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In vitro fermentation characteristics of calf grower diet incubated with essential oil blend and *Lactobacillus*-derived probiotic and postbiotic

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ABSTRACT

This study evaluated gas production and nutrient digestibility of calf grower feed containing *Lactobacillus plantarum* (LP) incubated with postbiotic *Lactobacillus acidophilus* (PLA) and an essential oil blend (EOB). Eight treatments were arranged in a 2 × 4 factorial arrangement with two feeds (with or without LP) and four additives (no additive, PLA, EOB and PLA+EOB), with eight replicates per treatment (4 bottles per run across two independent incubation runs). Treatments were randomly assigned to incubation bottles within each run, and each bottle was considered an experimental unit. PLA and EOB were administered at 5 and 20 μL/g of substrate, respectively. An *in-vitro* batch culture was conducted for 24 h. Data were analyzed using two-way ANOVA. Results showed that EOB increased microbial mass, while the combination of LP, PLA and EOB improved the partitioning factor. LP and PLA increased total gas ($P<0.05$), whereas EOB alone or with PLA reduced methane production and increased hydrogen sulfide. Feed digestibility was largely unaffected, although EOB increased neutral detergent fiber digestibility and reduced total volatile fatty acid concentration ($P=0.01$). It is concluded that probiotic *L. plantarum* and postbiotic *L. acidophilus* supplementation enhanced total gas production, while EOB reduced methane emissions, and additive combinations improved microbial and fermentative efficiency.

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INTRODUCTION

Dairy calf performance significantly impacts future reproductive efficiency and milk production. Higher growth rates in terms of average daily gain during both the preweaning (≥0.5 kg/day) and postweaning (≥0.8 kg/day) phases have been associated with earlier first calving and increased milk yield in first-lactation cows (Branco-Lopes et al., 2025). Optimizing rumen development and fermentation efficiency during early life is therefore critical for long-term productivity and sustainability of dairy systems. Over the years, antibiotics have been extensively used to accelerate growth performance in industrial livestock production (Choe et al., 2013); however, the development of antibiotic-resistant bacteria due to excessive use of antibiotics poses significant health risks to both livestock and humans (Izuddin et al., 2018). This growing concern has intensified global efforts to identify effective, non-antibiotic feed additives capable of enhancing rumen function while reducing environmental impacts.

Probiotics, also known as direct-fed microbials (DFM), are commonly used in U.S. dairy systems to promote health benefits. However, research on probiotic supplementation in dairy calves has yielded inconsistent results, with some studies showing improved growth rates while others reported no significant effects, highlighting the need for further investigation (Branco-Lopes et al., 2025). These inconsistencies may be attributed to differences in microbial strains, dosage, diet composition and interactions with the rumen ecosystem. Postbiotics, on the other hand, represent a newer class of feed additives composed of probiotic-derived metabolites, including organic acids and bacteriocins, that can exert biological effects without requiring viable microbial cells (Izuddin et al., 2018). Because postbiotics are more stable and less sensitive to environmental conditions than live microbes, they offer a promising alternative for modulating rumen fermentation in a more predictable manner. Both probiotics and postbiotics show potential in ruminant nutrition by enhancing ruminal fermentation and suppressing pathogenic microorganisms, supporting sustainable calf-rearing strategies (Choe et al., 2013).

Lactobacillus spp. improves gut health by producing organic acids and antimicrobial peptides, such as bacteriocins, that help stabilize microbial communities and inhibit pathogen growth (Huang et al., 2021). *Lactobacillus plantarum* (LP), commonly isolated from fermented foods and silages, has been associated with improved performance through enhanced gut health and microbial modulation (Casper et al., 2021). The postbiotic used in this study is a *Lactobacillus acidophilus* fermentation product containing spent bacterial cells, fermentation by-products and growth media (RumaCell, Pacer Technologies). It contains beneficial bacteriocins, including lactocin B, lactacin F, acidocin A, and acidocin B, which remain functional in the rumen environment (Hall et al., 2022). In addition, their effects on nutrient utilization, lactic acid bacteria (LAB) and their metabolites have been proposed as mitigation strategies for enteric methane (CH_4) production by altering hydrogen flow and suppressing methanogenic populations (Doyle et al., 2019).

Essential oils (EOs) are complex plant-derived compounds whose composition varies with plant species, plant part and environmental conditions (Kholif et al., 2026). They exhibit broad-spectrum antimicrobial activity and selectively influence rumen microbial populations (Davoodi et al., 2016; Kholif et al., 2026). Their ability to suppress methanogens and hydrogen-producing microbes has positioned essential oils as promising natural tools for reducing greenhouse gas emissions from ruminant systems. The effects of EOs on ruminal fermentation and gas production (GP) are closely linked to their chemical structure and inclusion rate, and blending essential oils is increasingly used to enhance synergistic antimicrobial and fermentation-modifying effects (Kholif and Olafadehan, 2021). A specific EO blend (EOB) containing garlic, lemongrass, cumin, lavender and nutmeg, has previously been shown to reduce gas and methane production while improving microbial efficiency and propionate formation (Ike et al., 2024b). However, limited information is available on how such blends interact with probiotics and postbiotics when applied simultaneously in calf grower diets.

