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Metabolic effects of nano-encapsulated extract of *Moringa oleifera* to reduce ruminal methane, carbon monoxide, and hydrogen sulfide outputs of high-concentrate diet studied *in vitro*

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ABSTRACT

The use of forage trees and their extracts at different levels, such as *Moringa oleifera*, has recently attracted the attention of many researchers as an alternative strategy to provide essential nutrients and reduce ruminant greenhouse gas emissions involved in global warming, using an *in vitro* gas production technique. Therefore, the present study aimed to evaluate increasing levels of *M. oleifera* extracts (methanolic or aqueous) chemically characterized using gas chromatography-mass spectrometry (GC-MS) processed as nanoencapsulation or not on biogas production such as methane, carbon monoxide, and hydrogen sulfide as well as ruminal fermentation kinetics *in vitro*. The nanoencapsulation process of *M. oleifera* was developed in two separate stages; 1 % acetic acid solution and 0.1 g of sodium tripolyphosphate were used for the first and second steps, respectively. Methane, carbon monoxide, hydrogen sulfide, and total gas production volumes were measured a long 48 h after inoculation. The main chemical compounds in the aqueous extract detected by GC-MS in *M. oleifera* leaves were oleic acid methyl ester (62.1 %), and cyclopentanetridecanoic acid methyl ester (11.9 %). In contrast, in the methanolic extract, they were oleic acid methyl ester (64.5 %), and methyl isostearate (10.1 %). Parameters related to gas production kinetics differed considerably within treatments, particularly with fractions b (P = 0.001, SEM = 11.12) and latency phase (P = 0.037, SEM = 0.15). The highest rates of fraction b (9.71 mL/g dry matter) and fermentation delay (1.92 h) were recorded for methanolic and nano-aqueous extracts, respectively. For both methanolic (10.56–10.68 mmol/g dry matter) and aqueous extracts (9.73–10.04 mmol/g dry matter), a linearly increasing trend was observed once the injection rate (0.25 and control groups, respectively) and metabolizable energy were elevated. During the incubation phase, 24 and 48 h, the types of extraction significantly impacted the amount of H_2S synthesis (P = 0.021 (SEM = 0.005) and P = 0.045 (SEM = 0.017), respectively). The ratio of CH_4: short-chain fatty acids had the highest efficiency (P = 0.277, SEM = 0.39), followed by CH_4: organic matter (P = 0.118, SEM = 0.16) and CH_4: metabolizable energy (P = 0.236, SEM = 0.068). Thus, it could be concluded that there is a possibility of selecting *M. oleifera* extracts to ameliorate greenhouse gas emissions, such as CH_4 production, without compromising fermentation kinetics and feed degradability.

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1. Introduction

Ruminant production is one of the most in-demand livestock industries, which is essential to a sustainable food supply and economic gains [1,2]. However, because ruminal fermentation of feeds is thought to be responsible for around 40 % of all anthropogenic emissions of greenhouse gases from the systems, with the result that digested feeds lose energy and CH_4 produced in ruminants as a byproduct of anaerobic microbial fermentation in the rumen has become an increasingly significant production shadow [3–5]. Consequently, there is an urgent need to develop and execute appropriate solutions to lower ruminal CH_4 production within livestock production and enhance dietary energy efficacy, which is attributable to the direct reduction of ruminal methanogenesis [6].

Several studies have demonstrated that certain plant species' secondary metabolites can enhance animal performance, lower CH_4 production, and lessen rumen the breakdown of proteins [7–9]. It has been known that certain secondary metabolites can reduce the amount of CH_4 produced during ruminal fermentation [10,11]. Accordingly, *M. oleifera*, a widely grown tree that is well suited to a variety of soil and irrigation conditions in tropical regions [12], is a plant rich in secondary metabolites, such as tannins, saponins, and numerous other phenolic compounds [11,13]. It has also been indicated that plant leaf meal, like that of *M. oleifera*, can be used as an economical source of protein for livestock [14,15]. According to several reports, rumen microbial activity stimulated by *M. oleifera* [16], thyme (*Thymus vulgaris*) [17], and rosemary (*Salvia rosmarinus*) [18]. Additionally, feeding tree leaves to ruminants has been shown to reduce intestinal methane emissions, and numerous researchers have promoted its usage as a substitute protein source for cattle [19].

Microencapsulation is an emerging technology widely employed in animal nutrition to create stable products of vitamins, minerals, and fatty acids [20]. This approach can be a physical barrier, protecting medications from the harsh external environment and increasing the substance's stability [21]. Furthermore, the technique traps bioactive molecules within a protective matrix, allowing for regulated release, targeted distribution, and preservation of the compounds until they reach their intended destinations [21]. Previous research has indicated that encapsulation technology has tremendous potential for improving cattle products. Researchers have investigated various encapsulation methods and materials to effectively deliver essential nutrients, improve feed efficiency, and improve animal welfare [22–26]. Furthermore, encapsulating volatile chemicals in animal feed can reduce feed waste and lower the environmental effect of livestock farming [27].

Although several research has looked into adding *M. oleifera* to ruminant diets, little is known about how the plant extracts of *M. oleifera* affect ruminal fermentation parameters and gas production kinetics in an *in vitro* experiment. Based on these findings, it was postulated that the leaves of *M. oleifera* may have additively reduced ruminal CH_4 generation and associative effects on ruminal fermentation and feed efficiency. Thus, the purpose of this experiment was to assess the impact of two types of extracts (methanolic or aqueous) processed or not as nanoencapsulation at varying concentrations of *M. oleifera* leaf on the fermentation profile, nutrient degradability, and *in vitro* ruminal generation of gasses such as CH_4, CO, and H_2S.

2. Materials and methods

2.1. Preparation of the aqueous extract

Leaves of *M. oleifera* (young and adults) were collected from different parts of more than 10 trees in Mexico in the period from November to December 2023. To prepare the aqueous extract, dried *M. oleifera* leaves were ground using a glass electric blender to achieve an average particle size of one mm. Then, 1 g of ground leaves was placed and submerged in 8 mL of distilled water. The ground leaves were placed in an individual closed bottle and placed in room temperature water for 72 h. After 72 h, it was filtered through Whatman 4 filter paper under vacuum, and the filtered extract was collected and subsequently stored at 4 °C, as described by Syeda and Riazunnisa [28].

2.2. Preparation of the methanolic extract

To obtain the methanolic extract of *M. oleifera*, the leaves were ground in a glass electric blender. Next, 125 g of the obtained powder was weighed and submerged in 100 mL of methanol (1:9), and this was made up to 1 L in a sealed container at ambient temperature (72 h) and then filtered through filter paper (Whatman 4, 20–25 μm pore size) under vacuum and stored at 4 °C temperature [29].

2.3. Nanoencapsulation of Moringa oleifera extract

The nanoencapsulation process of the *M. oleifera* extract was developed in two separate stages that were subsequently joined. In the first step of the procedure, 50 mL of a 1 % acetic acid aqueous solution was made, and 0.5 g of Pluronic F127® (a non-ionic surfactant, Sigma-Aldrich®, Toluca, Mexico) was progressively dissolved in it. After the Pluronic F127® was completely dissolved, 0.3 g of chitosan (Sigma-Aldrich®, Toluca, Mexico) was added, which would act as the encapsulating polymer. In the procedure's second phase, 0.1 g of sodium tripolyphosphate (STPP, CAS no. 7758-29-4, Sigma-Aldrich®, Toluca, Mexico) was added to the remaining 50 mL of the 1 % acetic acid solution prepared earlier, and 0.18 mg of *M. oleifera* liquid extract was added. Subsequently, the second stage was incorporated into the first stage under continuous magnetic stirring (600 rpm) till complete mixing. After 72 h, macroscopic observations were made after the formation of the nanoparticles to evaluate possible changes in the phases of the combination. For the development of empty chitosan nanocapsules, the method described by Ribeiro et al. [30] was used, but no *M. oleifera* extract was added in the second stage.

