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The Dose-Dependent Role of Sage, Clove, and Pine Essential Oils in Modulating Ruminal Fermentation and Biohydrogenation of Polyunsaturated Fatty Acids: A Promising Strategy to Reduce Methane Emissions and Enhance the Nutritional Profile of Ruminant Products

Mostafa Bokharaeian [1], Taghi Ghoorchi [1], Abdolhakim Toghdory [1] and Iman Janghorban Esfahani [2,*]

[1] Department of Animal and Poultry Nutrition, Animal Science Faculty, Gorgan University of Agricultural Science and Natural Resources, Gorgan 49189-43464, Iran; mostafa.bokharaeian_s99@gau.ac.ir (M.B.); toghdory@gau.ac.ir (A.T.)
[2] Glopex Co., Ltd., R&D Center, GeumGang Penterium IX Tower A2801, Dongtancheomdansaneop 1-ro 27, Hwaseong-si 18469, Gyeonggi-do, Republic of Korea
* Correspondence: john@glopex.kr; Tel.: +82-10-3437-2992

Featured Application: Livestock significantly contribute to greenhouse gas emissions, with methane production from animals like cows, sheep, and goats being a major concern. Reducing this methane output is crucial for environmental sustainability. There is also a growing interest in enhancing the nutritional quality of meat and milk from these animals. This study investigated the potential of natural additives—sage (SAG), pine (PIN), and clove (CLO) essential oils—to manipulate ruminal fermentation as well as biohydrogenation of polyunsaturated fatty acids (PUFA). The research focused on ruminal gas production, methane emissions, and fatty acid changes using in vitro methods. The essential oils led to lower gas and methane production. Additionally, they affected rumen pH, ammonia levels, and fatty acid profiles. These findings suggest that SAG, PIN, and CLO might reduce methane emissions and improve product quality in livestock. More research is needed to apply these findings practically.

Abstract: The livestock industry significantly contributes to greenhouse gas emissions, with ruminant animals, including cows, sheep, and goats, being responsible for a substantial share of these emissions due to methane production. Reducing methane emissions from ruminants is crucial for mitigating the environmental impact of livestock production. Additionally, there has been a growing interest in improving the nutritional quality of ruminant products through modifying their profile of fatty acids. The current study aimed to investigate the potential of sage (SAG), pine (PIN), and clove (CLO) essential oils as natural additives for modulating in vitro ruminal fermentation characteristics and biohydrogenation of polyunsaturated fatty acids (PUFA). Within the current experiment, three dose levels (300, 600, and 900 mg/L) of essential oils were evaluated using rumen inoculum from three mature Dalagh ewes (58 ± 2.84 kg body weight). The results revealed that the essential oils had a significant impact on gas production, methane and carbon dioxide production, ruminal fermentation parameters, and ruminal biohydrogenation of dietary PUFAs. The essential oil treatments resulted in reduced gas production compared with the control group. Methane production was significantly reduced by all doses of the essential oils, with the highest dose of CLO resulting in the lowest methane production. In addition, the essential oils affected ruminal fermentation parameters, including pH, ammonia concentration, and production of total volatile fatty acids. Promising modifications in ruminal biohydrogenation of PUFAs and the profile of fatty acids were also observed in the current study. These findings suggest that SAG, Pin, and CLO hold promise in mitigating methane emissions and improve the nutritional value of ruminant products. Further investigation is required to evaluate their effectiveness in practical feeding strategies for livestock.

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1. Introduction

The livestock industry significantly contributes to greenhouse gas emissions, comprising approximately 14.5% of total global emissions [1]. Ruminants, including cows, sheep, and goats, contribute substantially to these emissions, mainly due to the production of methane (CH_4) during enteric fermentation. Methane possesses a substantial greenhouse effect, with a global warming capacity 28-fold greater than the impact of carbon dioxide (CO_2) [2]. Consequently, decreasing methane emissions from ruminant animals is essential to mitigate the ecological consequences of livestock production.

In addition to the environmental concerns, there has also been increasing attention paid to improving the nutritional quality of ruminant-derived products, particularly milk and meat [3–6]. Modifying the biohydrogenation of polyunsaturated fatty acids (PUFA) within the rumen can lead to changes in the fatty acid composition of ruminant milk and meat. PUFA are essential fatty acids requiring dietary intake, as ruminants lack the capacity to synthesize them endogenously [7]. However, ruminal biohydrogenation converts these beneficial fatty acids into less desirable forms, such as saturated fatty acids (SFA).

Dietary essential oils have been put forward as a potential solution to decrease methane emissions and modify the composition of fatty acids in ruminant-derived products, such as milk and meat [8,9]. Essential oils are volatile compounds obtained from plants that possess various biological properties, such as antimicrobial [10], anti-inflammatory [11], immunostimulant [12], and antioxidant effects [13]. Some essential oils have also demonstrated the capacity to decrease methane emissions from ruminant animals by inhibiting the growth and activity of methanogenic archaea within the rumen [14]. Moreover, certain essential oils can modify the ruminal biohydrogenation of PUFA by inhibiting the activity of certain microbial populations and enzymes responsible for this process [15].

Several studies have investigated the potential of dietary essential oils to reduce methane emissions and modify the profile of fatty acids in ruminant-derived products [16–19]. For example, in an experiment involving dairy cattle, the inclusion of a blend of essential oils into the diet led to a notable decrease in methane emissions without affecting milk yield or composition [20]. In another experiment, when dairy cattle were supplemented with dietary essential oils, the composition of fatty acids in the milk was modified, characterized by an elevation in the levels of beneficial fatty acids, particularly conjugated linoleic acid (CLA), and a decrease in the level of less desirable fatty acids such as SFAs [21]. Similarly, the inclusion of essential oils into the diet of goats led to a decrease in methane emissions and an improvement in the composition of fatty acids in the meat [22,23]. Specifically, the levels of CLA and other beneficial fatty acids increased, whereas the levels of SFAs decreased [23].

Overall, incorporating essential oils into the diets of ruminant animals holds great potential for reducing greenhouse gas emissions and enhancing the nutritional value of ruminant products. However, further investigation is required to optimize the inclusion rate and type of essential oils utilized in ruminant diets and to achieve a deeper understanding of the underlying mechanisms responsible for their effects on methane emissions and ruminal biohydrogenation.

The objective of the current study was to investigate the potential of three essential oils, namely sage (SAG), pine (PIN), and clove (CLO), as natural additives for modulating in vitro ruminal fermentation and the biohydrogenation of PUFAs, in pursuit of mitigating methane emissions and improving the nutritional value of ruminant products.

2. Materials and Methods

2.1. Diet, Donor Animals, and Microbial Inoculum

Three mature Dalagh ewes (58 ± 2.84 kg body weight), equipped with cannulas for sampling purposes, were utilized to collect the rumen inoculum. Ewes were selected as suitable donor animals for the current study due to their availability and also the similarities observed between sheep and cattle inoculums in in vitro studies, as already indicated by Yáñez-Ruiz et al. [24]. To stabilize and regulate their rumen microbiota, the ewes were relocated to the University Research Farm and provided with the experimental diet for a duration of 14 days. The experimental diet was a total mixed ration (TMR) formulated to meet the daily maintenance requirements of ewes, using a forage to concentrate ratio of 70:30, according to the guidelines presented by the National Research Council [25]. Table 1 presents the chemical composition and fatty acid profile of the diet used in the current study. Rumen fluids were collected 4 h after the morning meal and samples were subsequently pooled. The pooled rumen contents were then filtered through double layers of muslin cloth. The remaining residue from the muslin was then mixed with an anaerobic buffer medium solution using Theodorou's Reading Pressure Technique (RPT), as outlined in their study [26]. The volume of the buffer medium solution was equivalent to the volume of removed rumen fluid. This mixture was homogenized for approximately 20 s and then gently squeezed to retain the fibrolytic bacteria that contribute to the ruminal biohydrogenation. Subsequently, the two rumen extracts were combined and transferred into a pre-warmed flask, flushed with CO_2, and immediately transported to the laboratory. In the laboratory the flasks were kept in a water bath at 39 °C. Additionally, a representative portion of the TMR already provided to the donor animals was freeze-dried, milled through a 1 mm mash screen, and stored in airtight containers after being flushed with CO_2. This freeze-dried sample served as the basal diet for the current in vitro experiment.

Table 1. Feed ingredients and chemical composition of the experimental diet.

