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Effects of Plant Essential Oils and Their Combinations on In Vitro Ruminal Fermentation Characteristics

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Abstract

This study aimed to evaluate the effects of eight plant essential oils on in vitro ruminal fermentation. The oils tested were verbena (VE), cinnamon (CE), rosemary (RE), Torreya grandis (TG), ginger (GE), fennel (SF), thyme (TE), and oregano (OE). Each was supplemented at five graded doses (0, 0.75, 1.5, 3, and 6% of the substrate DM) during 24 h incubations. The results demonstrated that adding 3% CE significantly increased the in vitro dry matter degradation rate (IVDMD) while enhancing microbial protein (MCP) synthesis efficiency and acetate concentration. The addition of 1.5% SF increased MCP and total volatile fatty acid (TVFA) concentrations without affecting ruminal ammonia nitrogen levels, and 0.75% TE enhanced nitrogen utilization efficiency while maintaining primary fermentation pathways and ammonia nitrogen levels. Although 0.75% OE did not alter overall fermentation intensity or nitrogen metabolism, it specifically regulated volatile fatty acid metabolic pathways, significantly promoting butyrate production. Through preliminary screening, four essential oils (CE, SF, TE, OE) that positively influenced rumen fermentation were selected and combined into five distinct blends: T1 (CE + SF + TE), T2 (CE + SF + OE), T3 (CE + TE + OE), T4 (SF + TE + OE), and T5 (CE + SF + TE + OE). In vitro 24 h fermentations with varying inclusion levels (0, 0.75, 1.5, 3% of the substrate DM), in comparison with the CON, showed that the T2 group significantly outperformed the other four formulations in nitrogen metabolism and IVDMD, while substantially reducing methane content. Simpson's index of microbial diversity was markedly elevated, alongside an increased relative abundance of key bacterial genera—*Selenomonas* and *Succinibacter*. Considering comprehensive performance metrics and economic viability, the 1.5% T2 was selected as the optimal formulation.

Keywords: plant essential oils; In vitro fermentation; ruminant; methane; microbial diversity

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1. Introduction

Methane (CH_4) emissions from ruminal fermentation represent a significant driver of agricultural greenhouse gases, acting as a potent contributor to global climate change [1]. Ruminal CH_4 is primarily produced by methanogenic archaea through the reduction of H_2 and CO_2 [2]. This emission level is influenced by critical factors like diet formulation, feed intake, and nutrient use efficiency [3].The excessive microbial proteolysis in the rumen degrades dietary protein into ammonia–nitrogen (NH_3-N) [4]. While a portion of this NH_3-N

is utilized for microbial protein (MCP) synthesis, a substantial proportion is absorbed and converted to urea for excretion [5], thereby leading to low nitrogen utilization efficiency and environmental pollution [6]. Furthermore, CH_4 production entails a substantial energy loss from the feed, which consequently lowers the efficiency of energy utilization and leads to suboptimal production outcomes in ruminants [7]. In addition to different feed processing [8], the use of feed additives which can also regulate rumen fermentation is equally important. The use of feed additives offers an effective approach to modulate rumen fermentation, thereby improving the utilization of dietary energy and protein [9]. Shifting the rumen fermentation pattern towards increased propionate and reduced acetate production [10] can enhance production performance [11] and reduce CH_4 emissions [12]. For example, supplementing with monensin has been shown to increase the proportion of propionate while decreasing the proportions of acetate and butyrate in the rumen [13]. However, since the European Union banned the use of antibiotics as growth promoters in animal feed in 2006 [14], there has been an urgent need to identify natural alternatives [15]. Essential oils (EOs)—aromatic volatile compounds derived from plant secondary metabolites—are generally recognized as safe for use in human and animal foods [16,17]. A key characteristic of essential oils is that their active components primarily fall into two major chemical classes: terpenoids and phenylpropanoids [18]. Plant essential oils, as natural alternatives, have garnered attention for their considerable potential in regulating ruminal processes [19]. Many commercial phytogenic feed additives are formulated with either single EOs or blends of several EOs. Numerous in vitro studies and a growing number of in vivo trials have indicated that specific plant essential oils and their active components can significantly modulate rumen fermentation [20]. For instance, Zhang et al. [21] reported that supplementing with 10 mL/kg of Zanthoxylum essential oil markedly modulated the ruminal microbial population in Small-tailed Han sheep. In contrast, Ranilla et al. [22] found that thyme essential oil, standardized to 75 mg/L carvacrol, reduced the molar proportion of propionate while increasing the concentration of NH_3-N in the rumen. Oregano essential oil is primarily characterized by its high content of carvacrol and thymol [23]. These bioactive compounds have been shown to mitigate localized oxidative stress in the intestinal tract, modulate the gut microbiota, and enhance natural antibody production [24]. Huang et al. [25] reported that including 20 g/d of oregano essential oil in the diet of beef cattle improved digestive health, which in turn bolstered growth performance and lowered susceptibility to diseases. The combination of single essential oils into blends can produce synergistic benefits, leveraging their distinct properties. For instance, Bach et al. [26] reported that a 1 g/d mixture of eugenol, geranyl acetate, and coriander oil mitigated CH_4 production without affecting dairy performance. In another study, Ma et al. [27] found that a blend high in cinnamaldehyde and apigenin improved growth performance in lambs. For decades, in vitro fermentation systems have been utilized in animal nutrition to mimic the rumen environment by maintaining critical parameters such as temperature, pH, osmolarity, and anaerobic conditions [28]. Among these, the in vitro batch culture technique has proven to be a cost-effective, efficient, and highly reproducible method, providing a standardized platform for evaluating ruminal fermentation kinetics and microbial community dynamics under controlled conditions [29]. Most plant essential oils contain a diverse array of bioactive compounds capable of modulating rumen fermentation patterns, improving feed digestibility and energy utilization efficiency, and influencing host metabolism [30]. Furthermore, they can suppress methanogenesis by directly inhibiting the metabolic activity of methanogenic archaea (methanogens) [31]. These attributes establish plant essential oils as a promising class of natural, safe, and effective modulators of rumen fermentation. To address this knowledge gap regarding the individual and combined effects of the eight selected plant essential oils, this study examined their influence on

rumen fermentation under in vitro batch culture conditions. The goal was to screen for promising single oils and synergistic blends that improve feed digestibility and enhance rumen fermentation, thereby supplying critical technical data and a theoretical basis for the practical application and commercial development of these formulations as feed additives.

2. Materials and Methods

2.1. Experimental Site

This experiment was conducted at the Ruminant Nutrition and Feed Laboratory, Department of Animal Science, Anhui Agricultural University (Hefei, China). Rumen fluid samples were collected from the rumen of three Simmental cattle with an average weight of 420 kg (±25 kg) using rumen fluid collectors. The experimental cattle were in good health, free from any diseases, and had unrestricted access to water and feed. This study was approved by the Animal Management and Animal Ethics Committee of the College of Animal Science and Technology, Anhui Agricultural University (Approval No.: SYDW-P20190600601).

Immediately after collection, the fluid was filtered through four layers of sterile gauze into a pre-warmed (40 °C) thermos flask and swiftly transported to the laboratory for subsequent procedures. All plant essential oils were sourced from Jiangxi Taicheng Natural Flavors Co., Ltd. in Jiangxi, China. The rumen donor cows and the TMR formula used for in vitro fermentation were the same. To ensure the authenticity and completeness of the experiment, the pre-feeding period for the three rumen donor cows was 7 days, and they had all adapted to the TMR. A total mixed ration (TMR) substrate for in vitro culture was prepared from corn, soybean meal, and corn silage as raw materials. All ingredients were dried in a constant-temperature forced-air drying oven at 65 °C for 48 h, ground and sieved through a 1 mm mesh. The specific composition and nutritional content of the diet are detailed in Table 1. The substrate was formulated with a forage-to-concentrate ratio of 60:40.

Table 1. Diet composition and nutrient levels (dry matter basis).

Composition	Content (%)	Nutrient Levels	Content (%)
Alfalfa hay	8.3	Ash	6.5
Corn silage	55.6	CP	15.6
Corn	21.7	EE	5.8
Wheat bran	5.4	NDF	40.4
Soybean meal	6.9	ADF	29.4
Saleratus	0.4	Ca	0.80
Premix	1.7	P	0.28
Total	100		

The chemical composition of the substrate was analyzed in five replicates. Dry matter (DM) was determined by oven-drying at 105 °C for 6 h. Ash content was measured by combustion in a muffle furnace at 550 °C for 5 h. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed sequentially according to Van Soest et al. [32]. Crude protein (CP) was determined by the Kjeldahl method, and crude fat (ether extract, EE) was analyzed by Soxhlet extraction. Calcium (Ca) and phosphorus (P) contents were measured by the EDTA complexometric method and spectrophotometry, respectively.