2.4. Emulsion characterization by particle size and polydispersity index

A Malvern laser particle size analyzer (Zetasizer Ver. 7.11, UK) was used to characterize the Chitosan + *M. oleifera* nanoencapsulation at 25 °C to determine the appropriate indices for the assessment of the determination of particle size and polydispersity index (PDI), which characterizes herterogeneity.

2.5. Gas chromatography-mass spectrometry (GC-MS) analysis; [31,32]

The chemical composition of *M. oleifera* extracts (methanolic and aqueous extracts) was performed using a Trace GC Ultra-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m × 0.25 mm × 0.25 μm film thickness). The same parameters as described by El-Fiki and Adly [33] were used. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral databases [28,34].

2.6. Chemical analysis of the diet

A proximate analysis of the diet was carried out following the procedures described in AOAC [35]. Using an ANKOM200 Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY) and according to the AOAC [36] methodological requirements, the fiber fraction was ascertained. The percentages of acid detergent fiber (ADF) and neutral detergent fiber (NDF) were calculated following Van Soest et al. [37].

2.7. Ruminal in vitro incubation

Incubation involves using *in vitro* measurement techniques to study

the effects of various additives and/or extracts on a sample of rumen fluid. The goal was to investigate the impact of gas formation on animal energy expenditure and emissions of CH_4, CO, and H_2S over a specified incubation period.

The experiments were carried out in amber glass vials (120 mL), containing 1.0 g of substrate each (high concentrate Table 1), varying doses (0 (negative control), 0.25, 0.5, and 1.0 mL) of methanolic and aqueous extracts, in crude and nonencapsulated forms, of *M. oleifera* extract, with nutrient solution, and rumen fluid (50 mL in a ratio of 4:1). The same doses of nano-chitosan were used as a positive control during the incubations (see Table 2).

A total of 225 bottles (triplicate samples, with 6 different *M. oleifera* extract types (negative control (without extract), positive control (chitosan), methanolic extract, nano-methanolic extract, aqueous extract, nano-aqueous extract) of 4 extract doses (0-, 0.25-, 0.5- and 1.0- mL) in 3 samplings in weeks, with 3 bottles as blanks (*i.e.,* solely rumen fluid) each run (week), were incubated for 48 h. After each bottle was filled, it was shaken, sealed, and put in the incubator (39 °C). Methane, carbon monoxide, hydrogen sulfide, and total gas production volumes were measured at 7 time points (2, 4, 6, 24, 28, 30, and 48 h after inoculation).

The rumen fluid had been obtained as a mix from four male bulls weighing 400 ± 25 kg live weight, and the nutritional solution was made using the technique outlined by Goering and Van Soest [37]. These animals were slaughtered at the municipal slaughterhouse in Toluca, State of Mexico, Mexico, following the Mexican Official Standard NOM-033-SAG/ZOO-2014, which outlines methods for the humane killing of domestic and wild animals. Before slaughter, the bulls were fed hay and commercial concentrate (Purina®, Toluca, State of Mexico, Mexico) in a 50:50 ratio and provided with constant access to water. The rumen contents from each animal were separately transferred to an airtight thermos and then filtered through four layers of gauze to obtain the rumen fluid, as described by Xue et al. [38], removing coarse particles while allowing larger microorganisms such as rumen protozoa to pass. The final mixture was created by combining the filtered rumen fluid.

2.8. Ruminal total gas, CH_4, CO, and H_2S productions

The treatments were put into vials and left in a water bath for 48 h, keeping the temperature constant at 39°C. Using the method outlined by Theodorou et al. [37], the total gas production (measured in psi) was measured. Simultaneously, CH_4, CO, and H_2S were determined (Dräger Safety X-am 20,500 MONITOR, Lübeck, Germany). Each treatment was subjected to incubation in triplicate in each run of incubation to ensure the accuracy of results. In addition, three blank (no substrate) negative controls per inoculum as well as the chitosan (same doses of extracts used) as a positive control, were included to allow for proper correction of the readings and to minimize any external interference in the data obtained.

Table 1
Feed ingredients and nutritive values of diet used as substrate.

Ingredients	%
Alfalfa hay	9.1
Wheat grains	25.0
Corn grains	25.0
Bran	13.9
Corn gluten	12.9
Soybean meal	2.0
Molasses	12.0
Vitamins/Minerals	0.1
Composition	
Crude protein (%)	14.66
Ether extract (%)	18.03
Acid detergent fiber	9.46
Neutral detergent fiber	24.51
Free nitrogen extract	66.41
Ca (g/kg)	1.58
P (g/kg)	3.75
Mg (g/kg)	1.76
Na (g/kg)	0.61
K (g/kg)	9.47
Cl (g/kg)	0.70
Zn (g/kg)	22.83
Cu (g/kg)	8.19
Fe (g/kg)	123.26

2.9. Ruminal pH and dry matter degradability

Following the fermentation process, the liquid portion of the diet was separated from the non-degraded portion by filtering the contents of the vials using filter bags (Filter bags F57, ANKOM Technology Corp., Macedonia, NY, USA) with a porosity of 25 μm. The filtrate was collected in beakers and used to measure the pH with a potentiometer (HI11102, Hanna® Instruments, Woonsocket, RI, USA). The bags with the non-degraded diet were washed and dried (60 °C, 48 h) to obtain the dry weight value. The dry matter degradability was obtained with the dry weight value.

2.10. Calculations and statistical analysis

The production volumes (mL/g dry matter (DM) incubated) of total biogas, CH_4, CO, and H_2S were used to estimate the maximum production, production rate, and lag phase time of each gas using the NLIN procedure of the Statistical Analysis System [38]. Metabolizable energy (ME; MJ/kg DM) was estimated using the equation proposed by Menke et al. [39]. Additionally, the CH_4 conversion efficiency was evaluated through the production of CH_4 per unit of short chain fatty acids (CH_4: SCFA), ME (CH_4: ME), and organic matter (CH_4: MO) in mmol/mmol, g/MJ, and mL/g, respectively.

The experimental design was completely randomized with a factorial arrangement (6 × 4), where factor 1 was the types of extracts used (negative control (without extract), positive control (chitosan), methanolic extract, nano-methanolic extract, aqueous extract, nano-aqueous extract), and factor 2 was the doses of each type of extract (0-, 0.25-, 0.5- and 1.0- mL extract/g DM), with three repetitions for each. The results for each treatment were determined by averaging the data from three repetitions in each treatment. Data analysis was performed using the statistical model mentioned below and SAS's GLM procedure:

$$Y_{jk} = \mu + TE_j + EX_k + (TE \times EX)_{jk} + \varepsilon_{jk}$$

Where, Y_{ijk} is the response variable, μ is the general mean TE_j is the effect of the type of extract, EX_k is the effect of extract doses, $(TE \times EX)_{jk}$ is the effect of the interaction between the type of extract and the extract doses, and ε_{ijk} is the experimental error. The comparison of means was performed using Tukey's test and were considered significantly different when $p \leq 0.05$. The contrast effect between nano extract and crude was also calculated.