Therefore, the present study addresses an important knowledge gap by systematically evaluating, within a single factorial design, the independent and interactive effects of a probiotic (*L. plantarum*), a postbiotic (derived from *L. acidophilus*) and an essential oil blend on rumen fermentation dynamics and environmental outputs. The objective of this study was to investigate the effects of these additives on ruminal fermentation kinetics, nutrient digestibility, greenhouse gas emissions and volatile fatty acid (VFA) production using an *in vitro* batch culture system. It was hypothesized that dietary supplementation with a probiotic (*L. plantarum*), a postbiotic (from *L. acidophilus*), or an EOB would (1) improve ruminal fermentation kinetics, (2) enhance nutrient digestibility, (3) increase the production of beneficial VFA, and (4) reduce greenhouse gas emissions compared to the control diet under *in vitro* conditions. To test these hypotheses, fermentation kinetics were evaluated by measuring total GP, microbial mass, partitioning factor and fermentation end-products (Sections 2.4 and 2.5); nutrient digestibility was assessed using *in vitro* apparent and true dry matter degradability and fiber fraction degradability (Section 2.5); beneficial fermentation products were evaluated by measuring individual and total volatile fatty acids (Section 2.6); and greenhouse gas emissions were assessed by measuring methane, carbon dioxide, ammonia, and hydrogen sulfide concentrations (Section 2.4).

MATERIALS AND METHODS

Study location and animals

The study was conducted at North Carolina A&T State University's Department of Animal Sciences, using four healthy, cannulated Holstein Friesian cows from the Dairy Unit as a source of rumen inoculum. Cows were selected based on health status, absence of metabolic disorders and normal rumen function, with no history of antibiotic treatment in the preceding 3 months. The animals were between 3-4 years of age and weighed 550–600 kg at the start of the study. The cows were cared for under IACUC protocol (LA22-0019) and fed a diet of free-choice grass hay with access to a mixed grass pasture.

Feed sample processing and chemical analysis

Samples of calf starter diets and alfalfa pellets were submitted to Dairyland Laboratories (Arcadia, WI) for nutrient analyses. Samples were analyzed for DM (#930.15), crude protein (CP; #990.03), neutral detergent fiber (NDF; #2002.04), acid detergent fiber (ADF; #973.18), lignin (#973.18), ether extract (#2003.05), and ash (#942.05) according to the AOAC official methods (AOAC, 2019). For both sample groups, chloride was extracted with 0.5% nitric acid and analyzed by potentiometric titration with silver nitrate (Metrohm 848 Titrino Plus, Metrohm, Riverview, FL, USA). Non-fiber carbohydrates (NFC = 100 − NDF − CP − EE − ash), cellulose (ADF−ADL), hemicellulose (NDF−ADF), and organic matter (OM = 1000 − ash) in feed ingredients were calculated. Metabolizable energy (ME; Mcal/kg) was calculated for grower pellet and alfalfa pellets using the equations of NRC (2001).

Ingredients and treatments

Two types of diets were prepared using calf grower pelleted grain diets formulated without (control) or with *L. plantarum* (LP) and pelleted alfalfa hay, as shown in Table 1. These pellets were ground and each separately mixed with ground pelleted alfalfa hay at a forage-concentrate ratio of 40:60 for use as incubation substrates. Clean 100 mL serum bottles were labelled accordingly using a permanent marker and arranged orderly for use. Ankom F57 (Ankom Technology Corp., Macedon, USA) filter bags were marked using a black Lumocolor permanent special pencil, soaked in acetone for 5 minutes, and placed under the fume hood to dry for 20 minutes.

Table 1. Chemical composition (%, DM basis) of the control diets (with and without LP) and alfalfa pellets (LP: *Lactobacillus plantarum*)

Nutrient	Control (Without LP)	Control (with LP)	Alfalfa pellets
Dry matter, wet basis	92.2	91.9	89.6
Organic matter	92.9	92.5	87.7
Crude protein	19.3	19.2	19.3
Acid detergent fiber	8.80	8.81	41.0
Neutral detergent fiber	18.4	19.2	47.4
Acid detergent lignin	1.54	1.30	9.40
Non-fiber carbohydrate	52.5	50.8	24.9
Ether extract	4.40	4.60	2.28
Total digestible nutrients	80.0	80.0	52.8
Metabolizable energy, Mcal/kg	2.98	2.98	2.02

The fiber bags were then oven-dried at 55°C for 48 h, after which they were placed in a desiccator for about 15 minutes. The individual weight of fiber bags was recorded by removing them from the desiccator using a tweezer and weighed using a digital scale. Bags were sealed using a heat impulse sealer (Model # AIE-200HR, American International Electric, City of Industry, CA, USA) before being inserted into 100 mL serum bottles (Cat# 223747; Wheaton Science Products, Millville, NJ, USA). Substrates (0.5 ± 0.05 g each of Control and LP) were put in filter bags and then sealed using a heat sealer. Immediately after sealing the bags, they were placed into their designated 100 mL serum bottles.