Ingredients	Value	Profile of Fatty Acids	Value
Barley, grain, % DM	29.0		
Alfalfa, hay, % DM	19.0	TFA (g/kg Feed DM)	41.4
Corn, grain, % DM	12.4	Individual FAs, % TFA	
Wheat straw, % DM	11.0	C14:0 (myristic acid)	3.0
Wheat bran, % DM	9.0	C16:0 (palmitic acid)	12.6
Soybean meal, % DM	7.2	C16:1 (palmitoleic acid)	2.9
Rapeseed meal, % DM	4.5	C18:0 (stearic acid)	2.7
Linseed, ground, % DM	3.0	C18:1 (*trans*-11 VA)	0.6
Fish Oil, % DM	2.0	C18:1 n-9 (oleic acid)	13.8
CaCo3, % DM	0.7	C18:2 n-6 (LA)	25.2
Min-vit premix *, % DM	0.7	C18:3 n-3 (LNA)	21.2
Bentonite, %, DM	0.5	C20:4 (Arachidonic acid)	0.3
Na-Bicarbonate, % DM	0.5	C20:5 n-3 (EPA)	6.7
Common Salt, % DM	0.5	C22:6 n-3 (DHA)	3.6
Chemical Composition		SFA	18.2
DM, % as fed	89.2	UFA	74.2
ME, Mcal/kg	2.7	MUFA	17.2
CP, % DM	14.6	PUFA	57.0
E.E., % DM	3.9	SFA:UFA	0.2
NDF, % DM	30.5	Remaining Fatty Acids	7.6
ADF, % DM	17.6		
Ca, % DM	0.8		
P, % DM	0.5		

* Each kilogram of Min-vit premix contains: 750,000 IU vitamin A, 100,000 IU vitamin D3, 5000 IU vitamin E, 155 g calcium, 30 g phosphorus, 3500 mg Mn, 4500 mg Zn, 15 mg Co, 25 mg Se, 45 mg I, 550 mg Cu, 4500 mg Fe, 1000 mg antioxidant. DM: dry matter; ME: metabolizable energy; CP: crude protein; E.E.: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; FAs: fatty acids; TFA: total fatty acids; VA: vaccenic acid; LA: linoleic acid; LNA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

2.2. Plant Material and Preparation

Sage leaves (*Salvia officinalis*), pine needles (*Pinus species*), and clove buds (*Syzygium aromaticum*) were purchased from the local market in Gorgan, Golestan, Iran. Moreover, the taxonomic classification of the genus and species was confirmed by experienced taxonomists who specialize in the herbarium of the Department of Plant Production at Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. The plant material was thoroughly cleaned, removing any dirt, debris, or damaged parts. Following the cleaning process, the plant material was freeze-dried for a duration of 72 h, ensuring the complete elimination of moisture.

2.3. Extraction of Essential Oils

The dried plant materials were finely ground and then subjected to hydro-distillation using a Clevenger-type apparatus. The plant materials were then transferred into a round-bottom flask, and distilled water was added to cover the plant materials. The flask was fitted with a condenser, and the hydro-distillation process was conducted for 240 min. The extracted oils were dehydrated using anhydrous sodium sulphate, flushed with N_2, and kept at 4 °C in sealed dark vials until they were analyzed.

2.4. Essential Oil Analysis

Gas chromatography was used to analyze the SAG, PIN, and CLO compounds. A 1 µL aliquot of each essential oil was diluted with n-hexane in a 1:20 (*v:v*) ratio and subsequently introduced into an Agilent HP-6890 gas chromatograph (GC; Agilent Technologies, Palo Alto, CA, USA) coupled with an Agilent HP-5973 mass selective detector equipped with an HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Restek, Bellefonte, PA, USA). The elution gradient commenced with initial isothermal mode at 60 °C for 1 min. The temperature was then increased at a rate of 5 °C per minute, reaching 180 °C, and then maintained in isothermal mode for 1 min. Subsequently, the temperature was increased to 280 °C at a rate of 40 °C per minute and kept at this temperature for 1.5 min in isothermal mode, resulting in a total run time of 30 min. The injection temperature was set at 200 °C, and the flow rate was consistently maintained at 1 mL per minute using helium gas in split mode, with a ratio of 50:1. The sweep range extended from 40 to 450 m/z, and the solvent cut was performed within 3 min. The temperature at the interphase was maintained at 270 °C, while the ion source temperature was set to 260 °C. The mass spectrometer was operated using 70 eV electron impact ionization. Reference standards, including 1,8-cineole, α-Pinene, and Eugenol (Merck, Darmstadt, Germany), were utilized for precise compound identification and quantification in the analysis of SAG, PIN, and CLO essential oils, respectively. For consistency, each analysis was conducted in triplicate. The chemical composition of the experimental essential oils is provided in Table 2.

Table 2. Chemical composition of SAG, PIN, and CLO.

Compounds	% of SAG	Compounds	% of PIN	Compounds	% of CLO
α-Thujone	43.68	α-Pinene	26.32	Eugenol	69.53
Borneol	9.04	Limonene	18.45	Eugenyl acetate	12.88
1,8-Cineole	8.21	Bornyl Acetate	16.76	β-Caryophyllene	12.67
β-Thujone	7.13	β-Pinene	15.90	α-Humulene	1.36
Viridiflorol	5.32	Camphene	6.40	α-Cubebene	0.48
Camphene	3.11	β-Myrcene	3.50	Caryophyllene oxide	0.32
α-Pinene	3.06	α-Terpinyl acetate	2.79	α-Copaene	0.33
α-Humulene	2.89	α-Terpinene	2.46	α-Farnesene	0.32
Humullene epoxide II	2.83	1,8-Cineole	2.23	Cahvicol	0.29
β-Pinene	2.54	O-Cymene	1.03	Chavicol	0.28

Table 2. *Cont.*

Compounds	% of SAG	Compounds	% of PIN	Compounds	% of CLO
Manool	2.02	δ-3-Carene	0.87	D-Germacrene	0.14
B-Caryophyllene	1.79	Camphore	0.61	γ-Muurolene	0.11
Limonene	1.47	B-Caryophyllene	0.53	Methyl salicylate	0.09
O-Cymene	1.54	p-Anis Aldehyde	0.42	β-Cadinene	0.09
B-Selinene	1.01	α-Terpinolene	0.41	Epicubenol	0.08
Trans-Sabinene acetate	0.88	1-Octan-3-ol	0.24	β-Copaene	0.07
Carvacrol	0.73	Tricyclene	0.15	Humuladienone	0.05
Caryophyllene oxide	0.69	α-Pinene oxide	0.13	2-Heptyl acetate	0.03
β-Myrcene	0.61	α-Terpineol	0.12	Cubenol	0.03
4-Terpineol	0.52	γ-Terpinene	0.11	Isocaryophyllene	0.02
Others	0.93	Others	0.57	Others	0.83

SAG: sage essential oil; PIN: pine essential oil; CLO: clove essential oil.

2.5. Experimental Design

The experiment was conducted in two simultaneous runs, with a total of ten treatments, each replicated eight times, resulting in a total sample size of 160 (n = 160). Moreover, an additional set of eight bottles per treatment (n = 80) was also employed, which were stopped at the onset of incubation (time 0 h), for subsequent analyses. Additionally, a set of four bottles (n = 4) was also incubated as blank bottles, devoid of added feed and essential oils. The basal diet was accurately weighed (1.000 g) into 125 mL serum bottles (Wheaton Scientific, Millville, NJ, USA) followed by the addition of 80 mL of RPT buffer medium, along with 20 mL of strained rumen fluid. The treatments were control (CON), which had no added EOC; *Salvia officinalis* (Sage oil; SAG); *Pinus sylvestris* (Pine oil; PIN); and *Syzygium aromaticum* (Clove oil; CLO) essential oils. Individual essential oils were introduced into the serum bottles to achieve the final concentrations of 300, 600, and 900 mg/L, resulting in a total of ten treatments. The bottles were placed in an incubator set at 39 °C, and the microbial fermentation of the basal diet was screened via measuring the gas pressure generated in the headspace. The measurements were taken at intervals of 3, 6, 12, 18, 24, 36, and 48 h of incubation, following the technique suggested by Theodorou et al. [26] and the adaptation described by Mauricio et al. [27].

2.6. Gas Production and Rumen Fermentation Kinetics

The measured gas production data were processed to fit with the model proposed by France et al. [28] as follows:

$$GP = B \times \left[1 - e^{-C(T-L)}\right]$$

where GP is the gas production, B is the ideal maximum gas production, C is the gas production rate, T is time of incubation, and L is lag time prior to gas production commencing. The parameters for the kinetics of rumen fermentation (B, C, and L) were estimated using the nonlinear regression procedure of Statistical Analysis Software version 9.4 (SAS 9.4; SAS Institute, Inc., Cary, NC, USA). Subsequently, the time taken to reach half of the ideal maximum GP (HT) and the average GP rate (AGPR) at HT was calculated using the following equations according to Kong et al. [29]:

$$HT = \log\left(\frac{2}{C}\right) + L$$

$$AGPR = \frac{A \times C}{2 \times (\log(2) + C \times L)}$$

2.7. Determination of Methane and Carbon Dioxide

Headspace gas (6 mL) was collected from each bottle using a 10 mL gas-tight syringe and was subsequently stored in a vacuum test tube (Vacutainer, Becton Dickinson, Franklin Laker, NJ, USA). The concentrations of CH_4 and CO_2 in the gas samples (24 h) were directly determined using a GC (GOW-MAC, G-M816, Bethlehem, PA, USA) equipped with an HP-PLOT Q capillary column (30 m × 0.53 mm × 40 µm) and a flame ionization detector (FID). The temperature of the column, injector, and detector were set at 50, 150, and 200 °C, respectively. Helium and hydrogen (H_2) gases were employed as carrier and combustion gases, respectively.