The specific types and their principal chemical components are detailed in Table 2.

Table 2. Plant essential oils main ingredients list.

Essential Oils	Main Ingredients
Verbena (VE)	Citral $\geq$ 50%
Cinnamon (CE)	Cinnamaldehyde $\geq$ 85%
Rosemary (RE)	1,8-Cineole $\geq$ 38%
Torreya grandis (TG)	Limonene $\geq$ 25%
Ginger (GE)	Zingiberene 25–35%
Fennel (SF)	Anethole 50–66%
Thyme (TE)	para-Cymene15–55%
Oregano (OE)	Carvacrol 60–85%

2.2. Experimental Design

The experiment was conducted in two consecutive phases employing in vitro ruminal fermentation systems. The first phase aimed to screen the effects of individual essential oils. It followed a single-factor design with eight plant essential oils (verbena, cinnamon, rosemary, Torreya grandis, ginger, fennel, thyme, and oregano). Each oil was tested at five inclusion levels (0, 0.75, 1.5, 3, and 6% of the substrate DM), resulting in 40 treatment groups with 3 repetitions in each group. A blank control (CON, substrate only) and an additional blank for gas calibration (no substrate, no oil) were included, with three replicates per group. All cultures were conducted over 24 h for the preliminary screening.

Based on the results of the first phase, the second phase also employed a single-factor completely randomized design. Five composite blends were assessed: T1 (cinnamon + fennel + thyme), T2 (cinnamon + fennel + oregano), T3 (cinnamon + thyme + oregano), T4 (fennel + thyme + oregano), and T5 (cinnamon + fennel + thyme + oregano). In groups T1–T4, the ratio of the compound essential oil is 1:1:1. In group T5, the ratio of the compound essential oil is 1:1:1:1. Each blend was tested at four inclusion levels (0, 0.75, 1.5, and 3% of substrate DM), resulting in 20 treatment groups. A substrate-only blank control and a baseline blank for gas calibration were included, all with three replicates. The 24 h fermentation was conducted to screen the composite essential oils.

Among the five essential oil combinations, T2 was selected for microbial profiling analysis at the end of the second phase of in vitro fermentation. This selection was based on T2 demonstrating the most favorable in vitro fermentation response at moderate addition levels, characterized by reduced NH_3-N, increased MCP, and enhanced IVDMD. Considering economic costs, we focused on in vitro fermentation broths from the T2 group of blended plant essential oils at different addition concentrations and conducted associated microbial analyses.

2.3. Batch Fermentation In Vitro

The entire process of preparing and conducting short-term batch fermentation was carried out according to the description by Cieslak [33] with modifications. Precisely 0.2 g of a total mixed ration (TMR) sample was weighed into a 100 mL fermentation flask. The flask containing the TMR was capped with a rubber stopper and placed into a 39 °C constant-temperature incubator for later use. The buffer solution was prepared 24 h in advance, sterilized by autoclaving, and kept ready for use. An 800 mL artificial rumen fluid was prepared by mixing 200 mL of fresh rumen fluid with the buffer solution. During the experiment, the corresponding type and dosage of plant essential oil was added to each fermentation flask. At the time of inoculation, 20 mL of preheated mixed artificial rumen fluid, heated to 39 °C in a constant-temperature water bath, was rapidly added to each fermentation bottle. A continuous CO_2 supply was maintained throughout the process. The bottles were immediately sealed with rubber stoppers and secured with

aluminum caps using a capper. Finally, the fermentation bottles were placed in a 39 °C constant-temperature incubator and continuously incubated at 100 rpm for 24 h. After the experiment, gas production was measured using a 50 mL glass syringe. Artificial saliva buffer solutions are prepared as shown in Tables 3 and 4.

Table 3. Buffer preparation table.

Buffer (A) 500 mL	Buffer (B) 500 mL	8% $NaCO_3$ 50 mL
KH_2PO_4 3 g distilled water 500 mL	$(NH_4)_2SO_4$ 6 g NaCl 6 g KH_2PO_4 3 g $MgSO_4 \cdot 7H_2O$ $CaCl_2 \cdot 2H_2O$ distilled water 500 mL	$NaCO_3$ 4 g distilled water 500 mL

Table 4. Medium preparation chart.

Final Buffer	Volumetric/L
Buffer (A)	40 mL
Buffer (B)	40 mL
Rumen liquid	200 mL
distilled water	720 mL
8% $NaCO_3$	50 mL
cysteine	0.6 g

After 24 h of fermentation, the gas volume was recorded. The culture medium was filtered for pH determination, and subsequently aliquoted: each aliquot was mixed with a 25% metaphosphoric acid solution, aliquoted, and stored at −20 °C for volatile fatty acid (VFA), ammonia nitrogen (NH_3-N), and MCP analysis. The filtered residue was dried according to the determination of dry matter (DM) degradability.

After the 24 h incubation, total gas volume was measured with a 50 mL glass syringe [34], and the collected gas was sampled for CH_4 determination. CH_4 concentration was determined by gas chromatography (Shimadzu, Kyoto, Japan) with an external standard.

After fermentation concludes, the filtered rumen fluid is immediately tested for final fermentation pH using a portable pH meter. Prior to testing, the pH meter is calibrated using pH standard buffers (pH = 4.00 and pH = 9.18).

The concentration of ammonia–nitrogen (NH_3-N) was quantified by the phenol-hypochlorite spectrophotometric method [35]. Microbial protein (MCP) concentration was determined using the Coomassie Brilliant Blue G-250 binding method [36]. The concentrations of volatile fatty acids (VFA) were analyzed by gas chromatography (Agilent, Santa Clara, CA, USA).

After fermentation, the entire contents of each fermentation flask were filtered through a 300-mesh nylon cloth. All residues were transferred onto the nylon cloth using distilled water. The nylon cloth containing the residues was then placed into a pre-weighed Petri dish and dried in a constant-temperature oven at 105 °C for 4–6 h until a constant weight was achieved. The total weight was recorded after cooling in a desiccator for 30 min. The in vitro dry matter degradation rate is calculated using the following formula:

$$\text{IVDMD (\%)} = [(W_1 - W_2)/W_1] \times 100 \quad (1)$$

where:

W_1 is the dry matter weight before fermentation (grams);

W_2 is the residual dry matter weight after fermentation (grams).

2.4. Rumen Microorganism DNA Extraction and PCR Amplification

For the microbial genome analysis, fermentation broth samples were collected from the four addition groups (0%, 0.75%, 1.5%, and 3%) of the T2 composite plant essential oil, with three replicates per group, resulting in a total of 12 samples. Total microbial genomic DNA was extracted from rumen liquid samples with the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. The quality and concentration of the extracted DNA were assessed by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), after which the DNA was stored at −80 °C until further use.

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the primer pair 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVG GGTWTCTAAT-3′) [37] on a T100 Thermal Cycler (BIO-RAD, Hercules, CA, USA).

The PCR mixture consisted of the following components: 4 μL of 5× Fast Pfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of Fast Pfu polymerase, 10 ng of template DNA, and ddH_2O added to a final volume of 20 μL. The cycle conditions for its polymerase chain reaction (PCR) were set as follows, referring to He S [38]: initial denaturation at 94 °C for 1 min; 30 cycles of denaturation at 94 °C for 20 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 10 min, and a final hold at 4 °C. The PCR products were excised from a 2% agarose gel, purified using the PCR Clean-Up Kit (YuHua, Shanghai, China) according to the manufacturer's instructions, and quantified with the Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Analysis of Sequencing Data

The purified amplicons were pooled in equimolar amounts and subjected to paired-end sequencing on an Illumina Nextseq2000 platform (Illumina, San Diego, CA, USA) following standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP659059.

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request. The data are also publicly accessible via the following link: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1395272 (accessed on 31 December 2025).

2.6. Statistical Analysis

The experimental data were processed in Microsoft Excel 2019 and then subjected to a one-way analysis of variance (ANOVA) in SPSS version 27.0 (IBM Corp., Armonk, NY, USA). Duncan's new multiple range test was employed to separate means when the ANOVA revealed a significant effect. All values are expressed as the mean, and a probability level of $p < 0.05$ was defined as statistically significant. Microbial bioinformatics was performed as follows: sequences were processed (FLASH v1.2.11), OTUs were clustered (UPARSE v11, 97% identity), and taxa were classified (RDP Classifier v2.13). Alpha diversity (ACE, Chao1, Shannon, Simpson, Coverage) was computed in mothur (v1.30.2) and compared using the Wilcoxon test. Beta diversity (PCA, PCoA, NMDS, UPGMA) and functional prediction (PICRUSt v1.1.0; KEGG) were analyzed in QIIME (v1.9.1). Differential species analysis was conducted with LEfSe, employing Kruskal–Wallis and Wilcoxon tests, with effect sizes estimated by LDA.