Four additive treatment types were prepared using three additives and a control (no additive). The first additive is RumaCell®, a fermentation product of *L. acidophilus* (PLA), which was added to the respective serum bottles at 5 µL/g of substrate. The second additive is an EOB formulated by combining EOs garlic, lemongrass, cumin, lavender, and nutmeg in the ratio 4:2:2:1:1, respectively. The blend was pipetted into the serum bottles at 20 µL/g of substrate. The third additive is a combination of PLA and EOB, individually added to serum bottles at 5 µL/g and 20 µL/g of substrate, respectively.

Batch culture, degradability and measurement of gases

This study utilized a randomized 2 × 4 factorial arrangement with 2 diets and 4 additive combinations. Preparation, dispensing of artificial saliva and rumen fluid, and initiation of fermentation have been extensively described (Alabi et al., 2024; Ike et al., 2024a). 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 $MgCl_2.6H_2O$, 0.0293 g $CaCl_2.2H_2O$. 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. Rumen fluid was collected from four cannulated Holstein Friesian cows and pooled within each incubation run to minimize individual cow variation, with all cows contributing equally to the inoculum. The pH was measured using a benchtop pH meter (Model B10P, VWR International, Radnor, PA, USA). Thereafter, 60 mL of artificial saliva and ruminal liquor were dispensed into serum bottles containing the substrate (Anele et al., 2014). The serum bottles were sealed with butyl rubber stoppers, crimped with aluminium seals and incubated in 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, resulting in eight replicates (n = 8) per treatment (4 bottles per run across the two independent incubation runs). Treatments were randomly assigned to incubation bottles within each run, and each bottle was considered an experimental unit. Additionally, during each incubation run, 4 bottles containing only the buffered inoculum (blanks) were included to establish baseline fermentation GP. This was a randomized *in vitro* experiment; however, blinding of laboratory personnel was not implemented because all outcome variables were measured using objective, instrument-based analytical procedures, thereby minimizing the risk of observer bias.

At the completion of 24 h, fermentation was stopped by placing the bottles on ice for 5 min. Gas pressure was measured at 6 h and 24 h by piercing the rubber stopper with a 22 mm gauge needle fitted to a digital gas pressure manometer (VWR International, Radnor, PA, USA). Gas pressure measurement at 6 h is important to prevent gas build-up that could inhibit fermentation. Subsequent to gas pressure measurements, concentrations of CH_4, carbon dioxide (CO_2), ammonia (NH_3), and hydrogen sulfide (H_2S) were measured using a table-top gas analyzer (Biogas 5000, Landtec, Dexter, MI,

USA). An aliquot of the gas from each bottle was introduced into the analyzer with a 22 G × 1.5 in (0.7 mm × 40 mm) gauge needle attached to the end of the inlet Tygon tube. The analyzer unit was purged between samplings to eliminate any residual gas from the previous sample. The analyzer was calibrated according to the manufacturer's instructions.

Nutrient degradability and microbial mass measurements

After measuring the gas pressure, liquid content was transferred into centrifuge tubes and centrifuged at 10,000 × g for 15 minutes at 4°C, and filter bags were removed from all 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 in 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. Degradability of NDF (*d*NDF), ADF (*d*ADF), ADL (*d*ADL), hemicellulose (*d*HEM) and cellulose (*d*CELL) was determined by subtracting the dried residue weight from the initial weight of the dried substrate.

Microbial mass (MM) was determined according to 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. MM and partitioning factor (PF_{24}) were calculated as described by Blümmel et al. (1997).

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 VFAs were determined following the protocol of Ruiz-Moreno et al. (2015) with modifications as previously described (Alabi et al., 2024; Ike et al., 2024a) using gas chromatography with flame ionization detection (FID). A metaphosphoric–crotonic acid mixture served as the internal standard. 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 × 4 factorial arrangement. An a priori sample size or power calculation was not conducted for this *in vitro* study. The number of incubation runs and replicates per treatment was determined based on established methodological standards in *in vitro* rumen fermentation experiments and previous studies using similar experimental designs (Anele et al., 2014; Blümmel et al., 1997), allowing adequate detection of treatment effects while accounting for between-run variability. The statistical model used was:

$$Y_{ijk} = \mu + A_i + D_j + AD_{ij} + e_{ijk},$$

where Y_{ijk} is the observation, μ is the mean, A_i is the additive, D_j is the diet, AD_{ij} is the interaction between additive and diet, and e_{ijk} represents error. The incubation bottle was considered the experimental unit. No a priori criteria were established for exclusion of data points, and all observations from valid incubation bottles were included in the analysis. Data were only subject to exclusion in cases of clear technical failure, such as leakage, etc. Prior to analysis, assumptions of normality and homogeneity of variance were assessed; such diagnostics are standard in *in vitro* fermentation research and often involve examination of residuals or formal tests such as Shapiro–Wilk and Levene's tests before model fitting. Because residuals were approximately normally distributed and variances were homogeneous, no data transformation was required. 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 statistically significant differences.