3. Results

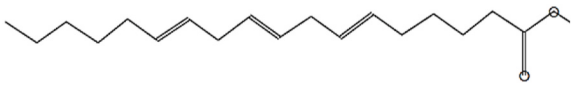
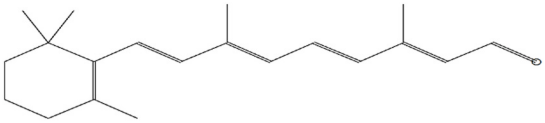
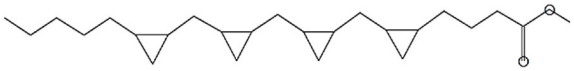
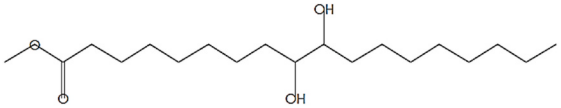
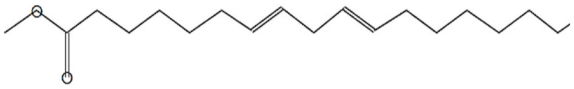
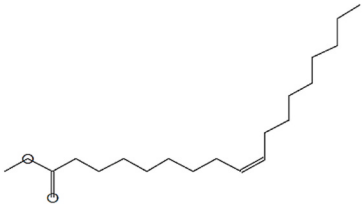
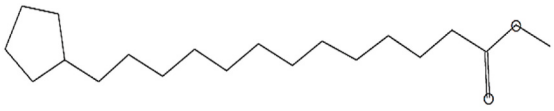
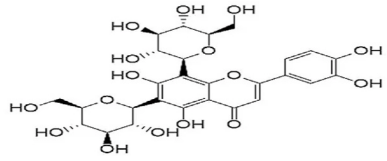
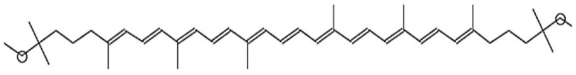
3.1. Particle size and PDI of chitosan nanoparticles

Mean diameter and polydispersity index (PDI) of synthesized chitosan nanoparticles were observed as 244.8 nm and 0.212, respectively.

3.2. GC-MS chemical compounds of the aqueous and methanolic extracts

Gas chromatography-mass spectrometry (GC-MS) analysis of the aqueous extract and methanolic extract of *M. oleifera* leaves are shown in Figs. 1 and 2, respectively. The main compounds were oleic acid methyl ester (62.10 %), cyclopentanetridecanoic acid methyl ester (11.87 %),

Table 2
Relative amounts of the chemical compounds in the aqueous extract of *Moringa oleifera* leaves as per GC-MS analysis.

RT[a]	Compound name	Area (%)	Molecular Formula	Molecular Weight	Chemical structure
6.46	6,9,12-Octadecatrienoic acid methyl ester	2.55	C19H32O2	292	
8.89	Retinal (Vitamin A aldehyde)	2.15	C20H28O	284	
27.76	Cyclopropanebutanoic acid 2-methyl ester	6.59	C25H42O2	374	
27.83	Methyl 9,10-Dihydroxystearate	2.22	C19H38O4	330	
30.67	7,10-Octadecadienoic acid methyl ester	8.98	C19H34O2	294	
30.84	Oleic acid methyl ester	62.10	C19H36O2	296	
31.38	Cyclopentanetridecanoic acid methyl ester	11.87	C19H36O2	296	
36.23	Luteolin 6,8-di-C-glucoside (Lucenin II)	2.66	C27H30O16	610	
36.71	Dimethoxylycopene	0.89	C42H64O2	600	

[a] RT: Retention time (min).

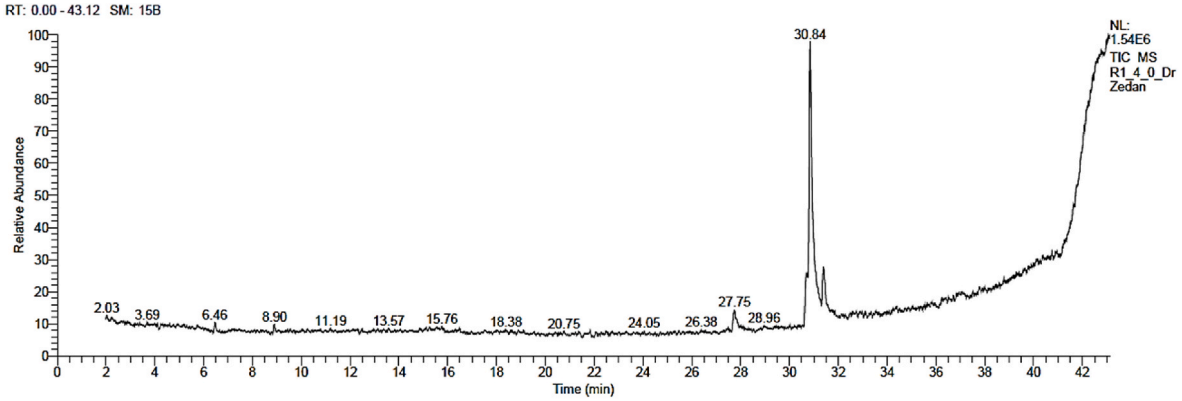


Fig. 1. GC-MS analysis of the aqueous extract of *Moringa oleifera* leaves: Retention times.

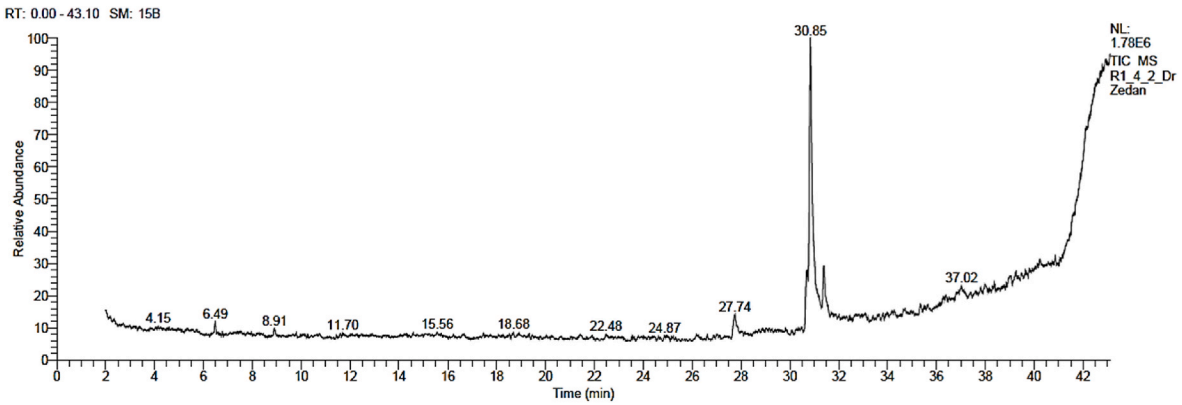


Fig. 2. GC-MS analysis of the methanolic extract of *Moringa oleifera* leaves: Retention times.