3. Results

3.1. Effects of Individual Essential Oils on the pH of In Vitro Ruminal Fermentation

As shown in Table 5, compared with the control, pH was significantly lowered ($p < 0.05$) by the addition of 0.75%, 1.5%, or 3% cinnamon oil and 1.5% rosemary oil. Conversely, a significant increase ($p < 0.05$) in pH was observed with 3% and 6% fennel oil, as well as 6% thyme oil.

Table 5. Effects of individual essential oils on the pH of in vitro ruminal fermentation.

Essential Oil	Concentration %DM					SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%	6%		
Verbena	6.47	6.37	6.37	6.5	6.47	0.021	0.119
Cinnamon	6.47 ^{a}	6.4 ^{b}	6.4 ^{b}	6.4 ^{b}	6.48 ^{a}	0.012	0.012
Rosemary	6.47 ^{a}	6.45 ^{ab}	6.4 ^{b}	6.47 ^{a}	6.5 ^{a}	0.011	0.009
Torreya grandis	6.47	6.48	6.45	6.49	6.49	0.00	0.291
Ginger	6.47	6.48	6.48	6.53	6.52	0.013	0.428
Fennel	6.47 ^{b}	6.43 ^{b}	6.46 ^{b}	6.6 ^{a}	6.62 ^{a}	0.024	<0.001
Thyme	6.47 ^{bc}	6.44 ^{c}	6.53 ^{bc}	6.55 ^{b}	6.67 ^{a}	0.026	0.002
Oregano	6.47	6.48	6.55	6.59	6.41	0.025	0.111

[1] CON = control group (0% essential oils); ^{a,b,c} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.2. Effects of Individual Essential Oils on NH_3-N Concentration in In Vitro Ruminal Fermentation

As presented in Table 6, compared with the control, a significant reduction ($p < 0.05$) in NH_3-N concentration was observed with 6% cinnamon, fennel, and thyme oils, as well as with 1.5%, 3%, and 6% oregano oil. Conversely, supplementation with 6% verbena, rosemary, or Torreya grandis oil significantly increased ($p < 0.05$) the NH_3-N concentration.

Table 6. Effects of individual essential oils on NH_3-N (mg/dL) concentration in in vitro ruminal fermentation.

Essential Oil	Concentration %DM					SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%	6%		
Verbena	30.90 ^{b}	28.43 ^{c}	33.28 ^{a}	34.61 ^{a}	34.46 ^{a}	0.664	<0.001
Cinnamon	30.90 ^{a}	29.30 ^{a}	29.38 ^{a}	28.76 ^{a}	24.57 ^{b}	0.650	0.003
Rosemary	30.90 ^{b}	29.05 ^{b}	31.62 ^{b}	31.72 ^{b}	35.98 ^{a}	0.690	0.003
Torreya grandis	30.90 ^{c}	31.58 ^{c}	31.68 ^{c}	34.70 ^{b}	38.71 ^{a}	0.836	<0.001
Ginger	30.90	32.34	33.21	30.94	31.24	0.329	0.075
Fennel	30.90 ^{a}	30.35 ^{a}	30.04 ^{a}	31.15 ^{a}	20.47 ^{b}	1.254	<0.001
Thyme	30.90 ^{a}	30.63 ^{a}	30.21 ^{a}	28.59 ^{a}	20.98 ^{b}	1.230	0.003
Oregano	30.90 ^{a}	29.04 ^{ab}	25.07 ^{c}	19.04 ^{c}	18.72 ^{c}	1.522	<0.001

[1] CON = control group (0% essential oils); ^{a,b,c} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.3. Effects of Individual Essential Oils on Microbial Protein (MCP) Concentration in In Vitro Ruminal Fermentation

As shown in Table 7, compared to the control group, adding 1.5% cinnamon oil and 0.75% rosemary oil, fennel oil, or thyme oil significantly increased ($p < 0.05$) MCP concentration. Conversely, MCP concentration significantly decreased ($p < 0.05$) when 6% cinnamon oil, rosemary oil, fennel oil, thyme oil, or oregano oil was added.

Table 7. Effects of individual essential oils on MCP (μg/mL) concentration in in vitro ruminal fermentation.

Essential Oil	Concentration %DM					SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%	6%		
Verbena	115.74	119.07	116.07	117.44	114.88	0.670	0.331
Cinnamon	115.74 ^{c}	119.43 ^{b}	124.27 ^{a}	118.07 ^{b}	113.93 ^{c}	1.002	<0.001
Rosemary	115.74 ^{c}	127.47 ^{a}	122.53 ^{ab}	119.44 ^{bc}	113.33 ^{c}	1.524	0.003
Torreya grandis	115.74	115.74	117.67	115.93	111.89	0.724	0.120
Ginger	115.74	116.30	116.48	117.10	111.87	0.983	0.517
Fennel	115.74 ^{c}	126.67 ^{a}	122.72 ^{b}	118.04 ^{c}	110.31 ^{d}	1.548	<0.001
Thyme	115.74 ^{b}	121.10 ^{a}	119.43 ^{ab}	115.20 ^{b}	106.48 ^{c}	1.451	<0.001
Oregano	115.74 ^{a}	122.04 ^{a}	118.52 ^{a}	100.06 ^{b}	99.26 ^{b}	2.676	<0.001

[1] CON = control group (0% essential oils); ^{a,b,c,d} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.4. Effects of Different Single-Plant Essential Oils on VFA Concentrations in In Vitro Rumen Fermentation Broth

As shown in Table 8, compared with the control group, the addition of 6% cinnamon oil, fennel oil, thyme oil, or oregano oil, as well as 3% fennel oil, significantly reduced ($p < 0.05$) the TVFA concentration in the in vitro fermentation broth. Conversely, TVFA concentrations significantly increased ($p < 0.05$) with the addition of 3% cinnamon oil, 3% and 6% ginger oil, and 1.5% fennel oil.

Table 8. Effects of different single-plant essential oils on VFA (mmol/L) concentrations in in vitro rumen fermentation broth.

Essential Oil		Concentration %DM					SEM	*p*-Value
		CON [1]	0.75%	1.5%	3%	6%		
TVFA	Verbena	64.61	63.67	63.3	70.71	64.80	1.673	0.662
	Cinnamon	64.61 ^{ab}	58.27 ^{b}	65.75 ^{ab}	74.43 ^{a}	42.09 ^{c}	3.141	0.009
	Rosemary	64.61	63.69	64.51	67.55	62.13	0.880	0.559
	Torreya grandis	64.61	62.8	64.06	74.69	71.59	1.609	0.051
	Ginger	64.61 ^{b}	67.44 ^{b}	67.34 ^{b}	78.73 ^{a}	77.33 ^{a}	2.070	0.023
	Fennel	64.61 ^{b}	68.96 ^{ab}	74.22 ^{a}	51.15 ^{c}	37.64 ^{d}	3.847	<0.001
	Thyme	64.61 ^{ab}	66.91 ^{ab}	71.69 ^{a}	62.77 ^{b}	37.01 ^{c}	3.867	<0.001
	Oregano	64.61 ^{a}	68.65 ^{a}	44.17 ^{b}	42.38 ^{b}	37.27 ^{c}	3.436	<0.001
Acetic acid	Verbena	45.46	45.53	44.15	51.03	52.12	1.236	0.174
	Cinnamon	45.46 ^{b}	39.53 ^{b}	45.69 ^{b}	60.45 ^{a}	35.99 ^{b}	2.790	0.025
	Rosemary	45.46	43.27	45.08	53.82	47.73	1.355	0.091
	Torreya grandis	45.46	44.89	46.21	57.48	51.07	1.987	0.167
	Ginger	45.46 ^{b}	49.68 ^{b}	48.64 ^{b}	62.7 ^{a}	59.29 ^{a}	1.910	<0.001
	Fennel	45.46 ^{bc}	49.47 ^{ab}	54.42 ^{a}	45.24 ^{c}	27.79 ^{d}	2.644	<0.001
	Thyme	45.46 ^{b}	49.52 ^{a}	48.94 ^{a}	42.37 ^{b}	27.62 ^{c}	2.331	<0.001
	Oregano	45.46 ^{a}	51.33 ^{a}	36.09 ^{b}	30.04 ^{bc}	26.30 ^{c}	2.777	<0.001
Propionic acid	Verbena	13.85 ^{b}	13.76 ^{b}	14.17 ^{b}	15.19 ^{b}	23.91 ^{a}	1.037	0.001
	Cinnamon	13.85 ^{a}	14.25 ^{a}	15.04 ^{a}	14.43 ^{a}	11.67 ^{b}	0.320	0.003
	Rosemary	13.85	15.87	14.34	13.39	15.61	0.434	0.31
	Torreya grandis	13.85	13.77	13.32	15.65	14.51	0.326	0.178
	Ginger	13.85	13.66	14.62	15.73	15.67	0.378	0.262
	Fennel	13.85 ^{a}	14.55 ^{a}	14.25 ^{a}	11.53 ^{b}	8.9 ^{c}	0.628	<0.001
	Thyme	13.85 ^{a}	14.13 ^{a}	15.94 ^{a}	15.81 ^{a}	10.18 ^{b}	0.666	0.04
	Oregano	13.85 ^{a}	13.43 ^{a}	11.29 ^{b}	11.97 ^{ab}	10.78 ^{b}	0.350	<0.001