2.8. Sampling, and Measurements

Following a 24-h incubation period, the bottles were removed from the incubator, uncapped, and the pH levels of the fluids were measured using a digital pH meter (HI-2210, HANNA Instruments, Cluj-Napoca, Romania). The contents from the first set of bottles (n = 40) were used to determine ammonia–nitrogen (NH_3-N) concentrations, as well as the total (TVFA) and individual volatile fatty acids (VFA), namely acetate, propionate, and butyrate. The contents of the second set of bottles (n = 40) were directly subjected to freeze-drying for subsequent calculations of dry matter (DM). The freeze-dried contents were further analyzed to determine neutral detergent fiber (NDF), linoleic acid (LA; C18:2*n*-6), α-linolenic acid (LNA; C18:3*n*-3), eicosapentaenoic acid (EPA; C20:5*n*-3), docosahexaenoic acid (DHA; C22:6*n*-3), *cis*-9, *trans*-11 CLA, *trans*-11 vaccenic acid (TVA, *trans*-11 C18:1), Stearic acid (SA; C18:0), and other fatty acid concentrations.

2.9. Chemical Analysis

The chemical composition of the basal diet was analyzed using the methods of AOAC [30]: DM, method 930.15; crude protein (CP), method 990.03; ether extract (EE), method 920.39; and ash, method 942.05. The dietary levels of NDF and acid detergent fiber (ADF) were determined in accordance with the methodology outlined by Mertens [31], utilizing amylase-treated NDF (aNDF). The NH_3-N concentrations in rumen fluid were determined via the phenol–hypochlorite colorimetric technique outlined by Weatherburn [32]. To analyze the VFA concentrations, samples of rumen fluid were centrifuged at 12,000× *g* for 15 min at 4 °C, and the resulting supernatant was collected. Concentrations of TVFA, acetate, propionate, and butyrate were measured using a High Performance Liquid Chromatography (HPLC) system (LaChrom, pump L-7100, Merck/Hitachi, Tokyo, Japan) equipped with a UV detector (L-2400; Hitachi) and a column (Metacarb 87H; Varian, Palo Alto, CA, USA), following the procedure outlined by Muck and Dickerson [33]. The in vitro DM digestibility (IVDMD) and in vitro NDF digestibility (IVNDFD) were estimated in bottles. Fatty acid methyl esters (FAME) in either basal diet or in vitro incubation samples were quantified following the procedure outlined by Wachira et al. [34], with Heneicosanoic acid-C21 (Restek, Ripley, UK) used as the internal standard. Subsequently, a GC (GOW-MAC, G-M816, Bethlehem, PA, USA) was employed to quantify the fatty acid profiles within the experimental samples. The GC system was equipped with an FID and a WCOT-fused silica 100 m × 0.25 mm CP-Sil 88 capillary column coated with a 0.2 µm film of cyanopropyl polysiloxane (Varian, Chrompack 7489, Middelburg, The Netherlands).

2.10. Data analysis

The data for GP were subjected to repeated measurement analysis using the PROC MIXED procedure in SAS 9.4. Results for pH, NH_3-N, TVFA, VFA, IVDMD, IVNDFD, and concentrations of fatty acids were also analyzed using the PROC MIXED procedure for a randomized block design. Statistical differences between means were assessed through the Tukey's multiple comparison test and considered significant at a threshold of $p < 0.05$, unless specified otherwise.

3. Results

3.1. Gas Production and Kinetics of Ruminal Fermentation

In the current study, the effects of three doses of SAG, PIN, and CLO on cumulative gas production were analyzed over a 48 h incubation period. Gas production was measured up to 48 h, with additional data points generated up to 120 h to demonstrate a comprehensive overview of the trend (Figure 1). The CON treatment exhibited the highest gas production at various time points throughout the incubation period, reaching a peak of 174.6 mL/g DM at 48 h.

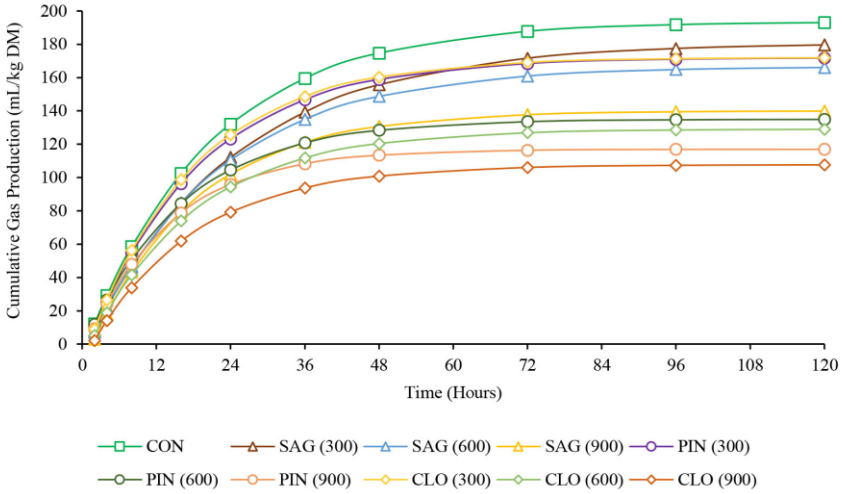

Figure 1. The effects of SAG, PIN, and CLO at inclusion levels of 0 (CON), 300, 600, and 900 mg/L on the in vitro cumulative gas production over 120 h. CON, SAG, PIN, and CLO represent control, sage essential oil, pine essential oil, and clove essential oil, respectively. In addition, 300, 600, and 900 represent corresponding dose levels in mg/L of each essential oils in the in vitro rumen culture.

Significant reductions in gas production were observed in the SAG, PIN, and CLO treatments ($p < 0.01$) at 48 h of incubation. In the SAG treatments, the dose of 900 mg/L demonstrated the lowest gas production (130.7 mL/g DM), followed by higher levels of gas production at doses of 600 mg/L and 300 mg/L (148.8 and 155.7 mL/g DM, respectively). Similarly, the PIN treatments exhibited a decrease in gas production, with the highest level of gas production observed at the dose of 300 mg/L (158.8 mL), and lower levels observed at doses of 600 mg/L and 900 mg/L (128.4 and 113.4 mL, respectively). Correspondingly, the CLO treatments also displayed a reduction in gas production, with the highest gas production observed at the dose of 300 mg/L (160.3 mL), and lower levels observed at doses of 600 mg/L and 900 mg/L (120.4 and 100.9 mL, respectively).

Table 3 presents the impact of different doses of SAG, PIN, and CLO on ruminal fermentation kinetics over a 48 h incubation period. The essential oil treatments significantly ($p < 0.01$) affected the cumulative gas production during the 48 h incubation period (GP_{48}). The highest dose of CLO (900 mg/L) resulted in a lower GP_{48} value (97.5 mL/g DM) compared to the CON group (176.4 mL/g DM), representing the lowest GP_{48} value among all treatments.

Table 3. The effects of increasing doses of three essential oils (SAG, PIN, and CLO) on the kinetics of ruminal fermentation in vitro.

ITEM	Treatments [1] (mg/L)										SEM	*p*-Value
	CON	SAG			PIN			CLO				
	0	300	600	900	300	600	900	300	600	900		
GP_{48} (mL/g DM)	176.4 ^{a}	155.6 ^{bc}	147.1 ^{c}	128.4 ^{de}	158.1 ^{b}	128.6 ^{d}	113.1 ^{f}	159.9 ^{b}	188.8 ^{ef}	97.5 ^{g}	1.92	<0.0001
B (mL/g DM)	191.9 ^{a}	180.6 ^{ab}	164.7 ^{c}	138.0 ^{d}	170.2 ^{bc}	132.9 ^{d}	114.9 ^{ef}	170.6 ^{bc}	128.4 ^{de}	106.1 ^{f}	2.86	<0.0001
C (h^{-1})	0.049 ^{cde}	0.042 ^{e}	0.047 ^{de}	0.058 ^{bc}	0.055 ^{bcd}	0.063 ^{b}	0.074 ^{a}	0.057 ^{bcd}	0.059 ^{bc}	0.060 ^{b}	0.00	<0.0001
L (h)	0.90 ^{ef}	1.15 ^{def}	1.34 ^{cd}	1.90 ^{ab}	1.22 ^{cde}	0.80 ^{f}	1.13 ^{def}	1.27 ^{cde}	1.57 ^{bc}	1.99 ^{a}	0.07	<0.0001
HT (h)	2.51 ^{de}	2.84 ^{cd}	2.96 ^{c}	3.43 ^{ab}	2.78 ^{cd}	2.30 ^{e}	2.56 ^{de}	2.82 ^{cd}	3.11 ^{bc}	3.52 ^{a}	0.07	<0.0001
AGPR (mL gas/h)	13.63 ^{a}	10.63 ^{d}	10.72 ^{d}	9.76 ^{e}	12.65 ^{bc}	11.99 ^{c}	11.06 ^{d}	12.97 ^{ab}	9.34 ^{e}	7.51 ^{f}	0.15	<0.0001

$^{a–f}$ Within a row, means without a common superscript are significantly different ($p < 0.05$). [1] CON, SAG, PIN, and CLO represent control, sage essential oil, pine essential oil, and clove essential oil, respectively. The numbers 0, 300, 600, and 900 represent corresponding dose levels in mg/L of each essential oil in the in vitro rumen culture. SEM: standard error of the means. GP_{48}, cumulative gas production at 48 h of incubation (mL/g DM); B, asymptotic gas production (mL/g DM); C, fractional fermentation rate (h^{-1}); L, lag (h); HT, time to reach half the ideal maximum gas production calculated as HT = log(2k) + Lag; AGPR, average gas production rate (mL gas/h) calculated as $(B \times k)/[(2 \times \ln 2) + (k \times L)]$.