7,10-octadecadienoic acid methyl ester (8.98 %), and cyclopropanebutanoic acid (6.59 %). Table 3 presents the chemical compounds in the methanolic extract of *M. oleifera* leaves, where the main compounds were oleic acid methyl ester (64.52 %), methyl isostearate (10.07 %), ethyl (9z, 12z) −9,12-octadecadienoate (7.81 %), and cyclopropanebutanoicacid (5.82 %) –.

3.3. Ruminal biogases production

Total gas production was significantly affected by the watery substrate and three different levels of the *M. oleifera* extract (Table 4).

In Table 5, the kinetics of CH_4 generation differed considerably within treatments, particularly with regard to fractions b ($P = 0.001$, SEM = 0.32) and latency phase ($P = 0.037$, SEM = 0.13). The highest rates recorded were 9.71 mL/g dry matter (DM) for fraction b and 1.92 h for fermentation delay, for methanolic and nano-aqueous extracts respectively. The amount of methane produced during the 48-h incubation period varied depending on the sample's exposure time to different extracts. It is evident that treatments administered during the entire 48-h phase of incubation profoundly affected the production of methane ($P = 0.0002$, SEM = 0.31). The ratio of milliliters of CH_4 accumulated per 100 mL of gas after 48 h showed a similar trend (Table 6).

Ruminal CO kinetics of production (b fraction) indicate an interaction between the crude and nano extractions ($P < 0.0001$, SEM = 0.014); the aqueous and nano-methanolic extracts had the maximum and minimum reported amounts, respectively. For all incubation intervals (4, 24, and 48 h), there are significant variations between all of the treatments in the case of CO production ($P < 0.0001$). In conclusion, it is unambiguous that applying all types of extracts enhanced the volume of gas produced as compared to the control, revealing the effect of extract efficiency on ruminal microorganism performance (Table 7).

Table 8 shows that no significant interactions were found between the various experimental treatments and control (CON) on the observed H_2S indices. However, fraction c ($P = 0.003$, SEM = 0.00003) and lag time ($P = 0.008$, SEM = 0.0026) notably differed depending on the type of extract. Similarly, during the incubation phase (24 h; $P = 0.021$ (SEM = 0.0044), and 48 h; $P = 0.045$ (SEM = 0.0167)), the types of extraction had an impact on the synthesis of H_2S.

3.4. Rumen fermentation parameters

Table 9 shows the influence of various types of extract on rumen parameters, including pH ($P < 0.0001$, SEM = 0.046), short-chain fatty acids (SCFA; $P = 0.0009$, SEM = 0.277), and Metabolizable energy (ME: $P = 0.0009$, SEM = 0.142), which was significant, except for the fermentation of dry matter degradability (DMD; $P = 0.902$, SEM = 11.19). For both methanolic and aqueous extracts, a linearly rising trend was observed once the injection rate and ME were elevated. As is obvious, Table 6 demonstrates that there was no discernible variation in the CH_4 conversion efficiency between treatments. The ratio of CH_4: SCFA had the highest efficiency value ($P = 0.277$, SEM = 0.39), followed by CH_4: OM ($P = 0.118$, SEM = 0.157) and CH_4: ME ($P = 0.236$, SEM = 0.068).

4. Discussions

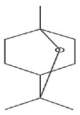
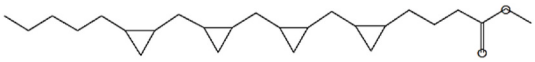
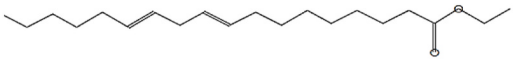
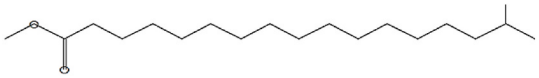
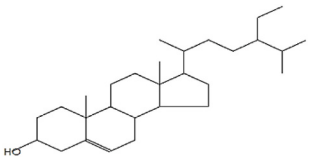
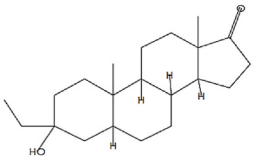
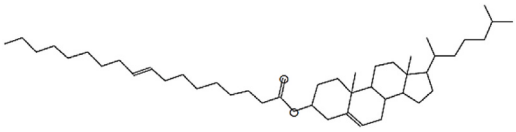
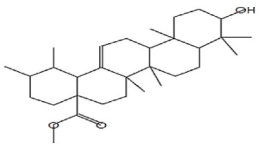
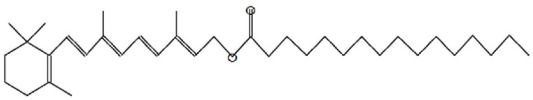
4.1. Total gas production

Diminished CO_2 and CH_4 production, as well as a slower rate of CH_4 production when using diets containing *M. oleifera*, are favorable from an environmental point of view. Additionally, diets containing *M. oleifera* were found to have a higher correlation with increased lag time in CH_4 and CO_2 production. These effects could potentially be caused by variations in the chemical structure of the treatments [39] and secondary metabolites (including tannins and phenolics) in *M. oleifera* [40]. In their investigations, Singla et al. [41] demonstrated how the microbial activity and availability of nutrients in the rumen were modulated through the chemical composition of the incubated substrates, which then affected the *in vitro* formation of CO_2 and CH_4.

Gas production is mitigated by secondary metabolites' protozoal and antibacterial properties [11]. Moreover, Goel and Makkar [39] noticed that secondary metabolites influence ruminal cellulolytic bacteria and decrease the creation of gases necessary for methanogenesis, such as CO_2 and H_2. In this regard, according to Bodas et al. [40], plants' secondary metabolites suppress the amounts of H_2 that are accessible for methanogenesis and inhibit the ruminal CH_4-producing bacteria. Moreover, Goel and Makkar [39] found that the administration of tannins and phenolic compounds resulted in a 50 % decrease in CH_4 production.

It was noticed a rise in biogas production specifically when using the watery substrate and three different levels of the *M. oleifera* extract. These findings indicate that the *M. oleifera* leaf extract had the most significant effect at lower concentrations of fibrous carbohydrates; which may be indicated by the notable interactions between the substrate type and the level of *M. Oleifera* extract. The results align with previous research, indicating that incorporating plant extracts with high plant secondary metabolites enhances the breakdown of substrates in the rumen, resulting in greater gas generation [42]. However, different parts of *M. oleifera* were reported to have an important content of minerals, protein, vitamins, β-carotene, amino acids, various phenolics, zeatin, quercetin, β-sitosterol (45.58 %), stigmasterol (23.10 %), caffeoylquinic acid, kaempferol, high levels of unsaturated fatty acids (oleic up to 71.60 %), saturated acids (palmitic and behenic up to 6.4 %), and campesterol [43,44], which could improve the activities of ruminal microorganisms and increase autumnal gas production with their low

Table 3
Relative amounts of the chemical compounds in the methanolic extract of *Moringa oleifera* leaves as per GC-MS analysis.

RT[a]	Compound name	Area (%)	Molecular Formula	Molecular Weight	Chemical structure
6.48	Eucalyptol	2.00	C10H18O	154	
27.74	Cyclopropanebutanoic acid, 2-methyl ester	5.82	C25H42O2	374	
30.67	Ethyl (9z,12z)-9,12-octadecadienoate	7.81	C20H36O2	308	
30.85	Oleic acid methyl ester	64.52	C19H36O2	296	
31.40	Methyl isostearate	10.07	C19H38O2	298	
36.38	Stigmast-5-en-3-ol	2.20	C29H50O	414	
36.96	3-Ethyl-3-hydroxy-5α-androstan-17-one	1.23	C21H34O2	318	
37.02	3beta-hydroxy-5-cholestene 3-oleate	2.77	C45H78O2	650	
37.81	Urs-12-en-28-oic acid, 3á-hydroxy-, methyl ester	1.99	C31H50O3	470	
37.99	Vitamin A palmitate (Retinol, hexadecanoate)	1.59	C36H60O2	524	

[a] RT: Retention time (min).

concentration detected in the *M. oleifera* leaves [42,45].