Table 8. *Cont.*

Essential Oil		Concentration %DM					SEM	*p*-Value
		CON [1]	0.75%	1.5%	3%	6%		
Butyric acid	Verbena	6.36 ^{bc}	6.37 ^{bc}	6.97 ^{b}	8.73 ^{a}	5.04 ^{c}	0.373	0.009
	Cinnamon	6.36 ^{b}	6.49 ^{b}	9.54 ^{a}	3.63 ^{c}	3.94 ^{c}	0.651	0.004
	Rosemary	6.36	6.55	7.10	7.06	8.00	0.284	0.583
	Torreya grandis	6.36	6.14	6.52	8.97	6.73	0.547	0.639
	Ginger	6.36 ^{a}	7.15 ^{a}	7.58 ^{a}	2.44 ^{b}	8.61 ^{a}	0.636	0.003
	Fennel	6.36 ^{a}	6.94 ^{a}	7.55 ^{a}	6.06 ^{a}	2.95 ^{b}	0.515	0.004
	Thyme	6.36	7.10	8.81	6.80	3.87	0.600	0.071
	Oregano	6.36 ^{b}	8.86 ^{a}	2.65 ^{c}	2.38 ^{c}	2.19 ^{c}	0.811	<0.001
A/P	Verbena	3.28	3.32	3.12	3.35	3.36	0.108	0.969
	Cinnamon	3.28 ^{b}	2.77 ^{b}	3.05 ^{b}	4.18 ^{a}	2.53 ^{b}	0.180	0.009
	Rosemary	3.28	2.75	3.14	4.02	3.13	0.166	0.13
	Torreya grandis	3.28	2.17	3.48	3.73	3.72	0.275	0.380
	Ginger	3.28 ^{b}	3.64 ^{ab}	3.35 ^{b}	4.00 ^{a}	3.97 ^{a}	0.098	0.016
	Fennel	3.28 ^{bc}	3.40 ^{bc}	3.82 ^{a}	3.57 ^{ab}	3.13 ^{c}	0.076	0.006
	Thyme	3.28	3.51	3.07	2.81	2.69	0.113	0.129
	Oregano	3.28 ^{b}	3.82 ^{a}	2.73 ^{c}	2.51 ^{c}	2.44 ^{c}	0.156	<0.001

[1] CON = control group (0% essential oils); ^{a,b,c,d} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error. A/P represents the ratio of acetic acid to propionic acid.

Compared to the control group, adding 3% cinnamon oil, 6% ginger oil, 1.5% fennel oil, and 0.75% and 1.5% thyme oil significantly increased ($p < 0.05$) acetic acid concentration. Conversely, the addition of 6% fennel oil, 6% thyme oil, and 1.5%, 3%, and 6% oregano oil significantly decreased ($p < 0.05$) acetic acid concentration.

Compared to the control group, adding 6% verbena oil significantly increased ($p > 0.05$) the propionic acid concentration in the in vitro fermentation broth. Conversely, adding 6% cinnamon oil, fennel oil, thyme oil, and oregano oil significantly decreased ($p < 0.05$) the concentration.

Compared to the control group, adding 6% verbena oil, fennel oil, cinnamon oil, or oregano oil significantly reduced ($p < 0.05$) the butyric acid concentration in the in vitro fermentation broth. Adding 3% verbena oil, 1.5% cinnamon oil, and 0.75% oregano oil significantly increased ($p < 0.05$) the butyric acid concentration in the in vitro fermentation broth.

Compared to the control group, adding 3% cinnamon oil, 3% and 6% ginger oil, 1.5% and 6% fennel oil, and 0.75% oregano oil significantly increased ($p < 0.05$) the acetate-to-propionate ratio in the in vitro fermentation broth. Conversely, the addition of 1.5%, 3%, and 6% oregano oil significantly reduced ($p < 0.05$) the acetic-acid-to-propionic-acid ratio.

3.5. Effects of Individual Essential Oils on Gas Production in In Vitro Ruminal Fermentation

As shown in Table 9, compared to the control group, the addition of 3% cinnamon oil significantly increased ($p < 0.05$) gas production. Conversely, the addition of 6% verbena oil, cinnamon oil, fennel oil, thyme oil, and oregano oil significantly reduced ($p < 0.05$) gas production.

3.6. Effects of Individual Essential Oils on In Vitro Dry Matter Degradability (IVDMD)

As shown in Table 10, compared to the control group, the addition of 0.75%, 1.5%, and 3% cinnamon oil, as well as 3% rosemary oil, significantly increased ($p < 0.05$) IVDMD. Conversely, 6% fennel oil and thyme oil significantly reduced ($p < 0.05$) IVDMD.

Table 9. Effects of individual essential oils on gas production (mL) in in vitro ruminal fermentation.

Essential Oil	Concentration %DM					SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%	6%		
Verbena	36.67 [b]	38.67 [b]	37.67 [b]	37.67 [b]	31.50 [a]	0.720	0.008
Cinnamon	36.67 [b]	37.67 [ab]	38.00 [ab]	40.67 [a]	24.67 [c]	1.000	<0.001
Rosemary	36.67	36.33	34.67	37.00	36.67	0.511	0.688
Torreya grandis	36.67	35.33	35.33	35.00	34.00	0.396	0.354
Ginger oil	36.67	34.33	36.00	38.00	37.00	0.486	0.165
Fennel oil	36.67 [ab]	37.00 [ab]	38.00 [a]	31.67 [b]	18.50 [c]	2.500	<0.001
Thyme oil	36.67 [ab]	38.33 [a]	34.33 [bc]	31.67 [c]	19.00 [d]	1.000	<0.001
Oregano oil	36.67 [a]	38.50 [a]	25.00 [b]	19.00 [c]	18.00 [c]	2.461	<0.001

[1] CON = control group (0% essential oils); [a,b,c,d] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

Table 10. Effects of individual essential oils on IVDMD (%).

Essential Oil	Concentration %DM					SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%	6%		
Verbena	65.68	71.48	70.79	67.71	63.02	1.195	0.090
Cinnamon	65.68 [b]	71.70 [a]	71.80 [a]	74.55 [a]	63.42 [b]	0.980	<0.001
Rosemary	65.68 [c]	70.19 [b]	69.82 [b]	75.00 [a]	70.93 [b]	0.857	0.002
Torreya grandis	65.68	67.60	65.77	71.79	68.32	0.836	0.062
Ginger	65.68	66.41	68.52	67.87	67.10	0.546	0.636
Fennel	65.68 [a]	69.36 [a]	66.53 [a]	60.39 [b]	56.51 [b]	1.523	<0.001
Thyme	65.68 [ab]	67.42 [a]	64.57 [ab]	61.93 [bc]	56.37 [c]	1.338	0.007
Oregano	65.68 [a]	65.65 [a]	62.06 [a]	49.64 [b]	43.89 [b]	3.005	0.003

[1] CON = control group (0% essential oils); [a,b,c] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.7. Effects of Essential Oil Formulations on In Vitro Ruminal Fermentation

As shown in Table 11, the addition of different combinations of plant essential oils had no significant effect on the pH of the rumen fermentation broth in vitro.

Table 11. Effect of essential oil blends on the pH of in vitro ruminal fermentation.

Item	Concentration %DM				SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%		
T1	6.57	6.46	6.51	6.57	0.022	0.172
T2	6.57	6.54	6.55	6.59	0.014	0.649
T3	6.57	6.51	6.55	6.56	0.014	0.454
T4	6.57	6.54	6.54	6.56	0.012	0.787
T5	6.57	6.53	6.54	6.60	0.015	0.358

[1] CON = control group (0% essential oils).

