cd}	0.011^{ab}	0.045^{abcd}	0.004^{defg}	3.16^{bcde}
HF	Control	85.5^{ab}	0.621^{bcd}	0.209^{ab}	0.138^{b}	0.008^{d}	0.021^{f}	0.003^{g}	2.99^{cde}
	Anise	80.1^{bc}	0.638^{ab}	0.180^{c}	0.139^{b}	0.009^{c}	0.027^{ef}	0.007^{abcd}	3.60^{a}
	Clove	60.6^{f}	0.626^{abcd}	0.207^{ab}	0.115^{cd}	0.011^{ab}	0.037^{cdef}	0.004^{efg}	3.03^{cde}
	Oregano	59.4^{f}	0.625^{bcd}	0.209^{ab}	0.116^{cd}	0.011^{ab}	0.033^{cdef}	0.007^{bcdef}	2.99^{cde}
	Peppermint	69.9^{de}	0.625^{abcd}	0.212^{a}	0.103^{ef}	0.009^{c}	0.041^{abcde}	0.010^{a}	2.96^{cde}
	EOB1	61.8^{ef}	0.611^{de}	0.205^{ab}	0.113^{cd}	0.011^{ab}	0.057^{a}	0.004^{defg}	2.99^{cde}
	EOB2	58.8^{f}	0.598^{e}	0.212^{a}	0.118^{cd}	0.011^{ab}	0.055^{ab}	0.006^{cdefg}	2.82^{e}
	EOB3	58.2^{f}	0.621^{bcd}	0.210^{ab}	0.119^{cd}	0.011^{a}	0.033^{cdef}	0.006^{cdef}	2.99^{cde}
	EOB4	59.6^{f}	0.613^{cde}	0.212^{a}	0.119^{c}	0.011^{ab}	0.037^{cdef}	0.007^{bcde}	2.90^{de}
	EOB5	60.4^{f}	0.626^{abcd}	0.210^{ab}	0.116^{cd}	0.011^{ab}	0.032^{cdef}	0.005^{cdefg}	2.99^{cde}
SEM		0.97	0.0020	0.0010	0.0010	0.0010	0.0010	0.0002	0.030
P value									
Diet		0.177	0.001	0.001	0.027	0.212	0.751	0.291	0.003
Additive		<0.001	0.078	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Diet × Additive		<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	0.002

Note: Means in the same column with different superscripts differ, $P < 0.05$. *P* value is the observed significance level of the *F*-test for diet×additive interactions; SEM = standard error of the mean. HC is high concentrate diet, HF is high forage diet, C_2 is acetate, C_3 is propionate, C_4 is butyrate, C_5 is valerate.

[1] The substrates were administered with no essential oils (control) or with individual anise, clove, oregano, and peppermint or their blends (EOB), respectively at 1:1:1:1 (EOB1), 1:2:3:4 (EOB2), 2:3:4:1 (EOB3), 3:4:1:2 (EOB4), or 4:1:2:3 (EOB5) at 100 µL/g DM.

[1] Individual anise, clove, oregano, and peppermint or their blends (EOB), respectively at 1:1:1:1 (EOB1), 1:2:3:4 (EOB2), 2:3:4:1 (EOB3), 3:4:1:2 (EOB4), or 4:1:2:3 (EOB5) at 100 µL·g^{-1} DM. Means with different superscripts within the same column differ, $P < 0.05$; SEM, Standard error of means.

The suppression of NH_3 as well could mean that oregano, being a phenol-based essential oil, has an inhibitory effect on the NH_3 hyperproducing bacteria or the inhibition of processes such as amino acid deamination, proteolysis, and peptidolysis, or an increase in the synthesis of microbial protein (Kholif and Olafadehan, 2021; Patra and Yu, 2012; Zhou et al., 2020).

Nutrient Disappearance

The HF diet had higher undegraded DM values compared to the HC diet, which could be due to components of the dietary substrates used as reported by Brice et al. (2022) with similar diets. Oregano in the HC diet and EOB3 in the HF diet had the highest numerical undegraded DM values (24.4% and 22.7%), and all the additives had higher undegraded DM than the control.

When compared to the control, higher MM values were consistently seen for the EOB and individual essential oils, indicating that their inclusion increased efficiency and microbial protein production efficiency. These results may be due to a reduction in gas production and methanogens (Molho-Ortiz et al., 2022). The results of MM are paralleled with those of NH_3 production in the rumen, which validate the inhibitory properties of oregano on ruminal proteolysis, peptidolysis, and amino acid deamination. Ike et al. (2024) observed an increase in MM with essential oil administration.

The addition of the EOs and their blend resulted in an observable decrease in IVDMD, which was predicted based on findings from earlier studies (Alabi et al., 2023; Beauchemin and McGinn, 2006). The negative impact of the essential oils and their blends on digestibility could be associated with a decrease in the activities of starch-digesting bacteria and protozoa that promote the production of CH_4 (Brice et al., 2022; Kurniawati et al., 2018). Oregano had the highest digestibility suppression in both diets, while peppermint and anise had the highest IVDMD in both diets compared to the other additives but lower than the controls by 11.4% (HC) and 8.7% (HF). In contrast to the results of the study for oregano, Zhou et al. (2020) observed a rise in NDF and ADF degradability when oregano was administered at increasing levels (13 to 130 mg/L incubation medium) in an *in vitro* system. The variations may be explained partially by the differences in the inclusion levels and diets. Higher digestibility observed for anise in both diets could be associated with lower undegraded DM and MM. This could be an indication that peppermint and anise promote the activities of fiber-degrading bacteria and protozoa (Ando et al., 2003).