Moreover, Morgavi et al. [46] speculated that the higher gas production obtained from adding moringa extracts might be explained by the high levels of secondary metabolites, which could potentially assist fibrolytic microbes in the rumen by providing substrates and microbes closer together, finally resulting in an accelerated the fermentation process of the substrates' following degradation. Still, it has been hypothesized that the simultaneous stimulatory and inhibitory actions of secondary metabolites on certain rumen microbes might account for their various impacts on producing gases [47]. The crude protein level of a substrate is known to negatively correlate with the amount of gas produced because protein stoichiometrically contributes less to gas production than carbohydrates [48]. In this regard, leaves of *M. oleifera* contain significant amounts of crude protein, but they are primarily insoluble and have low *in vitro* digestibility [47]. Nonetheless, Karásková et al. [49] found that the increased gas production is linked to ruminal microorganisms' increased availability of fermentable substrate, which is reflected in the increased gross energy of feed. When we compared the extracts (1 mL/g dry matter (DM) against 0.25 mL/g DM), similarly observed a dose-dependent rise with substantial nonlinear interactions with *M. oleifera* extract levels.

The availability of nutrients [50] and rapidly fermentable carbohydrates for rumen microorganisms [51] has been shown to increase biogas production. The higher biogas production indicated that *M. oleifera* provides significant amounts of nutrients and fermentable materials for the microbial community present in the *in vitro* model

Table 4
Effect of methanolic and aqueous *extracts* of Moringa oleifera *(in nano and crude forms)* at different doses of each extract (0.0, 0.25, 0.5, and 1.0 mL of extract/g dietary DM) on ruminal total gas production (mL/g DM) of high concentrate diets compared with nanoparticles of chitosan (as positive control) using male bulls as a source of ruminal inoculum.

pe of extract	Extract dose (mL/g DM)	Gas production kinetics[a]						Gras production (mL gas/g DM incubated)					
		b	±SD	c	±SD	Lag	±SD	4 h	±SD	24 h	±SD	Mean	±SD
Without extract	0	436.0	16.40	0.062	0.0066	1.255	0.3214	182.1	12.25	201.2	13.47	410.3	15.55
Nano-chitosan	0.25	463.4	27.05	0.055	0.0061	1.597	0.2279	195.0	5.84	213.7	3.35	446.1	5.11
	0.5	437.0	17.85	0.221	0.2731	1.110	0.1862	188.4	7.53	207.5	6.83	435.8	17.24
	1	447.5	13.88	0.231	0.2898	1.726	0.0959	191.8	1.84	211.9	1.32	444.4	11.49
Methanolic extract	0.25	507.4	22.10	0.071	0.0093	1.188	0.2028	216.6	20.88	238.3	21.65	504.2	25.78
	0.5	490.7	13.54	0.065	0.0031	1.142	0.3580	217.7	3.55	238.6	4.15	489.9	13.42
	1	490.6	3.48	0.070	0.0081	1.142	0.2349	220.1	3.19	240.9	3.72	490.4	3.51
Nano-methanolic extract	0.25	467.9	19.01	0.056	0.0044	1.398	0.2467	204.6	8.42	224.6	7.73	445.8	12.36
	0.5	455.8	14.08	0.059	0.0015	1.314	0.2052	205.5	7.62	225.0	8.35	448.5	11.81
	1	448.3	16.81	0.058	0.0075	1.505	0.2319	188.6	12.32	207.6	12.42	438.4	11.23
Aqueous extract	0.25	435.5	22.93	0.056	0.0064	1.728	0.3355	201.7	5.63	219.7	4.51	435.0	22.71
	0.5	451.2	17.23	0.060	0.0079	1.275	0.4154	202.4	11.39	221.9	12.34	450.2	17.20
	1	453.7	5.69	0.058	0.0090	1.311	0.3356	207.2	5.28	226.6	6.23	452.5	6.74
Nano-aqueous extract	0.25	439.0	7.02	0.053	0.0015	1.324	0.0701	196.9	6.45	213.0	9.04	437.8	7.73
	0.5	454.2	28.00	0.068	0.0091	1.200	0.1836	197.8	17.08	225.0	13.02	452.2	26.40
	1	431.7	62.68	0.069	0.0017	1.449	0.5249	171.3	37.34	189.1	44.26	441.0	48.33
SEM pooled [b]		11.118		0.0233		0.1508		6.019		6.228		9.271	
P value:													
Type of extract (TE)		0.0001		0.1053		0.1548		0.0005		0.0009		<0.0001	
Extract dose (ED)		0.6217		0.4958		0.0518		0.2857		0.2706		0.9571	
TE × ED		0.6832		0.7982		0.4621		0.4029		0.3027		0.8178	
Nano vs. Crude		0.0299		0.275		0.5095		0.0034		0.0072		0.0042	

[a] b = Asymptotic total gas production (mL/g DM); c = Rate of total gas production (mL/h); Lag = The initial delay before total gas production begins (h).
[b] SEM = Standard error of the mean; ±SD = Standard deviation.

Table 5
Effect of methanolic and aqueous *extracts* of Moringa oleifera *(in nano and crude forms)* at different doses of each extract (0.0, 0.25, 0.5, and 1.0 mL of extract/g dietary DM) on ruminal methane kinetics and production (CH_4, mL/g DM) of high concentrate diets compared with nanoparticles of chitosan (as positive control) using male bulls as a source of ruminal inoculum.