3.8. Effects of Essential Oil Blends on VFA Profiles in In Vitro Ruminal Fermentation

As shown in Table 12, compared with the control group, the addition of 0.75% T1 composite plant essential oil significantly increased ($p < 0.05$) the TVFA concentration, whereas the addition of 3% T2, T3, T4, and T5 composite plant essential oils significantly decreased ($p < 0.05$) the TVFA concentration.

Table 12. Effects of essential oil blends on VFA (mmol/L) profiles in in vitro ruminal fermentation.

Item		Concentration %DM				SEM	*p*-Value
		CON [1]	0.75%	1.5%	3%		
TVFA	T1	62.21 ^{b}	73.7 ^{a}	68.06 ^{ab}	64.27 ^{b}	1.617	0.027
	T2	62.21 ^{a}	67.68 ^{a}	68.16 ^{a}	49.27 ^{b}	2.804	0.007
	T3	62.21 ^{a}	69.64 ^{a}	61.14 ^{a}	49.14 ^{b}	2.837	0.022
	T4	62.21 ^{a}	62.60 ^{a}	62.42 ^{a}	40.64 ^{b}	3.384	<0.001
	T5	62.21 ^{a}	64.31 ^{a}	61.73 ^{a}	43.34 ^{b}	2.858	<0.001
Acetic acid	T1	44.62 ^{b}	54.10 ^{a}	48.89 ^{ab}	44.76 ^{b}	1.376	0.013
	T2	44.62 ^{a}	48.94 ^{a}	48.66 ^{a}	35.67 ^{b}	1.881	0.010
	T3	44.62 ^{b}	50.73 ^{a}	45.90 ^{b}	35.11 ^{c}	1.787	<0.001
	T4	44.62 ^{a}	43.63 ^{a}	43.51 ^{a}	26.48 ^{b}	2.522	<0.001
	T5	44.62 ^{a}	44.30 ^{a}	43.35 ^{a}	32.24 ^{b}	1.595	<0.001
Propionic acid	T1	14.13	14.54	15.09	15.02	0.229	0.526
	T2	14.13 ^{a}	14.93 ^{a}	14.39 ^{a}	10.31 ^{b}	0.644	0.002
	T3	14.13 ^{a}	14.74 ^{a}	14.79 ^{a}	10.58 ^{b}	0.648	0.002
	T4	14.13 ^{a}	15.14 ^{a}	14.48 ^{a}	10.68 ^{b}	0.672	<0.001
	T5	14.13 ^{a}	16.00 ^{a}	14.01 ^{a}	8.79 ^{b}	0.927	<0.001
Butyric acid	T1	3.55 ^{b}	5.06 ^{a}	4.08 ^{ab}	4.49 ^{ab}	0.211	0.042
	T2	3.55 ^{b}	3.81 ^{b}	3.29 ^{b}	5.11 ^{a}	0.231	0.001
	T3	3.55	4.17	5.39	3.44	0.326	0.109
	T4	3.55	1.88	4.44	3.47	0.509	0.394
	T5	3.55 ^{ab}	4.01 ^{a}	4.36 ^{a}	2.30 ^{b}	0.297	0.041
A/P	T1	3.16 ^{b}	3.72 ^{a}	3.24 ^{b}	2.99 ^{b}	0.101	0.010
	T2	3.16	3.28	3.38	3.47	0.069	0.522
	T3	3.16	3.44	3.12	3.33	0.065	0.280
	T4	3.16	2.89	3	2.5	0.101	0.058
	T5	3.16 ^{b}	2.78 ^{b}	3.09 ^{b}	3.73 ^{a}	0.133	0.024

[1] CON = control group (0% essential oils); ^{a,b,c} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error. A/P represents the ratio of acetic acid to propionic acid.

Compared with the control group, the addition of 0.75% T1, T3, and T4 composite plant essential oils significantly increased ($p < 0.05$) the acetic acid concentration. Conversely, the addition of 3% T2, T3, T4, and T5 composite plant essential oils significantly decreased ($p < 0.05$) the acetic acid concentration.

Compared to the control group, adding 3% T2, T3, T4, and T5 compound plant essential oils significantly reduced ($p < 0.05$) propionic acid concentration.

Compared to the control group, adding 0.75% T1 and 1.5% T2 significantly increased ($p < 0.05$) butyric acid concentration. Conversely, supplementing with higher concentrations of the T2 and T5 mixtures significantly decreased ($p < 0.05$) butyric acid concentration.

Compared with the control, supplementation with 0.75% T1 and 3% T5 significantly increased ($p < 0.05$) the A/P ratio.

3.9. Effects of Essential Oil Blends on NH_3-N Concentration in In Vitro Ruminal Fermentation

As shown in Table 13, compared with the control, supplementation with 3% T2, T3, and T5 blends significantly increased ($p < 0.05$) the NH_3-N concentration.

Table 13. Effects of essential oil blends on NH_3-N (mg/dL) concentration in in vitro ruminal fermentation.

Item	Concentration %DM				SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%		
T1	45.82	43.38	44.14	49.16	1.056	0.176
T2	45.82 [b]	42.18 [b]	44.97 [b]	51.64 [a]	1.405	0.036
T3	45.82 [b]	44.24 [b]	45.02 [b]	52.51 [a]	1.208	<0.001
T4	45.82	45.92	49.62	44.61	1.007	0.273
T5	45.82 [b]	45.50 [b]	47.16 [b]	53.00 [a]	1.000	<0.001

[1] CON = control group (0% essential oils); [a,b] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.10. Effects of Essential Oil Blends on MCP Concentration in In Vitro Ruminal Fermentation

As shown in Table 14, compared with the control group, the addition of 3% T2, 0.75% T4, and 3% T5 significantly decreased ($p < 0.05$) the MCP concentration. In contrast, supplementation with 1.5% T4 significantly increased ($p < 0.05$) the MCP concentration.

Table 14. Effects of essential oil blends on MCP (μg/mL) concentration in in vitro ruminal fermentation.

Item	Concentration %DM				SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%		
T1	181.48	175.74	168.1	182.41	2.351	0.061
T2	181.48 [a]	194.43 [a]	185.56 [a]	157.1 [b]	5.546	0.007
T3	181.48	174.08	189.82	173.45	2.765	0.087
T4	181.48 [b]	157.78 [c]	194.37 [a]	173.15 [b]	4.654	0.005
T5	181.48 [a]	168.83 [a]	179.63 [a]	145.74 [b]	5.048	0.001

[1] CON = control group (0% essential oils); [a,b,c] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.11. Effects of Essential Oil Blends on In Vitro Dry Matter Degradability (IVDMD)

As indicated in Table 15, compared with the control, IVDMD was significantly reduced ($p < 0.05$) by the addition of 3% T2, T3, T4, and T5 blends.

Table 15. Effects of essential oil blends on IVDMD (%).

Item	Concentration %DM				SEM	*p*-Value
	CON [1]	0.75%	1.5%	3%		
T1	67.19	66.92	66.62	64.41	0.511	0.118
T2	67.19 [a]	68.63 [a]	68.31 [a]	61.95 [b]	0.971	0.007
T3	67.19 [a]	65.19 [a]	66.34 [a]	61.60 [b]	0.784	0.006
T4	67.19 [a]	67.62 [a]	64.54 [a]	58.51 [b]	1.305	0.002
T5	67.19 [a]	66.06 [a]	67.13 [a]	59.31 [b]	1.088	<0.001

[1] CON = control group (0% essential oils); [a,b] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.12. Effects of Different Plant Essential Oil Combinations on Gas Production and CH_4 Yield in In Vitro Rumen Fermentation Broth

As shown in Table 16, compared with the control, the 3% inclusion level of the blends significantly reduced ($p < 0.05$) both gas production and CH_4 yield.

Table 16. Effects of different plant essential oil combinations on gas production (mL) and CH_4 yield (%) in in vitro rumen fermentation broth.