RESULTS

Nutrient disappearance

The degradability parameters were mainly influenced by the additive and LP × additive interaction (Table 2). Results are presented as least squares means with their associated standard error of the mean (SEM), based on eight *in vitro* observations per treatment combination (n = 8). The administration of EOB only in the LP diet, and the PLA+EOB combination in both the control with or without LP, increased ($p<0.001$) undegraded residuals, with the LP × additive interaction ($p=0.002$) showing a similar effect. Microbial mass values were higher ($P=0.019$) with the inclusion of PLA+EOB compared to PLA alone in the LP diet, except for PLA with the LP diet, which had the lowest value (0.077 g/kg DM). Higher ($P<0.001$) IVADDM and IVTDDM were generally observed in the control and PLA treatment with the LP diet; however, significant reductions were recorded with EOB treatments. The PF_{24} was affected by LP, additive and LP×additive, with the highest ($p<0.001$) values obtained for EOB (3.21 mg degraded DM/mL gas) and PLA+EOB (2.97 mg degraded DM/mL gas) in the control diet without LP.

Total gas production and greenhouse gas emission

Total GP and gas composition responses are summarized in Table 3 and expressed as least squares means ± SEM (n = 8 per treatment combination). The presence of LP and PLA increased ($P=0.003$) total GP, while the inclusion of EOB decreased total GP irrespective of LP or PLA.

Table 2. Effects of diet and additives on undegraded residual (g), dry matter disappearance, partitioning factor (mg degraded DM per mL gas) and microbial mass (g). Values are reported as least squares means with standard error of the mean (SEM); n = 8 observations per treatment combination derived from two independent incubation runs with four replicate bottles per run. IVADDM: *In vitro* apparent degradable DM, IVTDDM: *in vitro* true degradable DM, PF_{24}: partitioning factor at 24 h of incubation. [1]Diets included a control diet without *Lactobacillus plantarum* (control) and a diet supplemented with *L. plantarum* (LP). Additive treatments were as follows: no additive (–), a postbiotic derived from *Lactobacillus acidophilus* (PLA; 5 µL/g substrate), an essential oil blend (EOB; 20 µL/g substrate), and a combination of PLA and EOB at their respective doses.

Diet[1]	Additive	Undegraded	Microbial mass	IVADDM	IVTDDM	PF_{24}
Control	No additive	0.122^{b}	0.081^{ab}	56.4^{a}	73.7^{a}	2.51^{c}
	PLA	0.122^{b}	0.085^{ab}	55.2^{abc}	73.6^{a}	2.44^{cd}
	EOB	0.129^{ab}	0.094^{ab}	52.1^{bcd}	72.3^{abc}	3.21^{a}
	PLA+EOB	0.132^{a}	0.088^{ab}	52.5^{bcd}	71.5^{bc}	2.97^{b}
LP	No additive	0.123^{b}	0.082^{ab}	55.8^{ab}	73.4^{a}	2.38^{cd}
	PLA	0.126^{ab}	0.077^{b}	56.5^{a}	73.0^{ab}	2.29^{d}
	EOB	0.133^{a}	0.091^{ab}	51.6^{cd}	71.3^{c}	2.90^{b}
	PLA+EOB	0.131^{a}	0.100^{a}	50.1^{d}	71.7^{bc}	2.85^{b}
SEM		0.002	0.006	1.2	0.5	0.056
LP		0.235	0.908	0.515	0.208	<0.001
Additive		<0.001	0.019	<0.001	<0.001	<0.001
LP×Additive		0.002	0.410	0.001	0.001	<0.001

Table 3. Effects of diet and additives on total gas production (mL/g DM), CH_4 (mg/g DM), CO_2 (mg/g DM), NH_3 (mmol/g DM), and H_2S (mmol/g DM) production. Values are reported as least squares means with standard error of the mean (SEM); n = 8 observations per treatment combination derived from two independent incubation runs with four replicate bottles per run. GP: gas production, CH_4: methane, CO_2: carbon dioxide, NH_3: ammonia, H_2S: hydrogen sulfide. [1]Diets included a control diet without *Lactobacillus plantarum* (control) and a diet supplemented with *L. plantarum* (LP). Additive treatments were as follows: no additive (–), a postbiotic derived from *Lactobacillus acidophilus* (PLA; 5 µL/g substrate), an essential oil blend (EOB; 20 µL/g substrate), and a combination of PLA and EOB at their respective doses.

Diet[1]	Additive	Total GP	CH_4	CO_2	NH_3	H_2S
Control	No additive	139^{b}	12.5^{ab}	60.3	35.3^{b}	221^{c}
	PLA	140^{b}	13.1^{a}	68.0	68.3^{a}	246^{c}
	EOB	104^{d}	4.93^{c}	57.2	80.8^{a}	570^{a}
	PLA+EOB	112^{c}	4.80^{c}	59.4	66.3^{a}	478^{ab}
LP	No additive	143^{ab}	11.0^{b}	63.4	89.8^{a}	354^{bc}
	PLA	148^{a}	13.1^{a}	65.5	79.3^{a}	285^{c}
	EOB	114^{c}	3.70^{c}	52.2	80.3^{a}	551^{a}
	PLA+EOB	116^{c}	3.35^{c}	56.9	89.8^{a}	617^{a}
SEM		2.2	0.772	1.56	4.09	30.2
LP		0.003	0.021	0.560	0.002	0.038
Additive		<0.001	<0.001	0.047	0.222	<0.001
LP×Additive		<0.001	<0.001	0.799	0.032	<0.001

The additive (P<0.001) and the additive × LP interaction (P<0.001) generally affected total GP. Substantial reductions in CH_4 emissions were observed with EOB and PLA+EOB treatments across both diets, with the control supplemented with EOB (4.93 %) resulting in a 60% CH_4 reduction compared to the control without the additive. The LP diet supplemented with EOB (3.70 %) had an even greater reduction, and generally LP (P = 0.021), additive (P<0.001), and LP × additive (P<0.001) generally reduced CH_4. Significant variation in CO_2 emission was observed only with the additive (P=0.047), while LP (P = 0.002) and LP × additive (P = 0.032) affected NH_3 production, with the lowest value obtained in the control (35.3 ppm). Treatments generally resulted in elevated H_2S levels, with the highest values obtained for EOB and PLA+EOB in both the control and LP diets.