Parameter B, representing the asymptotic gas production per gram of dry matter (mL/g DM), was also significantly influenced by the treatments ($p < 0.01$). The highest dose of CLO (900 mg/L) led to a reduced B value (106.1 mL/g DM) compared to the CON group (191.9 mL/g DM), representing the lowest B value among the other treatments. The essential oil treatments had a significant influence on the fractional fermentation rate (C) ($p < 0.01$). The PIN treatment at the dose level of 900 mg/L exhibited the highest C value (0.074 h^{-1}), while the SAG treatment at the dose of 300 mg/L had the lowest C value (0.042 h^{-1}). Parameter L, representing the lag time (h) or the time required for gas production to initiate, exhibited significant variations among the treatments ($p < 0.01$). The highest dose of CLO (900 mg/L) increased the lag time (1.99 h) compared to the CON group (0.90 h), indicating the longest L value among the treatments. Parameter HT, representing the half-time or the time required to reach half of the maximum gas production in hours, was significantly affected by the treatments ($p < 0.01$). The highest dose of CLO (900 mg/L) increased the HT value (3.52 h) compared to the CON group (2.51 h), demonstrating the longest HT among the treatments. The essential oil treatments significantly influenced the AGPR ($p < 0.01$). The highest dose of CLO (900 mg/L) resulted in a lower AGPR (7.51 mL gas/h) compared to the CON group (13.63 mL gas/h), exhibiting the lowest AGPR among the other treatments.

3.2. Production of Methane and Carbon Dioxide

Table 4 presents the impact of different doses of SAG, PIN, and CLO on the production of two greenhouse gases, CH_4 and CO_2, in the rumen. The essential oil treatments had a significant effect ($p < 0.01$) on cumulative gas production at 24 h (GP_{24}). The highest GP_{24} was observed in the CON group (127.59 mL), while the highest dose of CLO (900 mg/L) resulted in the lowest GP_{24} (79.73 mL). Intermediate values were observed for the SAG and PIN treatments across their respective doses.

Table 4. The effects of increasing doses of three essential oils (SAG, PIN, and CLO) on the ruminal production of CH_4 and CO_2 in vitro.

ITEM	Treatments [1] (mg/L)										SEM	*p*-Value
	CON	SAG			PIN			CLO				
	0	300	600	900	300	600	900	300	600	900		
GP_{24} (mL)	127.59 ^{a}	111.20 ^{c}	109.90 ^{c}	100.90 ^{d}	120.51 ^{b}	100.41 ^{d}	91.72 ^{e}	122.94 ^{ab}	94.35 ^{de}	79.73 ^{f}	1.37	<0.0001
CH_4 (mL)	30.22 ^{a}	24.40 ^{c}	23.41 ^{c}	19.98 ^{d}	26.97 ^{b}	20.29 ^{d}	17.00 ^{e}	28.00 ^{b}	19.07 ^{de}	14.61 ^{f}	0.43	<0.0001
CH_4 (%)	23.73 ^{a}	22.03 ^{abc}	21.30 ^{bc}	19.84 ^{cd}	22.41 ^{ab}	20.20 ^{cd}	18.54 ^{d}	22.80 ^{ab}	20.19 ^{cd}	18.30 ^{d}	0.46	<0.0001
CO_2 (mL)	93.37 ^{a}	80.87 ^{c}	77.24 ^{c}	68.95 ^{d}	86.10 ^{b}	68.48 ^{d}	61.54 ^{e}	87.74 ^{a}	66.56 ^{d}	52.77 ^{f}	0.72	<0.0001
CO_2 (%)	73.20 ^{a}	72.80 ^{ab}	70.30 ^{cd}	68.41 ^{de}	71.50 ^{abc}	68.20 ^{de}	67.10 ^{e}	71.40 ^{abc}	70.58 ^{bcd}	66.20 ^{e}	0.50	<0.0001

$^{a–f}$ Within a row, means without a common superscript are significantly different ($p < 0.05$). [1] CON, SAG, PIN, and CLO represents control, sage essential oil, pine essential oil, and clove essential oil, respectively. The numbers 0, 300, 600, and 900 represent corresponding dose levels in mg/L of each essential oil in the in vitro rumen culture. SEM: standard error of the means. GP_{24}, cumulative gas production at 24 h of incubation (mL).

In terms of methane production, all doses of the essential oils led to a significant reduction compared to the CON group ($p < 0.01$). The CON group demonstrated the highest level of CH_4 production (30.22 mL), whereas the highest dose of CLO (900 mg/L) resulted in the lowest CH_4 production (14.61 mL). The percentage of methane in the total GP_{24} was also significantly affected by the essential oils ($p < 0.01$). The CON group exhibited the highest percentage of CH_4 (23.73%), while the highest doses of PIN and CLO (900 mg/L) showed the lowest percentages of 18.54% and 18.30%, respectively.

Carbon dioxide production was significantly influenced by the essential oil treatments ($p < 0.01$). The CON group demonstrated the highest CO_2 production (93.37 mL), whereas the highest dose of CLO (900 mg/L) resulted in the lowest CO_2 production (52.77 mL). Similarly, the percentage of CO_2 in the GP_{24} was significantly affected by the treatments ($p < 0.01$). The CON group had the highest CO_2 percentage (73.20%), while the highest doses of PIN and CLO (900 mg/L) showed the lowest percentages of 67.10% and 66.20%, respectively.

To evaluate the impact of SAG, PIN, and CLO at different doses on in vitro methane production, regression analysis was conducted, as demonstrated in Figure 2. The regression equation for SAG was determined as $y = -0.0354x + 30.213$, with an R^2 value of 0.9131, indicating a strong correlation between the essential oil dose (x) and methane production (y). For PIN, the regression equation was $y = -0.0026x + 30.213$, with an R^2 value of 0.9525, demonstrating a substantial level of predictability. Similarly, CLO exhibited a significant relationship, as evidenced by the equation $y = -0.0162x + 30.213$, with an R^2 value of 0.9546.

Likewise, in vitro CO_2 production was examined through regression analysis (Figure 2). SAG exhibited a negative correlation, as indicated by the equation $y = -0.0715x + 93.363$, with an impressive R^2 value of 0.9261, suggesting a strong relationship between the essential oil dose (x) and CO_2 production (y). PIN also had a significant impact, with the regression equation $y = -0.0164x + 93.363$ and an R^2 value of 0.9694, signifying a high level of predictability. Similarly, CLO exhibited a considerable influence on CO_2 production, with the equation $y = -0.0326x + 93.363$ and an R^2 value of 0.9886.

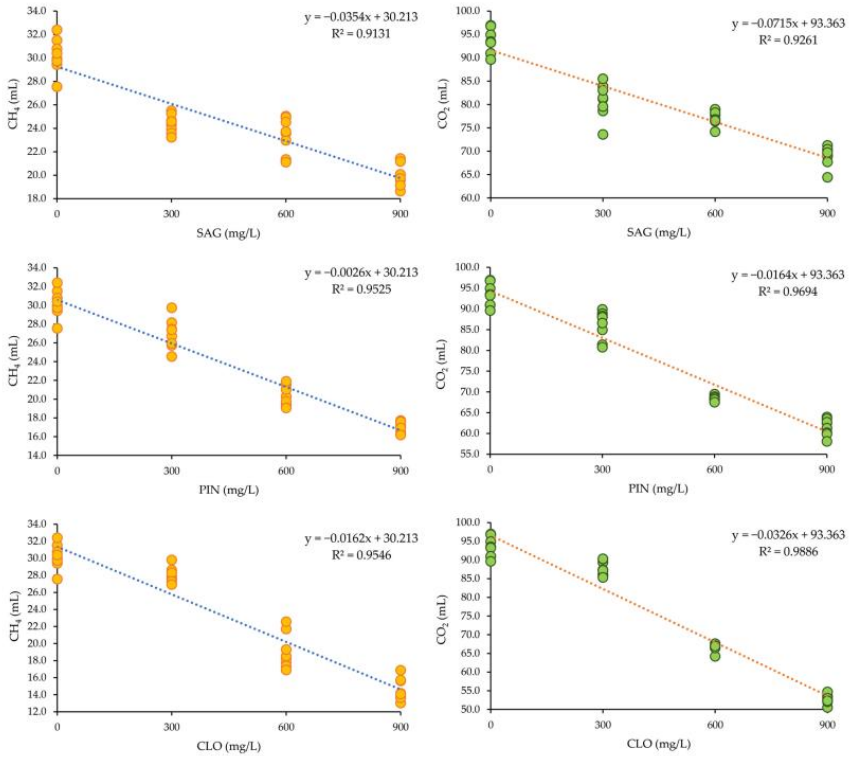

Figure 2. Regression analysis for the effects of various dose levels (0, 300, 600, and 900 mg/L) of SAG, PIN, and CLO on methane (CH_4) and carbon dioxide (CO_2) production (mL) in vitro.