Item		Concentration %DM				SEM	*p*-Value
		CON [1]	0.75%	1.5%	3%		
Gas production (mL)	T1	38.00 [a]	37.50 [a]	37.00 [a]	34.67 [b]	0.496	0.006
	T2	38.00 [a]	36.67 [a]	36.00 [a]	31.00 [b]	0.900	0.005
	T3	38.00 [a]	36.50 [a]	37.00 [a]	31.33 [b]	1.003	0.006
	T4	38.00 [a]	38.50 [a]	36.33 [a]	26.00 [b]	1.686	<0.001
	T5	38.00 [a]	37.67 [a]	37.67 [a]	28.67 [b]	1.215	<0.001
CH_4 production (mL)	T1	5.07	5.01	4.66	4.32	0.123	0.083
	T2	5.07 [a]	4.61 [a]	4.53 [a]	3.77 [b]	0.186	0.008
	T3	5.07 [a]	4.67 [a]	4.73 [a]	3.78 [b]	0.168	0.010
	T4	5.07 [a]	4.97 [a]	4.84 [a]	3.09 [b]	0.314	0.003
	T5	5.07 [a]	5.08 [a]	5.13 [a]	3.39 [b]	0.247	<0.001
CH_4 content (%)	T1	13.35 [ab]	13.79 [a]	13.19 [b]	12.47 [c]	0.166	0.002
	T2	13.35 [a]	12.97 [a]	12.63 [ab]	12.18 [b]	0.176	0.029
	T3	13.35 [a]	13.47 [a]	13.15 [a]	12.06 [b]	0.192	<0.001
	T4	13.35 [a]	12.89 [a]	13.07 [a]	11.46 [b]	0.286	0.008
	T5	13.35 [a]	13.48 [a]	13.62 [a]	11.79 [b]	0.258	0.001

[1] CON = control group (0% essential oils); [a,b,c] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error.

3.13. Effects of Essential Oil Blends on Population Size (OTU) and α Diversity Analysis of the Rumen Microbiome

Based on high-throughput sequencing of the 16S rRNA gene, a total of 864,679 high-quality sequences were obtained from the 12 rumen fluid samples. Operational Taxonomic Unit (OTU) clustering analysis of the non-redundant sequences (Figure 1) showed that the CON, B75 (0.75% T2), C15 (1.5% T2), and D3 (3% T2) groups contained 2663, 2365, 2424, and 1656 OTUs, respectively. A core set of 962 OTUs was shared across all four treatment groups. Alpha diversity indices are summarized in Table 17. Compared with the control (CON) group, supplementation with 3% of the T2 blended essential oil (D3 group) significantly decreased ($p < 0.05$) the ACE, Chao1, Shannon, and Sobs indices. Conversely, it resulted in a significant increase ($p < 0.05$) in the Simpson index.

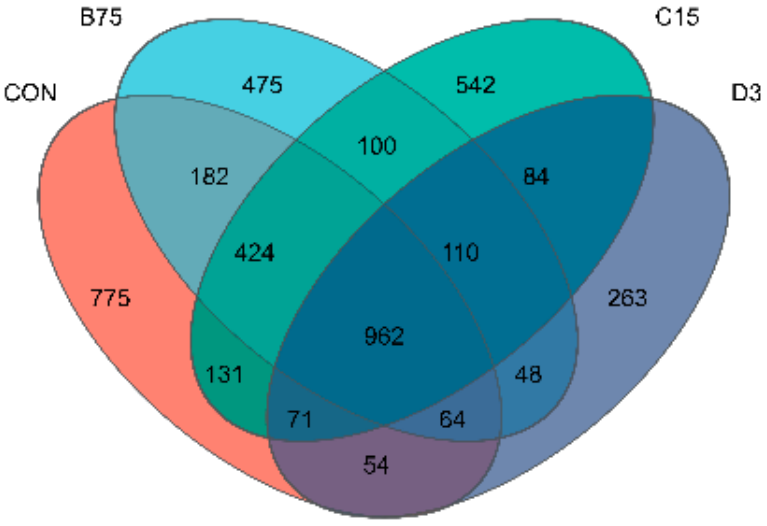


Figure 1. Shared OTUs across different groups.

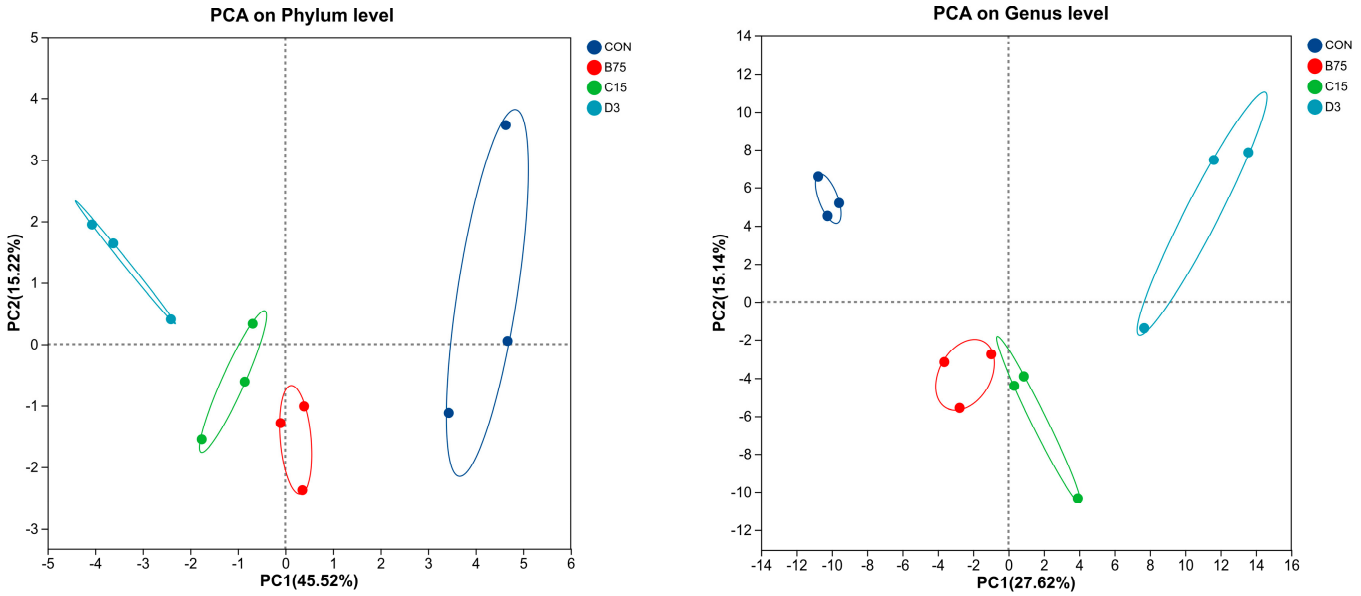
Table 17. Effect of different concentrations of essential oils of T2 complex plants on the alpha diversity index of rumen microbial communities.

Item	Treatment				SEM	*p*-Value
	CON [1]	B75	C15	D3		
Community richness						
Ace	1908.96 [a]	1799.33 [a]	1890.25 [a]	1264.88 [b]	86.157	0.001
Chao1	1837.28 [a]	1739.49 [a]	1802.81 [a]	1227.72 [b]	80.929	0.001
Microbial diversity						
Shannon	5.01 [a]	4.66 [a]	4.71 [a]	3.53 [b]	0.18	<0.001
Simpson	0.03 [b]	0.04 [b]	0.04 [b]	0.12 [a]	0.013	0.004
Sobs	1665.33 [a]	1493.33 [a]	1499 [a]	1024 [b]	76.841	<0.001
Coverage	0.991 [b]	0.990 [bc]	0.989 [c]	0.993 [a]	0	0.006

[1] CON = control group (0% essential oils); [a,b,c] denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error. CON, B75, C15, and D3 in the treatment groups denote additive concentrations of 0%, 0.75%, 1.5%, and 3%, respectively.

3.14. Comparison of Beta Diversity in Rumen Bacteria

As shown in Figure 2, principal component analysis (PCA) performed at both the phylum and genus levels revealed clear separation among the four treatment groups within the coordinate space. These results indicate that, compared to the control group, the addition of T2 at different concentrations has a certain effect on the abundance and diversity of rumen microorganisms.



(**a**) (**b**)

Figure 2. (**a**) Principal component analysis at the phylum level; (**b**) Principal component analysis at the genus level.

3.15. Effects of Essential Oil Blends on Species Abundance Analysis at the Phylum and Genus Levels of Rumen Bacteria

As shown in Figure 3 and Table 18, at the phylum level, the rumen bacterial communities in the CON, B75, C15, and D3 groups were consistently dominated by six major phyla: *Bacteroidota*, *Firmicutes*, *Proteobacteria*, *Patescibacteria*, *Verrucomicrobiota*, and *Actinobacteriota*. Compared with the control group, supplementation with different concentrations of the T2

blend significantly decreased ($p < 0.05$) the relative abundance of *Bacteroidota* and increased ($p < 0.05$) that of *Firmicutes*. Furthermore, while the addition of 0.75% T2 significantly increased ($p < 0.05$) the abundance of *Proteobacteria*, the 3% T2 treatment resulted in a significant reduction ($p < 0.05$) in this phylum.