Type of extract	Extract dose (mL/g DM)	CH_4 production kinetics[a]						CH_4 production (mL gas/g DM incubated)					
		b	±SD	c	±SD	Lag	±SD	4 h	±SD	24 h	±SD	48 h	±SD
Without extract	0	7.32	0.745	0.0088	0.00033	1.8157	0.05013	1.28	0.224	1.81	0.273	7.28	0.741
Nano-chitosan	0.25	8.93	0.160	0.0087	0.00013	1.8456	0.01416	1.43	0.133	2.07	0.104	8.88	0.158
	0.5	8.72	0.413	0.0088	0.00004	1.8475	0.00909	1.38	0.053	2.00	0.102	8.68	0.411
	1	8.75	0.447	0.0089	0.00003	1.8565	0.00555	1.34	0.013	1.98	0.118	8.71	0.445
Methanolic extract	0.25	9.71	0.550	0.0091	0.00012	1.8355	0.02727	1.58	0.038	2.29	0.076	9.67	0.549
	0.5	9.48	0.259	0.0091	0.00002	1.8510	0.00616	1.52	0.025	2.15	0.037	9.43	0.258
	1	9.31	0.281	0.0069	0.00405	1.5782	0.48318	1.39	0.119	2.01	0.148	8.55	1.216
Nano-methanolic extract	0.25	8.38	1.033	0.0091	0.00083	1.8400	0.06774	1.37	0.437	1.95	0.471	8.34	1.023
	0.5	9.68	1.178	0.0066	0.00368	1.4572	0.64258	1.43	0.313	2.39	0.395	9.04	0.164
	1	9.19	0.684	0.0068	0.00359	1.5299	0.56877	1.50	0.159	2.14	0.294	8.69	0.131
Aqueous extract	0.25	8.55	0.279	0.0046	0.00397	1.2037	0.59466	1.34	0.126	1.90	0.136	7.39	0.741
	0.5	8.23	0.434	0.0080	0.00012	1.7923	0.02562	1.35	0.158	2.15	0.211	8.18	0.430
	1	7.76	0.749	0.0062	0.00302	1.4908	0.49578	1.24	0.183	2.11	0.525	7.39	0.809
Nano-aqueous extract	0.25	8.02	0.303	0.0092	0.00026	2.1842	0.51829	1.31	0.091	1.70	0.157	7.98	0.299
	0.5	8.61	0.742	0.0090	0.00012	1.8521	0.01508	1.32	0.219	1.95	0.235	8.56	0.738
	1	9.29	0.513	0.0093	0.00064	1.9201	0.10779	1.21	0.364	1.80	0.597	9.25	0.502
SEM pooled [b]		0.317		0.00076		0.13121		0.096		0.140		0.311	
P value:													
Type of extract (TE)		0.0013		0.0448		0.0375		0.1767		0.118		0.0002	
Extract dose (ED)		0.6064		0.6673		0.6669		0.5997		0.3471		0.329	
TE × ED		0.0882		0.4581		0.4054		0.9632		0.7395		0.1052	
Nano vs. Crude		0.9404		0.2411		0.2179		0.5179		0.3152		0.4881	

[a] b = Asymptotic CH_4 production (mL/g DM); c = Rate of CH_4 production (mL/h); Lag = The initial delay before CH_4 production begins (h).
[b] SEM = Standard error of the mean; ±SD = Standard deviation.

system. The inclusion of secondary phenolic compounds in *M. oleifera* extracts may offer potent anti-free radical and anti-lipid peroxidation effects. Because *M. oleifera* contains phytochemicals, there may be a better capacity for substrate breakdown, which results in higher gas generation at high extract levels [51].

According to several studies, the energy content of a food is linked to the amount of gas released during *in vitro* incubation [48]. In addition, it is readily apparent that a portion of the substrate, containing soluble sugars, undergoes fermentation early in the fermentation process. Despite typically constituting a small proportion of potentially digestible materials, these substances ferment instantly [52]. Subsequently, an increase in gas production occurs as a result of the establishment of cellulolytic organisms and finally breaking down the fiber particles of the diet in the rumen [39]. More specifically, studies examining the gas generation of *M. oleifera* have revealed that the highest gas production occurs during the final phases of fermentation [40,53].

Theodorou et al. [54] and Mtui et al. [47] assessed *M. oleifera* and other woody forage species and discovered that during the preliminary

Table 6
Effect of methanolic and aqueous *extracts* of Moringa oleifera *(in nano and crude forms)* at different doses of each extract (0.0, 0.25, 0.5, and 1.0 mL of extract/g dietary DM) on ruminal methane production (ml CH_4/100 mL gas) of high concentrate diets compared with nanoparticles of chitosan (as positive control) using male bulls as a source of ruminal inoculum.

Type of extract	Extract dose (mL/g DM)	CH_4 (ml gas/100 mL gas)					
		4 h	±SD	24 h	±SD	48 h	±SD
Without extract	0	0.700	0.1000	0.900	0.1000	1.775	0.1639
Nano-chitosan	0.25	0.733	0.0577	0.967	0.0577	1.992	0.0577
	0.5	0.733	0.0577	0.967	0.0577	1.992	0.0577
	1	0.700	0.0000	0.933	0.0577	1.958	0.0577
Methanolic extract	0.25	0.733	0.0577	0.967	0.1155	1.917	0.0144
	0.5	0.700	0.0000	0.900	0.0000	1.925	0.0000
	1	0.633	0.0577	0.833	0.0577	1.742	0.2363
Nano-methanolic extract	0.25	0.667	0.2082	0.867	0.2082	1.875	0.2634
	0.5	0.700	0.1732	1.067	0.2082	2.017	0.0878
	1	0.800	0.1000	1.033	0.1528	1.983	0.0520
Aqueous extract	0.25	0.667	0.0577	0.867	0.0577	1.700	0.1561
	0.5	0.667	0.0577	0.967	0.0577	1.817	0.0577
	1	0.600	0.1000	0.933	0.2517	1.633	0.1665
Nano-aqueous extract	0.25	0.667	0.0577	0.800	0.1000	1.825	0.1000
	0.5	0.667	0.0577	0.867	0.0577	1.892	0.0577
	1	0.700	0.1000	0.933	0.1155	2.10833	0.1443
SEM pooled [a]		0.0449		0.0598		0.0605	
P value:							
Type of extract (TE)		0.3713		0.2776		0.0007	
Extract dose (ED)		0.9749		0.4086		0.3626	
TE × ED		0.5916		0.5127		0.0956	
Nano vs. Crude		0.3099		0.7207		0.0039	

[a] SEM = Standard error of the mean; ±SD = Standard deviation.

16 h of fermentation, *M. oleifera* and *Morus alba* had the highest values of gas accumulation, reaching 108.6 and 111.5 mL/g, respectively. In the final phase, 96 h, their production was also greater compared to the other species (162.4 and 197.6, respectively).

4.2. CH_4 production

The amount of CH_4 generated varied with the addition of different levels of extracts. It peaked at 9.43 mL/g DM after 48 h of incubation but started at 1.21 mL/g DM after 4 h (Table 5). In disagreement with the current trial, Zeru et al. [55] found that all extracts from the *M. oleifera* plant decreased the production of CH_4. This finding is consistent with multiple studies that have demonstrated the potency of *M. oleifera* in reducing enteric CH_4 from ruminants [19]. This may be because *M. oleifera* contains tannins and saponins, which are known as the plant's secondary chemicals. These chemicals limit methanogen activity and reduce ruminal methane generation, although this was not observed in their trial [19]. It is evident that the reaction of fermentation patterns depends on the harmony between different compounds in each extract [56,57].

In the presence of *M. oleifera* extract, asymptotic CH_4 production was increased with nano as compared to crude extracts, illustrating again significant interactions between substrate type and *M. oleifera* concentration. The secondary metabolites found in these extracts were thought to be responsible for the observed effects, and have previously been shown to inhibit the rumen ability to produce hydrogen and methane [19]. Additionally, considerations have been given to using tannins and phenolics as an alternative to effective methanogen inhibitors, such as chemical, biological, and natural animal feed, for the rumen fermentation pathways in animal guts. This is because they seem to have antimicrobial effects, which could be a major cause of methane reduction [58]. Nevertheless, it is impossible to know how comparable the accessions were in their study to the previously described investigations, even if the latter claim is consistent with our current findings Apart from the secondary metabolites effects, various mechanisms have been proposed to affect the rate of methane production, including: (a) decreased digestion of fiber [59], (b) suppression of methanogens [60], and (c) reduced digestion of protein [41].

Detected chemical metabolites compounds, in both *M. oleifera* leaf extracts, are very comparable in ruminal CH_4 production, in the present study. However, the hydroalcoholic extract showed the presence of heneicosane (35.69 %), 1,2-benzenedicarboxylic acid (22.89 %), heptacosane (18.26 %), pentatriacontane (4.77 %), and hexadecanoic acid ethyl ester (3 %) as predominant compounds in the leaves extract [61], which may have a high ability to reduce the CH_4 emission by ruminal microorganisms.