3.3. In Vitro Ruminal Fermentation

The effects of SAG, PIN, and CLO on ruminal fermentation were investigated in this study (Table 5). The pH of the ruminal fluid was significantly influenced by the treatments ($p < 0.01$). Among the essential oils, the highest pH value (7.06) was observed in CLO at the dose of 900 mg/L, followed by PIN at 900 mg/L (6.97), while the CON group exhibited the lowest pH level (6.80). SAG at all doses displayed intermediate pH values. Ammonia concentrations in the ruminal fluid showed a decreasing trend with increasing doses of essential oils ($p < 0.01$). The CON group had the highest ammonia concentration (15.4 mM), while CLO at the dose of 900 mg/L resulted in the lowest concentration (12.8 mM). TVFA production in the ruminal fluid was significantly influenced by the treatments ($p < 0.01$). The highest TVFA concentration (89.61 mM) was observed in the CON group, while the lowest concentration (82.83 mM) was found in CLO at the dose of 900 mg/L. PIN and SAG treatments showed intermediate values across their respective doses. When examining the individual VFA components, significant variations were observed in the acetate concentration among treatments ($p < 0.01$). The CON group exhibited the highest acetate concentration (59.9 mol/100 mol), while the lowest concentration (51.9 mol/100 mol) was observed in PIN at the dose of 900 mg/L. Different patterns were observed for propionate (P), with the CON group having the lowest concentration (29.8 mol/100 mol), and the highest dose (900 mg/L) of SAG, PIN, and CLO resulting in the highest concentrations of 35.7, 36.3, and 35.9 mol/100 mol, respectively. Butyrate concentrations were also significantly affected by the treatments ($p < 0.01$), with the CON group having the highest concentration (5.0 mol/100 mol), and SAG, PIN, and CLO at the highest dose (900 mg/L) resulting in the lowest concentrations of 2.0, 1.9, and 1.9 mol/100 mol, respectively. Other VFA components showed different patterns, with the CON group consistently exhibiting the lowest concentrations ($p < 0.01$). The A:P ratio, representing the ratio of acetate to propionate, was significantly influenced by the treatments ($p < 0.01$). The CON group had the highest A:P ratio (2.0), while the lowest ratio (1.4) was observed in PIN at the highest dose (900 mg/L). IVDMD and IVNDFD were significantly affected by the essential oil treatments ($p < 0.01$). Generally, higher doses of the essential oils led to decreased

digestibility. The CON group had the highest IVDMD value (0.703 g/kg DM), while the highest dose of CLO (900 mg/L) showed the lowest value (0.570 g/kg DM). Similarly, the CON group had the highest IVNDFD value (0.313 g/kg NDF), while the highest dose of CLO (900 mg/L) demonstrated the lowest value of 0.252 g/kg NDF.

Table 5. The effects of increasing doses of three essential oils (SAG, PIN, and CLO) on the ruminal fermentation in vitro.

ITEM	Treatments [1] (mg/L)										SEM	*p*-Value
	CON	SAG			PIN			CLO				
	0	300	600	900	300	600	900	300	600	900		
pH	6.80 ^{g}	6.87 ^{f}	6.98 ^{c}	7.01 ^{b}	6.87 ^{ef}	6.97 ^{cd}	6.96 ^{d}	6.81 ^{g}	6.89 ^{e}	7.06 ^{a}	0.003	<0.0001
Ammonia (mM)	15.4 ^{a}	14.3 ^{bcd}	13.7 ^{def}	13.1 ^{fg}	14.6 ^{abc}	13.4 ^{efg}	13.0 ^{fg}	14.9 ^{ab}	13.9 ^{cde}	12.8 ^{g}	0.161	<0.0001
Total VFA (mM)	89.61 ^{a}	87.78 ^{b}	84.98 ^{c}	81.85 ^{f}	87.50 ^{b}	83.88 ^{d}	80.52 ^{g}	89.10 ^{a}	85.77 ^{c}	82.83 ^{e}	0.176	<0.0001
VFA (mol/100 mol)												
Acetate (A)	59.9 ^{a}	58.0 ^{b}	55.2 ^{c}	51.9 ^{e}	57.0 ^{b}	53.9 ^{d}	51.0 ^{e}	59.0 ^{a}	56.0 ^{c}	53.0 ^{d}	0.200	<0.0001
Propionate (P)	29.8 ^{e}	31.9 ^{cd}	33.8 ^{b}	35.7 ^{a}	32.8 ^{c}	34.6 ^{b}	36.3 ^{a}	31.0 ^{d}	33.9 ^{b}	35.9 ^{a}	0.208	<0.0001
Butyrate	5.0 ^{a}	4.0 ^{b}	3.0 ^{c}	2.0 ^{d}	4.0 ^{b}	2.9 ^{c}	1.9 ^{d}	3.9 ^{b}	3.1 ^{c}	1.9 ^{d}	0.123	<0.0001
Others	5.3 ^{f}	6.2 ^{ef}	8.1 ^{cde}	10.5 ^{ab}	6.3 ^{ef}	8.7 ^{bcd}	10.9 ^{a}	6.1 ^{f}	7.1 ^{def}	9.4 ^{abc}	0.401	<0.0001
A:P	2.0 ^{a}	1.8 ^{b}	1.6 ^{d}	1.5 ^{ef}	1.7 ^{c}	1.5 ^{e}	1.4 ^{f}	1.9 ^{b}	1.7 ^{cd}	1.5 ^{e}	0.016	<0.0001
IVDMD (g/kg DM)	0.703 ^{a}	0.652 ^{cd}	0.640 ^{de}	0.621 ^{fg}	0.680 ^{b}	0.630 ^{ef}	0.602 ^{h}	0.660 ^{c}	0.610 ^{gh}	0.570 ^{i}	0.002	<0.0001
IVNDFD (g/kg NDF)	0.313 ^{a}	0.295 ^{abc}	0.278 ^{bcd}	0.268 ^{cd}	0.300 ^{ab}	0.280 ^{bcd}	0.269 ^{cd}	0.304 ^{ab}	0.283 ^{abc}	0.252 ^{d}	0.006	<0.0001

$^{a–i}$ Within a row, means without a common superscript are significantly different ($p < 0.05$). [1] CON, SAG, PIN, and CLO represent control, sage essential oil, pine essential oil, and clove essential oil, respectively. The numbers 0, 300, 600, and 900 represent corresponding dose levels in mg/L of each essential oil in the in vitro rumen culture. SEM: standard error of the means. TVFA: total volatile fatty acids; VFA: volatile fatty acids; IVDMD: in vitro dry matter digestibility; IVNDFD: in vitro neutral detergent fiber digestibility.

3.4. In Vitro Concentrations of Fatty Acids

Table 6 presents the effect of three inclusion levels of SAG, PIN, and CLO on the concentrations of fatty acids in the rumen. When compared to the CON group, myristic acid (C14:0) was significantly decreased ($p = 0.0035$) with the addition of 900 mg/L of PIN into the culture, resulting in a decrease from 3.84% to 3.31%. A notable reduction ($p = 0.0009$) in the concentrations of palmitic acid (C16:0) was also observed with the addition of 900 mg/L of SAG, PIN, and CLO into the cultures, as was also observed in 600 mg/L of CLO. Significant differences ($p = 0.0046$) in palmitoleic acid (C16:1) concentrations were observed among the treatments, with the highest value in the 300 mg/L SAG treatment and the lowest value in the treatment containing 900 mg/L of SAG. The essential oils had the strongest decreasing effect on the ruminal concentrations of stearic acid (C18:0) when 300 and 900 mg/L of CLO were added into the cultures ($p < 0.001$). The concentration of oleic acid (C18:1 *cis*-9) demonstrated a significant decreasing pattern ($p < 0.001$) as the doses of essential oils increased. The lowest value was recorded in the treatment with 600 mg/L of CLO, followed by 300 mg/L and 900 mg/L of CLO, as well as 600 mg/L of PIN. *Trans*-11 vaccenic acid (C18:1 *trans*-11) significantly increased ($p = 0.0002$) in the treatments of 300 mg/L of CLO and 600 mg/L in either PIN or CLO. Linoleic acid (C18:2 *n*-6) significantly increased ($p < 0.001$) in the 300 mg/L CLO treatment, followed by 600 mg/L of CLO and 600 mg/L of SAG. The highest concentrations of CLA (C18:2 *cis*-9, *trans*-11) were observed in the 300 mg/L CLO treatment, followed by 600 mg/L of CLO and 900 mg/L of SAG ($p < 0.001$). Among the treatments, 300 and 600 mg/L of CLO resulted in a significant increase ($p < 0.001$) in the concentrations of LNA (C18:3 *n*-3). The other treatments demonstrated either mild or no significant effects on the concentrations of LNA. Arachidonic acid (C20:4) did not show any significant differences ($p > 0.05$) among the treatments. Except for 600 mg/L of CLO, all the treatments resulted in a significant increase ($p < 0.001$) in the in vitro concentrations of EPA (C20:5 *n*-3), with the highest value observed in 900 mg/L of SAG, followed by 900 mg/L of PIN. The in vitro concentration of