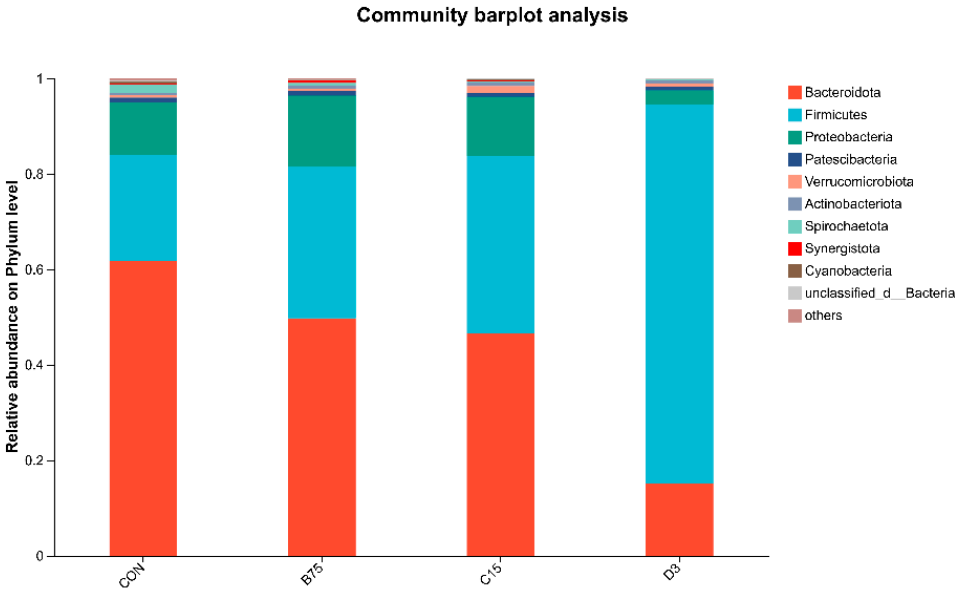


Figure 3. Histogram of rumen microbial community structure at the phylum level under different T2 blend concentrations (top 6 taxa).

Table 18. Effects of different concentrations of the T2 essential oil blend on the rumen microbial community at the phylum level (top 6 taxa).

Item	Treatment				SEM	*p*-Value
	CON [1]	B75	C15	D3		
Bacteroidota	61.72 ^{a}	49.74 ^{b}	46.53 ^{b}	15.16 ^{c}	0.053	<0.001
Firmicutes	22.25 ^{c}	31.70 ^{b}	37.17 ^{b}	79.39 ^{a}	0.068	<0.001
Proteobacteria	10.99 ^{b}	14.92 ^{a}	12.43 ^{ab}	2.92 ^{c}	0.014	<0.001
Patescibacteria	0.97	0.93	0.79	0.77	0.000	0.227
Verrucomicrobiota	0.53 ^{b}	0.50 ^{b}	1.44 ^{a}	0.58 ^{b}	0.001	0.017
Actinobacteriota	0.50	0.71	0.71	0.74	0.001	0.353

[1] CON = control group (0% essential oils); ^{a,b,c} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error. CON, B75, C15, and D3 in the treatment groups denote additive concentrations of 0%, 0.75%, 1.5%, and 3%, respectively.

As shown in Figure 4 and Table 19, analysis at the genus level identified several dominant taxa, including *Prevotella*, *Rikenellaceae*, *Pseudobutyrivibrio*, *Streptococcus*, *Succinivibrio*, *Succiniclasticum*, and *Selenomonas*, across all treatment groups (CON, B75, C15, D3). Compared to the control, supplementation with T2 at any concentration significantly reduced ($p < 0.05$) the abundance of *Prevotella*. The 3% T2 treatment significantly increased ($p < 0.05$) the abundances of *Pseudobutyrivibrio* and *Streptococcus*. Furthermore, while 1.5% T2 significantly increased ($p < 0.05$) *Succinivibrio*, the 3% inclusion significantly decreased ($p < 0.05$) it. The abundance of *Selenomonas* was also significantly elevated ($p < 0.05$) by 3% T2. In contrast, the abundances of *Rikenellaceae* and *Succiniclasticum* were not significantly affected ($p > 0.05$) by T2 supplementation at any concentration.

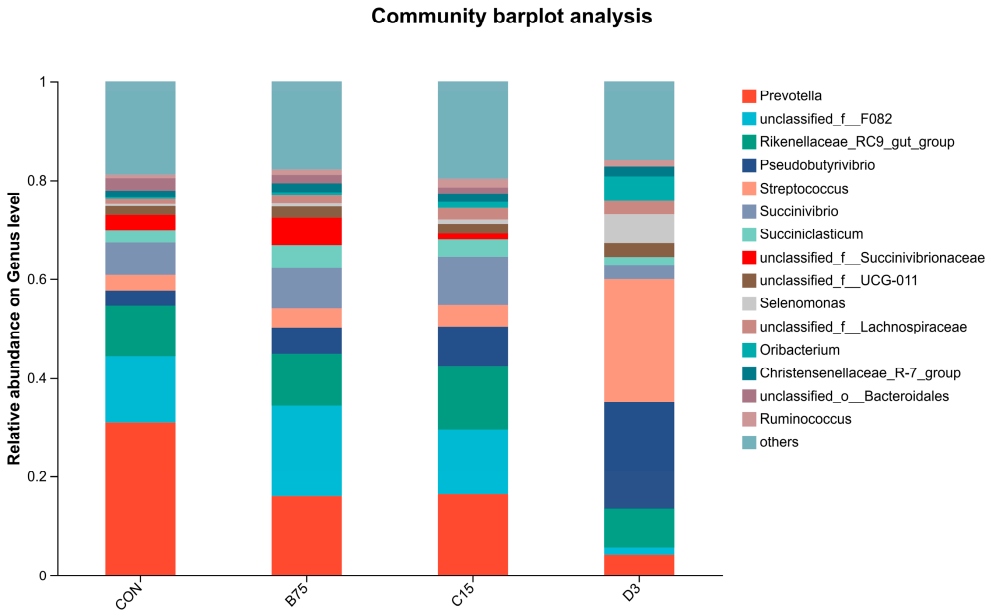


Figure 4. Histogram of genus-level rumen microbial composition under different T2 blend concentrations (top 10 genera).

Table 19. Effects of different concentrations of the T2 essential oil blend on the rumen microbial community at the genus level.

Item	Treatment				SEM	*p*-Value
	CON [1]	B75	C15	D3		
Prevotella	30.99 ^{a}	16.09 ^{b}	16.50 ^{b}	4.22 ^{c}	0.030	<0.001
unclassified	13.44 ^{b}	18.16 ^{a}	13.08 ^{b}	1.45 ^{c}	0.020	<0.001
Rikenellaceae	10.07	10.76	12.91	7.78	0.008	0.099
Pseudobutyrivibrio	3.10 ^{b}	5.24 ^{b}	7.86 ^{b}	21.55 ^{a}	0.023	<0.001
Streptococcus	3.19 ^{b}	3.79 ^{b}	4.27 ^{b}	24.93 ^{a}	0.029	<0.001
Succinivibrio	6.52 ^{b}	8.23 ^{ab}	9.91 ^{a}	2.79 ^{c}	0.009	0.003
Succiniclasticum	2.37	4.61	3.44	1.73	0.004	0.057
unclassified	1.76	2.27	2.02	2.77	0.002	0.228
Selenomonas	0.44 ^{b}	0.61 ^{b}	0.90 ^{b}	5.90 ^{a}	0.007	<0.001
others	0.25	0.25	0.28	0.27	0.006	0.164

[1] CON = control group (0% essential oils); ^{a,b,c} denote superscript letters. Within the same row, means labeled with the same superscript letter or no letter show no significant difference ($p > 0.05$). Means labeled with different superscript letters show significant differences ($p < 0.05$). The SEM value represents the standard error. CON, B75, C15, and D3 in the treatment groups denote additive concentrations of 0%, 0.75%, 1.5%, and 3%, respectively.

4. Discussion

Rumen fermentation is the anaerobic microbial degradation of ingested feed into energy and other metabolites [39]. In this process, bacteria, protozoa, and fungi collectively hydrolyze complex carbohydrates, yielding organic acids and gases as major metabolic end-products [40]. Although relatively stable, rumen fermentation can be influenced by dietary and environmental factors, which may shift the microbial community structure and ultimately affect fermentation efficiency and host health [41]. Therefore, maintaining microbial homeostasis in the rumen is essential for ruminant health and productivity [42]. Essential oils, as plant secondary metabolites, are considered promising natural alternatives to antibiotics in ruminant nutrition [43]. They exhibit the potential to modulate rumen fermentation [44] through antimicrobial properties [45], mitigate CH_4 emissions, and enhance propionate production [46].