Nutrient digestibility

Nutrient digestibility results are presented in Table 4 as least squares means ± SEM (n = 8). No significant effects of LP were observed for nutrient digestibility except for *d*NDF. Moreover, no additive or LP × additive interaction were observed (P > 0.05) for *d*ADF, *d*ADL, and *d*HEM. The additive affected DM digestibility (*d*DM) and *d*CELL. Higher (P<0.001) *d*DM was observed in the control with PLA and the LP diet with no additive, while EOB and the PLA+EOB combination reduced the *d*DM of both the control and LP diets. *d*NDF increased with the presence of LP (P=0.024), with the highest observed when the LP diet was combined with EOB (60.7 %). The inclusion of the additive reduced *d*CELL (P<0.001), as well as LP × additive (P=0.001) interaction, with either diet containing EOB showing the lowest values.

Volatile fatty acids

Volatile fatty acid concentrations are summarized in Table 5 and reported as least squares means ± SEM (n = 8). The additive and LP × additive interaction significantly affected total and individual VFA concentrations, while LP affected only butyric acid concentration. Decreased total VFA and C_2 concentrations were obtained with EOB and PLA+EOB treatments, while PLA was similar to the control which had higher values. Additionally, EOB and PLA+EOB also decreased (P<0.001) the C_2:C_3 ratio. Inclusion of PLA reduced (P<0.001) C_4 acid concentration, but the other treatments were higher and comparable with the control diet. The concentration of C_5 decreased with additives (P=0.025). The influence of additives (P=0.001) on iso-C_4 concentration showed that while the LP diet with PLA was highest, there was a decrease with the inclusion of EOB and PLA+EOB in both diets.

Table 4. Effects of diet and additives on *in vitro* nutrient digestibility (%). Values are reported as least squares means with standard error of the mean (SEM); n = 8 observations per treatment combination derived from two independent incubation runs with four replicate bottles per run. *d*DM: degradable DM, *d*NDF: degradable neutral detergent fiber, *d*ADF: degradable acid detergent fiber, *d*ADL: degradable acid detergent lignin, *d*HEM: degradable hemicellulose, *d*CELL: degradable cellulose. [1]Diets included a control diet without *Lactobacillus plantarum* (control) and a diet supplemented with *L. plantarum* (LP). Additive treatments were as follows: no additive (–), a postbiotic derived from *Lactobacillus acidophilus* (PLA; 5 µL/g substrate), an essential oil blend (EOB; 20 µL/g substrate), and a combination of PLA and EOB at their respective doses.

Diet[1]	Additive	*d*DM	*d*NDF	*d*ADF	*d*ADL	*d*HEM	*d*CELL
Control	No additive	54.6^{ab}	57.9^{b}	36.5	10.5	42.4	40.5^{a}
	PLA	55.3^{a}	59.0^{ab}	37.3	10.9	42.0	40.2^{a}
	EOB	53.1^{bc}	58.9^{ab}	37.4	10.4	41.8	39.5^{ab}
	PLA+EOB	51.0^{d}	58.1^{b}	37.4	10.1	41.6	38.6^{b}
LP	No additive	55.5^{a}	59.8^{ab}	37.4	11.1	41.8	40.3^{a}
	PLA	54.3^{abc}	58.9^{ab}	36.5	10.3	41.9	40.3^{a}
	EOB	52.9^{bcd}	60.7^{a}	38.0	10.5	41.2	39.2^{b}
	PLA+EOB	52.7^{cd}	59.6^{ab}	37.0	10.5	41.3	39.6^{ab}
SEM		0.618	0.780	0.625	0.394	0.379	0.326
LP		0.435	0.024	0.826	0.564	0.146	0.508
Additive		<0.001	0.539	0.608	0.549	0.219	<0.001
LP×Additive		<0.001	0.032	0.521	0.452	0.895	0.001

Table 5. Effects of diet and additives on total and individual volatile fatty acid concentrations (mmol/g DM). Values are reported as least squares means with standard error of the mean (SEM); n = 8 observations per treatment combination derived from two independent incubation runs with four replicate bottles per run. VFA: volatile fatty acids, C_2: acetate, C_3: propionate, C_4: butyrate, C_5: valerate. [1]Diets included a control diet without *Lactobacillus plantarum* (control) and a diet supplemented with *L. plantarum* (LP). Additive treatments were as follows: no additive (–), a postbiotic derived from *Lactobacillus acidophilus* (PLA; 5 µL/g substrate), an essential oil blend (EOB; 20 µL/g substrate), and a combination of PLA and EOB at their respective doses.