DHA (C22:6 *n*-3) was also increased ($p < 0.001$) in the 900 mg/L SAG treatment, followed by 600 mg/L of SAG. In terms of overall fatty acid composition, the lowest ($p < 0.001$) concentration of SFA was observed in the 600 mg/L CLO treatment, followed by 600 mg/L of PIN and 900 mg/L of either SAG or CLO. Among the treatments, 300 and 600 mg/L of SAG, as well as 300 mg/L of CLO, showed the highest ($p = 0.0014$) in vitro concentrations of unsaturated fatty acids (UFA). Except for 300 mg/L of SAG and PIN, all the other treatments resulted in a significant decrease ($p < 0.001$) in the in vitro concentrations of monounsaturated fatty acids (MUFA). The highest in vitro concentrations of PUFA were observed in 600 mg/L of SAG and 300 mg/L of CLO, followed by 900 mg/L of SAG and 600 mg/L of CLO. Among the experimental treatments, only CLO, at all three dose levels, significantly ($p < 0.001$) reduced the SFA:UFA ratio, with 600 mg/L of CLO showing the lowest ratio.

Table 6. The effects of increasing doses of three essential oils (SAG, PIN, and CLO) on the ruminal concentrations of fatty acids in vitro.

Fatty ACIDS	Treatments [1] (mg/L)										SED	*p*-Value
	CON	SAG			PIN			CLO				
	0	300	600	900	300	600	900	300	600	900		
Individual FAs, % TFA												
C14:0 (Myristic acid)	3.84 ^{a}	3.77 ^{ab}	3.64 ^{abc}	3.62 ^{abc}	3.62 ^{abc}	3.49 ^{abc}	3.31 ^{c}	3.71 ^{abc}	3.33 ^{bc}	3.45 ^{abc}	0.089	0.0035
C16:0 (Palmitic acid)	12.20 ^{a}	11.58 ^{ab}	11.05 ^{ab}	10.75 ^{b}	12.19 ^{a}	11.33 ^{ab}	10.84 ^{ab}	11.52 ^{ab}	10.42 ^{b}	10.63 ^{b}	0.282	0.0009
C16:1 (Palmitoleic acid)	2.60 ^{ab}	2.70 ^{a}	2.08 ^{bc}	1.95 ^{c}	2.56 ^{abc}	2.33 ^{abc}	2.14 ^{abc}	2.25 ^{abc}	2.40 ^{abc}	2.36 ^{abc}	0.123	0.0046
C18:0 (Stearic acid)	7.82 ^{a}	6.60 ^{abc}	6.47 ^{abc}	6.12 ^{bc}	6.94 ^{ab}	5.68 ^{bc}	6.96 ^{ab}	5.42 ^{c}	5.42 ^{c}	6.10 ^{bc}	0.270	<0.0001
C18:1 (Oleic acid)	5.47 ^{a}	4.58 ^{b}	3.51 ^{c}	3.63 ^{c}	5.25 ^{ab}	3.23 ^{cd}	3.40 ^{c}	3.17 ^{cd}	2.51 ^{d}	2.97 ^{cd}	0.149	<0.0001
C18:1 (*trans*-11 VA)	3.47 ^{b}	3.90 ^{ab}	3.77 ^{ab}	3.50 ^{b}	3.75 ^{ab}	4.14 ^{a}	3.79 ^{ab}	4.11 ^{a}	4.31 ^{a}	3.96 ^{ab}	0.111	0.0002
C18:2 n-6 (LA)	1.98 ^{de}	2.42 ^{abcd}	2.60 ^{abc}	2.20 ^{bcde}	2.05 ^{de}	2.10 ^{cde}	1.81 ^{e}	2.92 ^{a}	2.64 ^{ab}	2.47 ^{abcd}	0.104	<0.0001
C18:2 (*cis*-9, *trans*-11 CLA)	0.67 ^{cd}	0.81 ^{bc}	0.92 ^{abc}	0.94 ^{ab}	0.67 ^{cd}	0.67 ^{cd}	0.56 ^{d}	1.06 ^{a}	0.96 ^{ab}	0.89 ^{abc}	0.046	<0.0001
C18:3 n-3 (LNA)	1.80 ^{e}	2.56 ^{cd}	2.99 ^{abc}	2.59 ^{cd}	1.89 ^{e}	2.21 ^{de}	1.95 ^{e}	3.31 ^{a}	3.28 ^{ab}	2.75 ^{bcd}	0.108	<0.0001
C20:4 (Arachidonic acid)	0.12	0.15	0.26	0.14	0.15	0.11	0.18	0.13	0.14	0.09	0.036	0.2584
C20:5 n-3 (EPA)	1.02 ^{f}	1.35 ^{ed}	1.69 ^{abc}	1.81 ^{a}	1.50 ^{cd}	1.68 ^{abc}	1.80 ^{ab}	1.51 ^{bcd}	1.08 ^{ef}	1.48 ^{cd}	0.057	<0.0001
C22:6 n-3 (DHA)	0.79 ^{ef}	1.13 ^{bcd}	1.34 ^{ab}	1.43 ^{a}	0.93 ^{def}	1.19 ^{bc}	0.92 ^{def}	0.98 ^{cdef}	1.01 ^{cde}	0.78 ^{f}	0.044	<0.0001
SFA	23.86 ^{a}	21.94 ^{abc}	21.16 ^{bcd}	20.49 ^{cd}	22.75 ^{ab}	20.51 ^{cd}	21.11 ^{bcd}	20.65 ^{bcd}	19.17 ^{d}	20.18 ^{cd}	0.425	<0.0001
UFA	17.92 ^{ab}	19.58 ^{a}	19.15 ^{a}	18.18 ^{ab}	18.74 ^{ab}	17.64 ^{ab}	16.54 ^{b}	19.44 ^{a}	18.31 ^{ab}	17.73 ^{ab}	0.444	0.0014
MUFA	11.54 ^{a}	11.17 ^{a}	9.36 ^{b}	9.07 ^{b}	11.55 ^{a}	9.69 ^{b}	9.33 ^{b}	9.53 ^{b}	9.22 ^{b}	9.28 ^{b}	0.259	<0.0001
PUFA	6.38 ^{d}	8.41 ^{abc}	9.79 ^{a}	9.10 ^{ab}	7.18 ^{cd}	7.95 ^{bc}	7.21 ^{cd}	9.91 ^{a}	9.09 ^{ab}	8.45 ^{abc}	0.308	<0.0001
SFA:UFA	1.65 ^{a}	1.51 ^{abc}	1.44 ^{abcd}	1.46 ^{abcd}	1.63 ^{ab}	1.51 ^{abc}	1.61 ^{abc}	1.41 ^{cd}	1.26 ^{d}	1.41 ^{bcd}	0.043	<0.0001
Remaining Fatty Acids	58.23 ^{c}	58.48 ^{bc}	59.69 ^{abc}	61.34 ^{abc}	58.51 ^{bc}	61.85 ^{ab}	62.36 ^{a}	59.91 ^{abc}	62.53 ^{a}	62.09 ^{a}	0.671	0.0001
TFA (% Feed DM)	80.69 ^{ab}	74.35 ^{b}	77.03 ^{ab}	77.28 ^{ab}	74.67 ^{b}	77.40 ^{ab}	79.41 ^{ab}	75.58 ^{b}	83.57 ^{a}	80.97 ^{ab}	1.524	0.0040

$^{a–f}$ Within a row, means without a common superscript are significantly different ($p < 0.05$). [1] CON, SAG, PIN, and CLO represent control, sage essential oil, pine essential oil, and clove essential oil, respectively. The numbers 0, 300, 600, and 900 represent corresponding dose levels in mg/L of each essential oil in the in vitro rumen culture. SEM: standard error of the means. FAs: fatty acids; TFA: total fatty acids; VA: vaccenic acid; LA: linoleic acid; CLA: conjugated linoleic acid; LNA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

The highest in vitro concentration of total fatty acids (TFA) was observed in 600 mg/L of CLO, while the lowest concentrations were observed in 300 mg/L of SAG, PIN, and CLO ($p = 0.0040$). The CON group showed an intermediate concentration of TFA among the treatments.