The inhibition of produced CH_4 took place with a ratio of dosages of *M. oleifera* leaf extracts to distilled water at 4.5 % to 100, 5.2 % to 75, 28.7 % to 50, and 29.3 % to 25 mg/L, respectively. These results were consistent with the antimethanogenic potential of *M. oleifera* reported by Zeru et al. [55], which ranged from 18 % to 29 %. Nonetheless, the disparity in extract dose levels may account for the lower CH_4 inhibition potentials of 4.5 % and 5.2 % in the aforementioned earlier investigation.

Numerous researches have indicated that the application dosages of the metabolites and their thresholds of lowest and maximum activities have a significant impact on the bioactivities of plant extracts [10,62]. Thus, biological differences across Moringa species, varieties, and accessions, in addition to variations in substrate types, application methods, and inclusion levels, are important factors contributing to the observed CH_4 inhibition in various investigations. Besides, the various antibacterial activities identified among the aforementioned parameters, as well as the direct impacts of ecotypes, cultivars, individual plants, and plant sections of Moringa on antimethanogenic potential and digestibility, were not indicated by prior studies [17,19]. Furthermore, as mentioned earlier, the secondary metabolites and antioxidant properties of *M. oleifera* can enhance the proliferation and function of ruminal fibrolytic microbes [63], leading to an accelerated rate and extent of substrate breakdown [39].

The inhibition of methanogenic activity by secondary metabolites from *M. oleifera* may be the primary cause of the reduction in CH_4 production with *M. oleifera*, rather than the decrease in DM digestibility [45]. At 25 and 50 mg/L in distilled water, Akanmu and Hassen [7] discovered that the secondary metabolites in *M. oleifera* extract decreased the *in vitro* production of CH_4. Moreover, the phenolic compounds in *M. oleifera* leaves, due to their antiprotozoal properties, have

Table 7
Effect of methanolic and aqueous extracts of Moringa oleifera (in nano and crude forms) at different doses of each extract (0.0, 0.25, 0.5, and 1.0 mL of extract/g dietary DM) on ruminal carbon monoxide (CO, mL/g DM) of high concentrate diets compared with nanoparticles of chitosan (as positive control) using male bulls as a source of ruminal inoculum.

Type of extract	Extract dose (mL/g DM)	CO production kinetics[a]						CO production (mL/g DM incubated)					
		b	±SD	c	±SD	Lag	±SD	4 h	±SD	24 h	±SD	48 h	±SD
Without extract	0	0.0685	0.0063	0.0010	0.000078	0.2162	0.00650	0.0042	0.00059	0.0091	0.00170	0.0341	0.00316
Nano-chitosan	0.25	0.1970	0.0217	0.0007	0.000391	0.1732	0.07024	0.0148	0.00237	0.0278	0.00198	0.0946	0.01065
	0.5	0.2010	0.0262	0.0010	0.000050	0.2128	0.00434	0.0166	0.00268	0.0287	0.00183	0.1000	0.01306
	1	0.2168	0.0304	0.0005	0.000442	0.1322	0.07460	0.0179	0.00217	0.0285	0.00300	0.1005	0.02134
Methanolic extract	0.25	0.2161	0.0131	0.0010	0.000021	0.2103	0.00129	0.0187	0.00155	0.0329	0.00135	0.1075	0.00650
	0.5	0.2142	0.0380	0.0010	0.000012	0.2141	0.00116	0.0157	0.00276	0.0304	0.00491	0.1067	0.01893
	1	0.2304	0.0389	0.0007	0.000399	0.1608	0.08031	0.0203	0.00304	0.0372	0.00785	0.1113	0.02496
Nano-methanolic extract	0.25	0.1244	0.0390	0.0008	0.000486	0.1727	0.08066	0.0100	0.00222	0.0155	0.00390	0.0595	0.02047
	0.5	0.1465	0.0205	0.0008	0.000468	0.1632	0.09032	0.0096	0.00222	0.0207	0.00297	0.0703	0.01478
	1	0.1461	0.0124	0.0008	0.000453	0.1746	0.07872	0.0107	0.00031	0.0188	0.00074	0.0704	0.01006
Aqueous extract	0.25	0.2222	0.0084	0.0010	0.000031	0.2151	0.00080	0.0178	0.00305	0.0309	0.00062	0.1107	0.00414
	0.5	0.2225	0.0267	0.0008	0.000474	0.1518	0.10915	0.0191	0.00081	0.0329	0.00430	0.1057	0.02215
	1	0.2685	0.0276	0.0011	0.000031	0.2184	0.00181	0.0234	0.00312	0.0345	0.00449	0.1339	0.01371
Nano-aqueous extract	0.25	0.1418	0.0143	0.0010	0.000051	0.2128	0.00501	0.0106	0.00014	0.0201	0.00212	0.0706	0.00714
	0.5	0.1594	0.0249	0.0696	0.119345	0.1451	0.06728	0.0134	0.00131	0.0225	0.00169	0.0769	0.01496
	1	0.1670	0.0339	0.0009	0.000046	0.2111	0.00711	0.0135	0.00294	0.0244	0.00514	0.0830	0.01691
SEM pooled [b]		0.013812		0.004436		0.024542		0.00113		0.001756		0.008054	
P value:													
Type of extract (TE)		<0.0001		0.389		0.8116		<0.0001		<0.0001		<0.0001	
Extract dose (ED)		0.0354		0.3567		0.6092		0.0031		0.0608		0.1367	
TE × ED		0.898		0.416		0.405		0.2612		0.5989		0.852	
Nano vs. Crude		<0.0001		0.3287		0.434		<0.0001		<0.0001		<0.0001	

[a] b = Asymptotic CO production (mL/g DM); c = Rate of CO production (mL/h); Lag = The initial delay before CO production begins (h).
[b] SEM = Standard error of the mean; ±SD = Standard deviation.

exhibited potent antibacterial effects on various microbial species, such as *Salmonella typhi*, *Escherichia coli*, and *Staphylococcus aureus* [56]. These phenolic compounds have also been found to affect CH_4-producing archaea in the rumen [29]. According to Ku-Vera et al. [19], phenols cause damage to the rumen archaea membrane and attach to portions of the cell envelope or proteinaceous adhesin, which hinders the formation of the methanogen-protozoa complex, reduces interspecies hydrogen transfer, and inhibits methanogen development. When considered collectively, it is worthwhile to further investigate the impact of various extraction methods of *M. oleifera* on *in vitro* CH_4 production and ruminal fermentation profile, considering the paucity of published research in this area.

4.3. CO and H_2S production

Ruminal CO produced as a byproduct of incomplete feed decomposition, signifying a decrease in rumen microbial activity [64]. It is also regarded as a secondary greenhouse gas, yet little research has been conducted to determine the quantity of CO produced by ruminants [65,66]. Nonetheless, it has been documented that the breakdown of organic matter occurs under anaerobic circumstances and results in the production of CO [67]. Since it has been reported that the ruminal microbiota and the concentration of organic matter influence CO production, the variation in CO between different forages can be explained by the degree and digestibility of organic matter [65,66]. Consequently, it is important to note that the fermentation potential of each inoculum's rumen and microorganisms, as well as microbial activity, are responsible for its production.