Diet[1]	Additive	VFA	C_2	C_3	C_2:C_3	C_4	C_5	Iso-C_4	Iso-C_5
Control	No additive	83.3^{a}	56.9^{a}	16.6	3.47^{a}	8.67^{a}	0.915^{a}	0.134^{a}	0.067
	PLA	79.7^{ab}	56.0^{a}	15.1	3.71^{a}	7.61^{b}	0.809^{b}	0.124^{a}	0.059
	EOB	76.1^{bc}	50.7^{b}	15.9	3.21^{b}	8.68^{a}	0.780^{b}	0.063^{b}	0.046
	PLA+EOB	74.5^{bc}	49.4^{b}	15.7	3.16^{b}	8.50^{a}	0.802^{b}	0.057^{b}	0.048
LP	No additive	78.9^{abc}	55.5^{a}	15.1	3.68^{a}	7.33^{b}	0.812^{b}	0.107^{ab}	0.058
	PLA	78.8^{abc}	55.3^{a}	15.1	3.68^{a}	7.34^{b}	0.843^{b}	0.138^{a}	0.059
	EOB	75.2^{bc}	50.0^{b}	15.8	3.18^{b}	8.39^{a}	0.804^{b}	0.082^{ab}	0.051
	PLA+EOB	73.6^{c}	48.0^{b}	16.2	2.97^{b}	8.53^{a}	0.818^{b}	0.061^{b}	0.051
SEM		1.83	1.32	0.58	0.081	0.245	0.0240	0.0200	0.0081
LP		0.170	0.264	0.520	0.873	0.009	0.670	0.848	0.989
Additive		0.001	<0.001	0.457	<0.001	<0.001	0.025	0.001	0.175
LP×Additive	0.010	<0.001	0.381	<0.001	0.041	0.016	0.01	0.835	

DISCUSSION

Nutrient digestibility

The inclusion of EOB increased the undegraded substrate portion, particularly when administered in the LP diet, which was also evident when PLA+EOB was included in either the control or LP diet. This increase in undegraded residuals consequently decreased the values of IVADDM and IVTDDM. Additionally, significant LP × additive interaction effects confirmed that the degradability response is diet-dependent. The impact of EOs supplementation includes a shift in fermentation pattern and a reduction in *d*DM due to altered microbial populations. This effect is consistent with the antimicrobial properties of EOs against cellulolytic bacteria, as previously reported with EO blends containing garlic and lemongrass (Alabi et al., 2024; Kholif and Olafadehan, 2021). This confirms the antimicrobial effects of EOBs on both Gram-positive and Gram-negative bacteria which form the bulk of rumen fibrolytic flora (Caroprese et al., 2023; Ike et al., 2025).

Greater *d*DM was obtained from the diet containing LP and the control diet incubated with postbiotic fermentation products derived from *L. acidophilus*. This observation is similar to the report of Izuddin et al. (2018), who recorded a significant linear increase in OM digestibility with incremental additions of *L. plantarum* postbiotic additive. Consistent with our findings, improved feed digestibility observed with microbial additives has often been linked to enhanced *in vitro* GP, which reflects increased microbial degradation and provides a reliable proxy for estimating feed utilization by rumen microbes (Parchami et al., 2024). Supplementation with microbial additives including *L. plantarum* or *Enterococcus faecalis* resulted in improved *d*DM and *d*NDF relative to the control (Guo et al., 2020).

The inclusion of EOB or its combination with postbiotics slightly but significantly reduced *d*DM, which is consistent with previous studies reporting similar effects with either individual or EOBs. Singh et al. (2018) observed that lemongrass EOs supplementation reduced IVTDDM at doses higher than 10 µL/40 mL buffered rumen fluid. Gray et al. (2025) reported reduced DM digestibility in high-forage and high-concentrate diets incubated with various EOs and EOBs. Our results support this observation, as EOB inclusion decreased both *d*DM and *d*CELL across diets, suggesting its antimicrobial potency extended to cellulolytic microbes. In contrast to the foregoing observations, some studies have reported improved DM digestibility with EO supplementation. Yang et al. (2007) reported increased DM and OM digestibility with garlic and juniper berry EOs, emphasizing potential nutrient availability improvement.

Fiber digestibility was mostly stable across treatments, although additive inclusion improved *d*NDF while *d*CELL was slightly reduced by EOB. Interestingly, the highest *d*NDF was observed when LP was combined with EOB, suggesting a potential synergistic effect between probiotic-driven microbial modulation and the selective antimicrobial activity of EOs. Such interactions may suppress non-fibrolytic competitors, favoring fibrolytic microbial populations and enhancing fiber utilization. Significant interactions between diet and additive for both parameters showed that fiber degradation responses varied depending on the base diet. Diao et al. (2019) reported improvements in *d*DM and *d*NDF with cumin and lemongrass EOs. The potential improvement in NDF digestibility can be a result of the selective antimicrobial action of EOs, which suppressed CH_4-producing bacteria, possibly allowing for the proliferation or enhanced activity of beneficial fiber-digesting bacteria and fungi (Caroprese et al., 2023). Kurniawati et al.