3.5. In Vitro Biohydrogenation of Ruminal PUFA

Table 7 presents the impact of different experimental groups on the biohydrogenation of long-chain PUFA (LA and LNA) and very long-chain PUFA (EPA and DHA). The biohydrogenation of LA was the lowest ($p < 0.001$) in 300 mg/L of CLO, followed by the treatment containing 600 mg/L of SAG. Correspondingly, the biohydrogenation of LNA was the lowest ($p < 0.001$) in 300 mg/L of CLO, followed by treatment containing 600 mg/L of CLO. Among the experimental treatments, 600 mg/L of CLO resulted in the lowest biohydrogenation of EPA, followed by 900 mg/L of CLO ($p < 0.001$). Likewise, the biohydrogenation of DHA was significantly decreased ($p < 0.001$) in 600 mg/L of CLO, followed by 900 mg/L of PIN and 900 mg/L of CLO.

Table 7. The effects of increasing doses of three essential oils (SAG, PIN, and CLO) on the ruminal biohydrogenation of long chain and very long chain PUFAs in vitro.

Fatty Acids	Treatments [1] (mg/L)										SED	*p*-Value
	CON	SAG			PIN			CLO				
	0	300	600	900	300	600	900	300	600	900		
C18:2 n-6 (LA)	73.4 ^{a}	70.0 ^{a}	59.9 ^{bc}	64.6 ^{b}	73.2 ^{a}	70.6 ^{a}	72.5 ^{a}	57.7 ^{c}	60.7 ^{bc}	63.0 ^{b}	0.81	<0.0001
C18:3 n-3 (LNA)	77.7 ^{a}	72.1 ^{b}	55.7 ^{de}	59.2 ^{cd}	76.9 ^{a}	72.5 ^{b}	73.5 ^{ab}	53.7 ^{e}	57.3 ^{de}	63.0 ^{c}	0.77	<0.0001
C20:5 n-3 (EPA)	65.8 ^{a}	57.1 ^{b}	56.5 ^{bc}	56.1 ^{bc}	58.5 ^{b}	56.7 ^{bc}	53.3 ^{cd}	56.6 ^{bc}	49.3 ^{e}	52.0 ^{de}	0.72	<0.0001
C22:6 n-3 (DHA)	51.5 ^{a}	47.7 ^{ab}	41.9 ^{bc}	35.8 ^{cd}	50.4 ^{a}	47.5 ^{ab}	34.1 ^{d}	47.1 ^{ab}	30.8 ^{d}	31.3 ^{d}	1.40	<0.0001

$^{a–e}$ Within a row, means without a common superscript are significantly different ($p < 0.05$). [1] CON, SAG, PIN, and CLO represent control, sage essential oil, pine essential oil, and clove essential oil, respectively. The numbers 0, 300, 600, and 900 represent corresponding dose levels in mg/L of each essential oil in the in vitro rumen culture. SEM: standard error of the means. LA: linoleic acid; LNA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

4. Discussion

The current study aimed to investigate the impact of SAG, CLO, and PIN on the reduction of greenhouse gas emissions (CH_4 and CO_2 production) and the enhancement of the nutritive quality of ruminant products by examining the in vitro biohydrogenation of ruminal PUFAs. This study elucidated the potential implications of these interventions on ruminal fermentation processes and the bioconversion of PUFAs, both of which play pivotal roles in methane emissions and the nutritional composition of ruminant-derived foods. The acquired findings offer valuable insights into the modulation of these processes, which are vital for the development of sustainable strategies to address environmental concerns without compromising the nutritional value of animal products.

4.1. Gas Production and Kinetics of Ruminal Fermentation

The evaluation of gas production and the kinetics of ruminal fermentation provide valuable insights into the effects of essential oils on rumen microbial activity and fermentation processes. Within the current study, the supplementation of essential oils into the cultures of rumen fluid resulted in the inhibition of gas production at various time points, indicating a reduction in microbial activity within the rumen. This finding aligns with previous studies reporting reduced gas production when treatments were supplemented with sage, pine, and clove essential oils [35–37]. The authors reported dose-dependent effects of essential oils on cumulative gas production. Generally, higher doses of sage, pine, and clove essential oils resulted in lower gas production levels compared to the control group. The reduction in gas production could be attributed to the antimicrobial properties of the essential oils [38]. Known for their antimicrobial activity, essential oils have been shown to effectively target a wide range of microorganisms, including rumen bacteria [19,38,39]. Therefore, decreased gas production may be attributed to the stronger inhibitory effects exerted on rumen microbial populations by higher doses of essential oils.

4.2. Kinetics of Rumen Fermentation

The significant variations observed in asymptotic gas production (B) values among the essential oil treatments indicate differences in the maximum gas production achieved per gram of dry matter. SAG, PIN, and CLO exhibited dose-responsive reduced B values compared with the CON group, suggesting the limiting effects of these essential oils on ruminal fermentation and gas production. This could be due to the presence of bioactive compounds, such as α-Thujone, α-pinene, and eugenol in SAG, PIN, and CLO, respectively, which has been reported to inhibit microbial fermentation and reduce gas production [40–43]. Moreover, the dose-dependent increase in the fractional fermentation rate (C) observed in the present study was also in accordance with the study of Kahvand and Malecky [37]. Furthermore, the delayed initiation of fermentation and gas production, as indicated by the higher lag time (L) observed in most treatments, can be attributed to the inhibitory effects of certain compounds in essential oils and the time required for microorganisms to adapt to these essential oils. The longest HT was observed in the 900 mg/L CLO treatment, indicating slower fermentation kinetics and delayed attainment of the peak gas production. This observation suggests that certain components in CLO might have inhibitory effects on rumen microbial populations, thereby impacting their metabolic activity and the efficiency of gas production. Finally, the supplementation of 900 mg/L of CLO resulted in the lowest AGPR, indicating a decrease in fermentation rate and gas production efficiency. This observation suggests that the bioactive compounds present in CLO may have inhibitory effects on rumen microorganisms, as discussed earlier.

4.3. Production of Methane and Carbon Dioxide

In our study, we examined the significant effects of various doses of SAG, PIN, and CLO on the production of CH_4 and CO_2 during ruminal fermentation. The inclusion of these essential oils led to a noteworthy reduction in greenhouse gas emissions, specifically CH_4, which is a potent greenhouse gas known to contribute significantly to global warming [44]. In our findings, it was revealed that all doses of essential oils significantly reduced CH_4 production compared to the CON group. This indicates the methane-reducing properties of SAG, PIN, and CLO in ruminal fermentation. Our findings were consistent with the study of Zmora et al. [45], which observed a reducing effect of sage essential oil on in vitro ruminal methane production. However, a study by Kahvand and Malecky [37] did not find any significant effects of sage essential oil on methane production within the rumen. This discrepancy may be attributed to variations in the chemical composition of essential oils and the dose levels implemented in their study. Supporting our observations, Vera et al. [35] reported a decrease in the ruminal production of CH_4 when using pine essential oil, aligning with our findings regarding the reduction in CH_4 production in our PIN treatments. Furthermore, Günal et al. [46] reported a drop in ruminal CH_4 production when clove essential oil was applied, further supporting our findings. These studies offer additional evidence of the methane-mitigating effects of sage, pine, and clove essential oils in ruminal fermentation. Furthermore, our study demonstrated a significant dose-dependent effect of the essential oils on methane production. Specifically, the highest dose of CLO (900 mg/L) led to the lowest CH_4 production, underscoring the remarkable potential efficacy of CLO in effectively mitigating methane emissions. The decrease in methane production observed in our study can be attributed to the bioactive compounds found in these essential oils. These compounds likely inhibit methane-producing microorganisms in the rumen. In their study, Patra and Saxena [47] reported that the addition of clove oil, with its antimicrobial properties, significantly reduced ruminal methane production by inhibiting the growth and activity of methanogenic microorganisms. Kurniawati and Muhsin Al Anas [48] similarly attributed the reduction in methane emissions to the antimicrobial and antioxidant properties of pine essential oil. They suggested that these properties modulate ruminal fermentation and methane production.

In addition to the significant decrease in methane production, our study also revealed a substantial reduction in CO_2 production following the addition of essential oils, particularly

in the 900 mg/L CLO treatment. This finding indicates that CLO has the potential to effectively reduce CO_2 emissions as well. The breakdown of cellulose and hemicellulose, major components of ruminant feed, occurs during ruminal fermentation. This breakdown is facilitated by the activity of glucanases, which lead to the production of monomers such as hexoses and pentoses. Following this, the monomers undergo partial oxidation, resulting in the formation of volatile fatty acids. Simultaneously, CO_2 and hydrogen are released [49]. These findings further support the growing body of evidence that highlights the potential of essential oils as natural additives for mitigating greenhouse gas emissions from ruminant animals.