The IVTDDM and IVDMD showed a similar pattern, with the highest IVTDDM depression being noted when oregano was added to the HC diet. In contrast, there was no significant difference in the EOBs. When compared to the control, the EOs and EOBs for the HF diet decreased IVTDDM. The EOs used in this study reduced the IVTDDM and IVADDM for both substrates, though the HF was more affected than HC.

The *d*NDF, *d*ADF, and *d*ADL values in both diets were suppressed by the inclusion of individual essential oils or their blends. However, the addition of peppermint in the HF diet improved the *d*NDF and *d*ADF by 4.2% and 7.2%, respectively, while anise inclusion had no effect. Improved fiber digestibility in the HF diet with the addition of peppermint agrees with previous studies (Benchaar et al., 2006; Yang et al., 2007). In consistence with the findings of Günal et al. (2017), anise did not influence the digestibility of fiber in either diet in the current investigation. This result is crucial as most essential oils depress fiber degradability (Kholif and Olafadehan, 2021). Both oregano and EOB3 suppressed *d*NDF in HC diet by 23.8% and 35.9%, respectively. This could be due to the phenolic nature of oregano which has been reported to have adverse effect on *d*NDF by reducing fibrolytic bacteria activities (Castillejos et al., 2006; Fraser et al., 2007; Patra and Yu, 2012). However, others (Zhou et al., 2019) reported an increase in ruminal fungi with oregano inclusion at 7 g daily resulting in enhanced fiber digestion in sheep. As mentioned before, different sources of the same essential oil could result in different effects on ruminal fermentation (Mora-Zúñiga et al., 2022).

Volatile Fatty Acids

These are produced during ruminal fermentation and are crucial for the survival of ruminants as they provide the bulk (more than 70%) of energy needed by this class of animals (McDowell and Annison, 1991). Higher total VFA noted for HC *versus* HF could imply a lower fermentation efficiency with feed containing high forage and a reflection of HC having better DM digestibility. This observation aligns with the report of Wang et al. (2020).

Individual essential oils and EOBs reduced the total VFA, which implied that carbohydrate fermentation and microbial activities were negatively affected. The influence of individual essential oils on the VFA of both diets was in the order anise<peppermint<clove<oregano. This is an indication that anise had the least negative impact on VFA, and oregano had the most negative impact on VFA. Reduction in VFA is considered nutritionally detrimental to ruminant animals (Günal et al., 2017), because it is a primary energy source. The results of gas production for this study followed the same trend as the VFA and agreed with some previous studies with positive correlations between gas and VFA (Getachew et al., 2004; Kubelková et al., 2018).

Anise inclusion resulted in a significant propionate reduction in both HC and HF diets whereas peppermint and EOB2 and EOB4 had the highest propionate production. Günal et al. (2017) reported lower propionate proportion with the administration of anise essential oil at 250 mg/L of culture fluid, while Wanapat et al. (2013) reported increased ruminal propionate with the administration of peppermint powder to beef cattle. The addition of peppermint to HC diet in this study resulted in the highest ruminal acetate proportion; however, the administration of EOB2 (containing 40% peppermint) to the HF diet resulted in the lowest proportion, highlighting the effects of inclusion level and diet. According to Ozkan et al. (2015), the concentration of acetate increased linearly as the amount of peppermint in a feed consisting of barley grains increased.

Conclusion

The individual essential oils and EOB had different effects on nutrient digestibility and greenhouse gas mitigation. Both can be used as additives to benefit digestibility and greenhouse gas emissions, which would in turn improve animal health, environmental health and livestock production. Oregano essential oil or EOB based mainly on oregano demonstrated the highest

potential for significantly reducing greenhouse gas emissions, while anise had minimal impact on digestibility, total gas and CH_4 production. Anise essential oil is beneficial as it effectively reduces CH_4 production without negatively affecting nutrient digestibility. Despite the efficacy of essential oils, additional studies are needed to find suitable diets and dosages to lower greenhouse gas emissions and improve nutrient digestibility. Further research should involve conducting *in vivo* experiments by directly infusing essential oils into cannulated cows.

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CRediT Authorship Contribution Statement

DeAndrea Gray, Peter Dele, Joel Alabi, and Oludotun Adelusi: Formal Analysis, investigation, methodology and writing – original draft. **Michael Wuaku, Deborah Okedoyin, Chika Anotaenwere, and Kelechi Ike:** Formal Analysis and investigation. **Olatunde Oderinwale:** Writing – review & editing. **Ahmed E. Kholif:** Data curation, formal analysis, methodology and writing – review & editing. **Uchenna Anele:** Conceptualization, funding acquisition, project administration, resources, supervision and writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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