(2018) reported that high EOs concentrations impacted fiber and protein degradability, highlighting the importance of optimal inclusion rates. Ahmed et al. (2021) indicated that garlic did not significantly interfere with fiber degradability but potentially improved nutrient digestibility when combined with other feed components. Taken together, these studies and our findings demonstrate the complex and context-specific interactions between probiotics, postbiotics, EOs and rumen microbial communities in shaping nutrient digestibility.

Microbial additives improve ruminal fermentation, which can be attributed to high bacterial growth activity leading to increased microbial fermentation during incubation (Miguel et al., 2019). In this study, calf pellets with *L. plantarum*-based additive combinations produced more gas compared to the control. This observation is consistent with a previous study (Astuti et al., 2018), which reported a significantly higher total GP from 24 h *in vitro* rumen fermentation of 14 strains of *L. plantarum*. Zhang et al. (2023) reported that alfalfa inoculated with *L. plantarum* strains increased GP, likely due to the rapid degradation of soluble substrates.

Enhanced GP observed with the fermentation product of *L. acidophilus* shows that microbial additives are not limited to live organisms. Postbiotic inclusion from *L. plantarum* RG14 culture administered from 0.6% based on the rumen fluid media increased GP from soluble fraction fermentation (Izuddin et al., 2018). Postbiotic metabolites and cell wall components enhance nutrient degradation by optimizing the rumen environment and promoting beneficial microbial activity (Guo et al., 2024). In the present study, both LP and PLA increased total GP, confirming their role in stimulating microbial fermentation through enhanced growth and enzymatic activity. However, EOB supplementation consistently reduced GP irrespective of LP inclusion, consistent with its antimicrobial activity against fibrolytic and methanogenic microbes (Davoodi et al., 2016; Kholif et al., 2026).

The observed additive × LP interaction indicates that GP response depends on both microbial inoculation and EO compounds. Essential oil blends significantly affect GP, often reducing total gas (Alabi et al., 2024; Gray et al., 2025; Ike et al., 2024a), an effect which is likely due to the antimicrobial properties which can inhibit rumen microbial activity. Treatments containing EOB consistently decreased GP, and this reduction was not affected by diet or postbiotic inclusion. Such reductions are not necessarily detrimental, since lower GP coupled with higher PF_{24} in EOB treatments indicate improved microbial efficiency and redirection of fermentation toward energetically favorable pathways.

Microbial mass values were enhanced by the PLA+EOB treatment compared to PLA alone in the LP diet, suggesting a potential synergistic effect of postbiotics with EO when combined with probiotics. Postbiotics supply bioactive metabolites such as organic acids and bacteriocins (Hall et al., 2022), which may stabilize microbial growth under EO-induced stress. However, PLA with LP yielded the lowest microbial mass, potentially due to excessive acidification limiting microbial protein synthesis (Branco-Lopes et al., 2025). While EOB slightly reduced digestibility parameters, its inclusion resulted in enhanced microbial efficiency, reflected by higher PF_{24} and microbial mass, especially in the control diet. The PF_{24} was strongly influenced by LP, additive, and their interaction, with the highest values obtained for EOB and PLA+EOB in the control diet without LP. This suggests EOs enhanced microbial efficiency despite lower digestibility. The PF_{24} represents the ratio of truly digested matter to gas produced (Blümmel et al., 1997), indicating how efficiently microbes convert feed into biomass and fermentation products, with implications for ruminant production and environmental impact (Jackson et al., 2010).

Greenhouse gas emissions

In vitro fermentation changes in total GP due to additive inclusion are accompanied by shifts in greenhouse gas composition, including CH_4, CO_2, NH_3 and H_2S (O'Donnell et al., 2024). In the present study, additives generally reduced CH_4, with the strongest effect observed for EOB and PLA+EOB. The control diet supplemented with EOB reduced CH_4 output by 60%, while the LP diet supplemented with EOB achieved a 73.2% reduction, confirming the strong antimethanogenic capacity of EO blends through suppression of archaea and hydrogen-producing bacteria, and redirection of hydrogen toward propionate production (Doyle et al., 2019; Ike et al., 2024a). By contrast, PLA inclusion alone did not substantially mitigate CH_4, suggesting that postbiotics may enhance rumen fermentability without conferring antimethanogenic benefits. This supports previous reports that certain postbiotics can increase substrate availability for methanogens (Fernández et al., 2023). However, the combination of PLA with EOB consistently reduced CH_4, highlighting the capacity of EO to override the CH_4-promoting effects of postbiotics.

The LP diet reduced CH_4 by 11% compared to the control, similar to previous studies on *L. plantarum* and other lactic acid bacteria. The mechanisms include competition for hydrogen utilization with methanogens, increased fibrolytic and H_2-incorporating bacteria (Astuti et al., 2018; Doyle et al., 2019; Zhang et al., 2023), and production of antimicrobial H_2O_2 by *L. plantarum* that inhibits methanogens (O'Brien et al., 2013). The synergistic reduction observed in the LP × EOB treatments suggests that EO antimethanogenic effects are strengthened in the presence of probiotics (Kholif et al., 2024). The effect of EOs on CH_4 production has been reported to be dependent on specific ratios and concentrations of the active compounds (Gray et al., 2025), with blends often more effective due to synergism (Ike et al., 2024b; Kilic et al., 2011). Reduction of CH_4 in EOB treatments also elevated H_2S, indicating a potential trade-off in hydrogen utilization. In the rumen, H_2S is produced by sulfate-reducing bacteria (SRB) using lactate and sulfate (Zhao and Zhao, 2022). Methanogens and SRB compete for hydrogen, with SRB thermodynamically advantaged, especially under moderate temperatures (Drewnoski et al., 2012). Approximately 70-85% of H_2S eructated from the rumen can be inhaled (Pogge et al., 2016) and subsequently absorbed into the bloodstream, potentially leading to polioencephalomalacia, a syndrome associated with sulfur toxicity resulting from high sulfur intake.