In this study, supplementation with 3% cinnamon essential oil significantly increased the in vitro dry matter degradation rate, markedly elevated the acetate proportion along with the acetate-to-propionate (A/P) ratio, and substantially reduced the butyrate proportion. These results suggest that cinnamon essential oil may suppress protozoal activity [47], thereby indirectly promoting fibrolytic bacterial activity while inhibiting butyrate-producing bacteria. Consequently, rumen fermentation shifted toward a fiber-fermentative pattern, and the increased acetate proportion could benefit energy supply and rumen health [48]. In line with this, Castillejos et al. [19] reported in an in vitro study that 500 mg/L eugenol reduced the propionate proportion without affecting total VFA levels. Moreover, a 3% concentration of cinnamon oil significantly increased microbial protein (MCP) concentration without affecting ammonia nitrogen (NH_3-N), suggesting improved nitrogen utilization may occur by suppressing the activity of bacteria incapable of producing MCP. This implies that cinnamon essential oil may promote synchronized nitrogen uptake in the rumen [49], potentially reducing the need for exogenous nitrogen supplementation and lowering nitrogen emissions into the environment.

Compared to the control group, the addition of 1.5% fennel essential oil significantly increased MCP concentration without affecting NH_3-N, suggesting it may inhibit bacteria that do not synthesize MCP; this phenomenon is consistent with the addition of 3% cinnamon essential oil. Concurrently, it significantly elevated total volatile fatty acid concentration, acetate proportion, and A/P ratio. This implies that fennel oil may more effectively suppress microorganisms involved in rapid starch fermentation, such as Prevotella species with strong deamination activity [50], while simultaneously protecting or stimulating fibrolytic bacteria.

The addition of 0.75% thyme essential oil increased nitrogen utilization efficiency by enhancing MCP without altering primary fermentation pathways (stable VFA composition, gas production, and degradation rate) or nitrogen metabolism fundamentals (unchanged NH_3-N). Benchaar et al.'s in vivo trial [51] demonstrated that adding 50 mg/kg DM of thyme oil or thymol to dairy cow diets did not affect rumen fermentation, nutrient utilization, or milk production. This suggests thyme may rebalance rumen microbial communities by reducing bacteria and protozoa that waste nitrogen [52], thereby steering fermentation toward more efficient and healthier patterns. However, in vitro experiments by Onel et al. [53] found that 60 mg/L thymol significantly reduced ammonia nitrogen, potentially inhibiting ammonia-producing bacteria growth.

In experiments evaluating oregano essential oil in vivo and in vitro, Fangyu Zhang [54] found no significant effects on TVFA, acetate, or propionate, consistent with the findings of this study. In this experiment, the addition of 0.75% oregano essential oil altered the overall fermentation intensity (degradation rate, gas production, and total VFA remained unchanged) and nitrogen metabolism basis (NH_3-N and MCP remained unchanged), but specifically modified the terminal metabolic pathways of volatile fatty acids, significantly promoting butyrate production.

In the T1–T5 combined essential oil mixture, the addition gradients of each group had no significant effect on pH, which was consistent with the results of Khateri et al. [55], who added a 1.6 mL (50–100% thyme, 30% cinnamon, 20% cloves) combined plant essential oil to the lamb feed. Adding 3% of T2, T3, T4, and T5 combined plant essential oils all significantly reduced the dry matter digestibility of the in vitro fermented substrate. This finding was consistent with that of Tager and Krause [56], indicating that high concentrations of plant essential oils directly affect the degradation of the substrate by rumen microorganisms and the production of VFA in rumen fluid, which is not conducive to improving rumen fermentation. Some combined plant essential oils had no effect or increased the NH_3-N content in the rumen, which might be because the rumen microorganisms adapted to

the combined plant essential oils, and the normal physiological metabolism of the rumen microorganisms was no longer affected [57]. The addition of each group of 3% combined plant essential oils significantly reduced the CH_4 content. It is speculated that the essential oils would interfere with specific enzymes or metabolic pathways for methane production, thereby reducing the efficiency of methane generation [58].

At the phylum level, we observed significant alterations in the *Proteobacteria* phylum across all addition gradients in group T2. As *Proteobacteria* serves as a key indicator of microbial community shifts [59], the varying essential oil concentrations applied at each T2 gradient induced pronounced changes in microbial community composition and relative abundance. This may stem from the diverse bioactive compounds within essential oils and the differential sensitivity of various microorganisms to EO concentrations, thereby driving shifts in rumen microbial populations [60]. Furthermore, *Bacteroidota*, *Firmicutes*, and *Proteobacteria* were the dominant phyla. The relative abundances of *Bacteroidota* and *Firmicutes* accounted for over 80% of the total abundance across all T2 groups. *Firmicutes* exhibited the lowest abundance in the control group and the highest in the highest-dose group, while *Bacteroidota* showed the opposite trend. This aligns with the findings of Zhengwen Wang [10]. Thus, it is speculated that plant essential oils in the T2 group may regulate carbohydrate metabolism by influencing the *Firmicutes* abundance [61].

Supplementation with 1.5% T2 suppressed microorganisms associated with inefficient nitrogen metabolism (e.g., *Bacteroidetes* and *Prevotella*), while promoting the abundance of taxa linked to efficient metabolic pathways (e.g., *Firmicutes* and *Succinivibrio*). The significant increase in the butyrate proportion was likely attributable to enhanced activity of fibrolytic bacteria within the *Firmicutes* phylum. Shifts in TVFA, acetate, and propionate levels indicated a shift toward more efficient energy metabolism, and the elevated acetate-to-propionate ratio suggested a healthier fermentation pattern. The marked reduction in CH_4 production may be explained by a redirected hydrogen flow: *Succinivibrio* species utilize hydrogen for propionate synthesis, thereby competing with methanogens for available hydrogen and reducing methanogenesis. These findings align with those of Peng et al. [62], who reported that a high-dose essential oil blend lowered CH_4 production and modulated rumen fermentation parameters both in vivo and in vitro in goats.

At the genus level, *Prevotella* exhibited the highest abundance at 0% addition and the lowest abundance at 3% addition. *Prevotella* showed a positive correlation with both propionic acid production and gas yield. Therefore, we speculate that high concentrations of T2 essential oil inhibited *Prevotella* growth, leading to a significant decrease in propionic acid concentration and gas yield. This finding is consistent with the results of Idowu et al. [63] on rapeseed essential oil in in vitro fermentation. *Pseudobutyrivibrio* exhibits a positive correlation with butyrate synthesis [64]. A 3% T2 essential oil concentration significantly increased *Pseudobutyrivibrio* abundance, resulting in markedly elevated butyrate levels at this addition rate. *Streptococcus* produces lactic acid [65] rather than VFA, altering rumen fermentation pathways. This may inhibit methanogens, thereby reducing methane content [66]. *Selenomonas* exhibits positive correlation with propionic acid production [67] and negative correlation with methane production [68]. Thus, a 3% T2 essential oil blend significantly increased the *Selenomonas* abundance, reducing methane content. However, compared to the control group, 3% T2 significantly suppressed MCP concentration, TVFA concentration, acetic acid, and propionic acid concentration. Considering economic costs, the 3% T2 addition doubles the dosage compared to 1.5%. Therefore, considering all indicators, we select the 1.5% T2 addition gradient as the optimal composite essential oil formulation.

5. Conclusions

(1) Regarding these eight single-plant essential oils, adding 3% CE optimizes rumen microbial fermentation. It enhances the dry matter degradation rate, gas production, and MCP synthesis efficiency, while shifting fermentation toward an acetate-dominant pattern to improve nutrient utilization. Adding 1.5% FE promotes acetate production without affecting ammonia nitrogen content, enabling more efficient conversion of fermentation energy into MCP and total volatile fatty acids; 0.75% TE enhances nitrogen utilization efficiency without altering primary fermentation pathways or ammonia nitrogen levels; and adding 0.75% OE did not alter overall fermentation intensity or nitrogen metabolism, but specifically modified the volatile fatty acid metabolic pathway, significantly promoting butyrate production.

(2) Regarding these five composite plant essential oil blends, Blend T2 demonstrated superior performance compared to the other four blends in nitrogen metabolism and the in vitro dry matter degradation rate. Furthermore, it significantly reduced CH_4 content. Considering all indicators alongside economic costs, the 1.5% T2 blend emerged as the optimal formulation.

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