4.4. In Vitro Ruminal Fermentation

The assessment of ruminal pH is a critical parameter for evaluating the stability and equilibrium of the rumen environment in ruminant animals, typically falling within the range of 5.0 to 7.5 [50]. In our investigation, we observed a dose-dependent effect of essential oils on ruminal pH, with the strongest impact observed at higher doses. Our findings align with the results reported by Busquet et al. [51], who noted no significant changes in rumen pH when low doses of clove bud oil were included into the cultures. However, at higher doses, a substantial increase in pH levels was observed. Similarly, Mulyandari et al. [52] conducted a study using a lower dose of clove essential oil (100 µL/L) and did not observe any noteworthy alterations in rumen pH levels. This lack of effect may be attributed to the relatively low concentration of clove essential oil used in their study. The observed increase in ruminal pH in our study can be attributed, at least in part, to the decrease in TVFA production and NH_3-N concentrations. It is well-established that ruminal pH is influenced by the production and utilization of VFAs, with lower pH values associated with higher VFA concentrations [53].

The advantageous dose-dependent effects of essential oils on in vitro concentrations of NH_3-N observed in our study hold promise for improving protein metabolism and reducing nitrogen waste in ruminal fermentation. Excessive ammonia production in the rumen has been linked to inefficient utilization of dietary protein and elevated nitrogen excretion [54]. Our findings demonstrate that essential oils, particularly at higher doses, have the potential to optimize protein metabolism and minimize nitrogen waste. The reduction in ammonia concentrations observed in our study is consistent with previous research highlighting the ammonia-lowering effects of essential oils in ruminal fermentation. For example, Patra and Yu [55] conducted a study investigating the impact of various essential oils on ruminal fermentation and found a significant decrease in ammonia production upon the addition of clove oil. This reduction in ammonia levels can be attributed to the bioactive compounds present in essential oils, which have the ability to inhibit the activity of ruminal microbial enzymes responsible for ammonia release.

The observed decrease in TVFA concentration in the rumen is accompanied by a concomitant reduction in pH levels and NH_3-N concentration, indicating a decrease in overall diet fermentation [56]. Since VFAs serve as the primary source of energy for ruminants, a reduction in ruminal VFA production could potentially have negative nutritional implications if this effect manifests in live animals. In our study, the inclusion of SAG, PIN, and CLO in the cultures resulted in a significant reduction of acetate, which is the primary VFA associated with methane production. This finding suggests that the addition of these essential oils has the potential to modulate rumen fermentation, leading to a decrease in methanogen activity [57]. Moreover, our study revealed that all the treatments employed exhibited an increase in propionate concentrations, consequently leading to a notable reduction in the A:P ratio. These findings further support the hypothesis put forward by Fouts et al. [58] that the application of essential oils enhances propionate production, thereby limiting the availability of H_2 for methane production.

The observed dose-dependent reducing effects of essential oils on IVDMD and IVNDFD in our study are consistent with previous findings that have reported on the negative impact of essential oils on ruminal nutrient digestibility. For example, Roy et al. [59] investigated

the effects of clove essential oil on ruminal fermentation and nutrient digestibility and observed a decrease in dry matter digestibility at a dose level of 300 ppm. Similarly, in another study conducted by Benchaar et al. [43], the addition of clove leaf oil to the culture at a dose of 200 mg/L did not significantly alter in vitro dry matter digestibility but led to a significant reduction in in vitro NDF digestibility. The slight discrepancy between these studies may be attributed to variations in the dose levels of the oils and the specific plant parts utilized. Nevertheless, the decrease in in vitro digestibility observed in our study suggests that essential oils, particularly at higher doses, have the potential to hinder ruminal nutrient digestibility. This inhibitory effect can be attributed to the potential antimicrobial properties exhibited by the bioactive compounds present in the essential oils, which may negatively impact the ruminal microbial population responsible for efficient nutrient breakdown and digestion.

4.5. In Vitro Biohydrogenation and Fatty Acid Concentrations

The present study demonstrated the advantageous reducing effects of SAG, PIN, and CLO at specific dose levels on the in vitro concentrations of C14:0 and C16:0. The saturated fatty acids, particularly C14:0 and C16:0, have been extensively studied and are known to possess atherogenic and thrombogenic properties, as documented by Carneiro et al. [60]. Notably, various studies, including those by Al-Amiri et al. [61] and Praagman et al. [62], have consistently reported their ability to elevate cholesterol levels and promote platelet aggregation. The consumption of atherogenic and thrombogenic foods, which exhibit a propensity to induce platelet aggregation, is associated with an increased risk of cardiovascular disease and, consequently, offers limited nutritional benefits.

During the ruminal fermentation process, LA undergoes an initial isomerization step, resulting in the formation of *cis*-9, *trans*-11 CLA. Subsequently, CLA is converted to *trans*-11 C18:1 and further to C18:0 [63]. In our study, the addition of 300 mg/L and 600 mg/L of CLO resulted in a significant reduction in the biohydrogenation of LA and LNA. Interestingly, we observed an accumulation of *cis*-9, *trans*-11 CLA and *trans*-11 C18:1, along with decreased concentrations of C18:0 in these treatments, indicating partial biohydrogenation of LA and LNA in the presence of CLO at both inclusion levels. This accumulation of CLA and *trans*-11 C18:1, coupled with the decreased concentration of C18:0, suggests that the second step of biohydrogenation is inhibited by CLO at 300 mg/L and 600 mg/L. Several studies have previously reported the hydrogenation of C18:1 isomers to C18:0 or their isomerization into various positional and geometric isomers [64,65].

The inhibitory effects of herbal essential oils on the growth of microorganisms involved in the biohydrogenation of *trans*-11 C18:1 to C18:0 have been reported [66,67]. This observation could potentially elucidate the decreased levels of C18:1 *n*-9 observed in several treatments examined in our study. Additionally, the concurrent reduction of C18:0 alongside the elevation of *trans*-11 C18:1 concentrations suggests that the essential oils primarily facilitated the conversion of C18:1 *n*-9 to *trans*-11 C18:1. The available evidence strongly indicates that the elevation of *cis*-9, *trans*-11 CLA levels in ruminant tissues and milk predominantly originates from an augmented availability of *trans*-11 C18:1 [68].

Several studies have investigated the efficacy of marine lipid supplements (fish oil and microalgae products) as potential sources of 20:5 *n*-3 and 22:6 *n*-3 for dairy ruminants [67,69,70]. However, the transfer rates of these very long chain PUFAs into meat and milk are constrained due to the extensive biohydrogenation in the rumen. In the context of ruminal metabolism, the conversion of C20:5 *n*-3 and C22:6 *n*-3 is thought to undergo a preliminary isomerization process, similar to the behavior observed for the C18 PUFAs. This isomerization step is believed to give rise to conjugated intermediates consisting of 20 and 22 carbon atoms [71]. In the current study, the level of biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 was comparatively lower in contrast to the ruminal biohydrogenation of C18 fatty acids such as C18:2 *n*-6 and C18:3 *n*-3. In addition, it is important to acknowledge that a complete understanding of the biohydrogenation pathways of C20:5 *n*-3 and C22:6 *n*-3 is still lacking. Our results align with those of Chow et al. [72], who demonstrated a

biohydrogenation rate of 61.6% for EPA and 46.2% for DHA following 24 h of incubation when a 2% fish oil inclusion was incorporated into the diets.

According to compelling evidence, both general dietary recommendations and guidelines from the World Health Organization (WHO) propose that replacing SFA with PUFA can effectively lower the risk of cardiovascular disease (CVD) by reducing LDL-cholesterol levels [73,74]. Our study's fatty acid analysis revealed significant changes that have important implications for the nutritional profile and health implications of ruminant-derived products. Specifically, the significant reduction in SFA levels across the majority of treatments and the constant concentrations of UFA in all experimental treatments, particularly in the 600 mg/L of CLO, demonstrated a marked decrease in the SFA:UFA ratio accompanied by an increase in PUFA. This rise in PUFA is especially desirable given its established correlation with reduced markers of cardiovascular disease risk [75].

5. Conclusions

In conclusion, the findings of this study highlight the in vitro potential of SAG, PIN, and CLO as effective interventions to reduce greenhouse gas emissions, specifically methane, during ruminal fermentation. The addition of these essential oils resulted in a significant decrease in gas production and methane production, which can be attributed to their antimicrobial properties and the inhibition of methane-producing microorganisms in the rumen. However, it is imperative to acknowledge the limitations of in vitro research and the necessity for validation through in vivo studies. Future work should prioritize translating these findings into practical applications. Moreover, the inclusion of essential oils led to a substantial reduction in carbon dioxide emissions, indicating their potential to mitigate overall greenhouse gas emissions. Additionally, the study demonstrated that essential oils, particularly CLO, effectively reduced the concentrations of atherogenic and thrombogenic fatty acids, such as C14:0 and C16:0, while promoting the accumulation of beneficial fatty acids, such as *cis*-9, *trans*-11 CLA and *trans*-11 C18:1. Since some essential oils exert their effects at high dose levels, we encourage researchers to consider novel techniques, such as nano-encapsulation, to enhance the effectiveness of plant chemical compounds in the rumen, even at lower doses, in their future studies. Overall, these findings provide valuable insights into the modulation of ruminal fermentation processes and the bioconversion of fatty acids, emphasizing the potential of essential oils as sustainable strategies to address environmental concerns without compromising the nutritional value of ruminant-derived products.

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