Volatile fatty acids

Volatile fatty acids are the primary end-products of carbohydrate fermentation and provide energy precursors for ruminant metabolism. These acids also support ruminal epithelium development which is crucial for nutrient absorption and metabolism (Miguel et al., 2019). Inclusion of the additives reduced total VFA in this study, with the LP diet incubated with

PLA and EOB having the lowest levels, indicating significant additive × diet interaction. This suggests partial suppression of fermentation activity, although higher GP with LP and PLA indicates that not all fermentation pathways were equally inhibited. The study by Monteiro et al. (2020) also showed that incremental administration of *L. plantarum* tended to cause a linear reduction in total VFA concentrations. The effect of EOB confirms the positive correlation reported between VFA and GP (Molho-Ortiz et al., 2022), with both parameters being significantly reduced. However, it is crucial to consider that there are conditions like substrate and microbial community dynamics which affect the interplay between VFA molar concentrations and gas yield (Maurus et al., 2023).

Acetate values were reduced by EOB inclusion regardless of diet, which is consistent with previous studies that reported a decrease in C_2 and the C_2:C_3 ratio with the inclusion of thyme, cinnamon, or lemongrass oils (Diao et al., 2019; Singh et al., 2018). The decrease in C_2 concentration reduces hydrogen availability for methanogens (Waters et al., 2025), which is consistent with the observed reduction in GP. A reduction in C_2:C_3 is linked with lower CH_4 production and better energy utilization (Parchami et al., 2024; Phupaboon et al., 2025). Although C_3 production did not increase, C_4 levels were higher with EOB, suggesting beneficial acetogenic hydrogen utilization, supporting fatty acid synthesis (Miguel et al., 2019). The PLA alone reduced C_4, likely due to lactic acid accumulation and bacteriocin activity that suppressed C_4-producing microbes, consistent with findings that *L. plantarum* can lower C_4 through shifts in microbial competition (Casper et al., 2021). Combined LP and PLA also reduced C_4, consistent with previous reports that *L. plantarum* increases lactic acid production and reduces hydrogen availability for CH_4 production (Astuti et al., 2018). Isoacids (iso-C_4 and C_5) were affected by the treatments. The decline in C_5 with additives suggests reduced amino acid deamination, supporting protein-sparing effects (Kholif et al., 2026). The LP diet with PLA had the highest iso-C_4 concentration, indicating stimulation of branched-chain VFA production. EOB and PLA+EOB reduced iso-C_4, suggesting suppression of proteolytic bacteria (Davoodi et al., 2016).

CONCLUSION

This study demonstrates the potential of *L. plantarum* (LP), a postbiotic derived from *Lactobacillus acidophilus* and essential oil blends (EOB) to modulate rumen fermentation, reduce methane production, and improve microbial efficiency. LP increased gas production and dry matter digestibility, confirming its role in enhancing ruminal fermentation kinetics and nutrient utilization, while the EOB decreased gas production and methane emissions by up to 73.2%, highlighting its antimethanogenic effect. The combination of LP and EOB resulted in a synergistic reduction in methane production and maintained microbial efficiency, suggesting a promising strategy for mitigating greenhouse gas emissions from ruminants. However, the effects on nutrient digestibility and volatile fatty acid production varied depending on the diet and additive combination, reflecting the complex interactions between probiotics, postbiotics, essential oils, and the basal diet. The results highlight the importance of considering the complex interactions between additives, diet, and the rumen microbiome in optimizing ruminant nutrition and reducing environmental impact. Further research is needed to determine the optimal inclusion rates and combinations of *L. plantarum* and the essential oil blend for specific dietary contexts.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Oludotun Adelusi: Conceptualization, formal analysis, investigation, methodology and writing – original draft. **Olatunde Oderinwale, Joel Alabi, Michael Wuaku, James Enikuomehin, Chika Anotaenwere, Deborah Okedoyin, Kiran Subedi, Kelechi Ike,** and **Ahmed Kholif**: Investigation, formal analysis and methodology. **Oludotun Adelusi**, and **Ahmed Kholif**: writing – review & editing. **Uchenna Y. 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.

ETHICS AND PERMIT APPROVALS

The experimental procedures and animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at North Carolina Agricultural and Technical State University, Greensboro (IACUC Protocol No: LA22-0019; approved 31 July 2022).

DATA AVAILABILITY STATEMENT

Data are contained within the article.

USE OF ARTIFICIAL INTELLIGENCE (AI) TOOLS

The authors declare that no AI tools were used.

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