Biogas contains H_2S, which if produced in the rumen can be hazardous to animals and can change their metabolism, resulting in disorders [68]. Sulfate-reducing bacteria convert sulfur to H_2S during ruminal feed fermentation [61]. As a result, the difference in H_2S production between ruminal inoculum sources (nano *vs.* crude extracts) is thought to be the consequence of a high population of sulfate-reducing bacteria in the bovine inoculum. Animal gastrointestinal tract maintenance and physiological function depend heavily on H_2S [64], which also helps to lower ruminal CH_4 generation. Rumen microorganisms have been seen to catabolize amino acids with sulfur groups, such as cysteine and methionine, during the breakdown process, resulting in the production of H_2S [58]. According to this theory, the concentration and destruction of the amino acids that make up the *M. oleifera* genotype, particularly those that include sulfur, may be related to the generation of H_2S with the methanolic and aqueous extracts of the genotype [69]. In summary, this is the first experiment that compares the quantity of H_2S generated by applying different extracts that we are aware of, thus it would be worthwhile to thoroughly assess it in future research.

4.4. Rumen fermentation profile and CH_4 conversion efficiency

When it comes to the evaluation of animal feed, the rate of degradation is considered to be a significant component [70]. Various extracts resulted in varying dry matter disappearance of *M. oleifera*, indicating different nutritional values. In the present study, only the nano-aqueous extract had a negative impact on the feed digestion features, while the other *M. oleifera* extracts did not affect parameters such as dry matter digestibility, metabolizable energy, short-chain fatty acids concentrations, and kinetics of fermentation.

Given that none of the *M. oleifera* accessions exhibited a discernible increase in dry matter digestibility or adverse effects, it is plausible that moringa leaf extracts may have stimulatory effects on the microorganisms responsible for feed digestion. The results are consistent with the prior conclusion drawn by Parra-Garcia et al. [10] have shown that the *M. oleifera* extract had a greater effect at lower fibrous carbohydrate levels. Additionally, Kolif et al. [11] have indicated that supplementing the diet with 10-, 20-, and 40- mL of moringa extract can enhance nutrient digestibility. In a study by Kolif et al. [8], it was found that

Table 8
Effect of methanolic and aqueous extracts of Moringa oleifera (in nano and crude forms) at different doses of each extract (0.0, 0.25, 0.5 and 1.0 mL of extract/g dietary DM) on ruminal hydrogen sulfide (H_2S, mL/g DM) of high concentrate diets compared with nanoparticles of chitosan (as positive control) using male bulls as a source of ruminal inoculum.

Type of extract	Extract dose (mL/g DM)	H_2S production kinetics[a]						H_2S production (mL/g DM incubated)					
		b	±SD	c	±SD	Lag	±SD	4 h	±SD	24 h	±SD	48 h	±SD
Without extract	0	0.4664	0.0413	0.00096	0.000017	0.22561	0.000859	0.0307	0.00456	0.0484	0.00493	0.2322	0.02054
Nano-chitosan	0.25	0.4823	0.0771	0.00099	0.000067	0.23013	0.005358	0.0231	0.01121	0.0449	0.01283	0.2403	0.03829
	0.5	0.4912	0.0082	0.00095	0.000079	0.22697	0.004426	0.0321	0.00186	0.0494	0.00544	0.2445	0.00433
	1	0.4761	0.1240	0.00103	0.000127	0.23432	0.012281	0.0216	0.01578	0.0422	0.02170	0.2372	0.06158
Methanolic extract	0.25	0.4930	0.0909	0.00097	0.000021	0.22880	0.001875	0.0307	0.00453	0.0500	0.00512	0.2588	0.02735
	0.5	0.5398	0.0368	0.00095	0.000057	0.22416	0.000760	0.0341	0.00445	0.0582	0.00321	0.2688	0.01843
	1	0.5275	0.0670	0.00095	0.000047	0.22433	0.003052	0.0322	0.00853	0.0566	0.01016	0.2626	0.03331
Nano-methanolic extract	0.25	0.4904	0.0481	0.00095	0.000026	0.22274	0.007132	0.0353	0.00211	0.0549	0.00432	0.2441	0.02398
	0.5	0.4731	0.0278	0.00089	0.000025	0.21752	0.002715	0.0364	0.00191	0.0592	0.00246	0.2354	0.01386
	1	0.4318	0.0480	0.00093	0.000133	0.22483	0.011244	0.0252	0.00729	0.0446	0.00832	0.2149	0.02425
Aqueous extract	0.25	0.4697	0.0305	0.00094	0.000031	0.22595	0.001039	0.0279	0.00279	0.0488	0.00319	0.2339	0.01518
	0.5	0.4329	0.0862	0.00090	0.000098	0.22440	0.003164	0.0243	0.00273	0.0463	0.00481	0.2154	0.04318
	1	0.4430	0.0767	0.00085	0.000053	0.21774	0.007065	0.0342	0.00634	0.0550	0.00804	0.2203	0.03835
Nano-aqueous extract	0.25	0.4710	0.0866	0.00099	0.000012	0.23062	0.001219	0.0287	0.00680	0.0431	0.00859	0.2346	0.04317
	0.5	0.4173	0.0502	0.00102	0.000042	0.22439	0.002091	0.0303	0.00351	0.0439	0.00507	0.2080	0.02497
	1	0.4313	0.0631	0.00104	0.000046	0.23293	0.007914	0.02143	0.00534	0.0375	0.01304	0.2149	0.03133
SEM pooled [b]		0.03477		0.00003		0.00261		0.00324		0.00438		0.01669	
P value:													
Type of extract (TE)		0.1082		0.003		0.0083		0.1339		0.0214		0.0456	
Extract dose (ED)		0.7303		0.5308		0.1259		0.1968		0.4224		0.568	
TE × ED		0.9251		0.6461		0.3801		0.1672		0.531		0.9747	
Nano vs. Crude		0.1416		0.0586		0.543		0.6277		0.0755		0.0895	

[a] b = Asymptotic H_2S production (mL/g DM); c = Rate of H_2S production (mL/h); Lag = The initial delay before H_2S production begins (h).
[b] SEM = Standard error of the mean; ±SD = Standard deviation.

moringa extract positively impacted ruminal digestion. This conclusion is in the same line with earlier studies demonstrating that thyme and moringa extracts increase the *in vitro* digestibility of organic and dry matter [34]. Herbal extracts containing secondary metabolites have been shown to improve ruminal microbes' ability to break down feed components [71]. Dey et al. [62] also noted that the addition of *M. oleifera* leaves to wheat straw resulted in higher levels of total-tract dry matter digestibility and total-tract organic matter digestibility. According to Cohen-Zinder et al. [63], adding *M. oleifera* improves digestibility, maintains exceptional conditions, and enhances feeding value. In this case, Li et al. [64] indicated that feeding dairy Holstein cows with *M. oleifera* may improve rumen fermentation, nutritional digestibility, and nutrient intake. However, previous studies have suggested that adding moringa to the diet could have a negative impact on ruminal fermentation, particularly on cellulolytic bacteria and nutrient digestibility due to the high secondary metabolites content [72], which has been linked to adverse effects on dry matter digestibility [61]. Similarly, in a study conducted by Gunal et al. [66], it was observed that the levels of dry matter digestibility and microbial crude protein decreased when large dosages of rosemary oil (500 mg/L) were used in an *in vitro* batch culture. However, Khorrami et al. [17] found no significant differences in digestibility when thyme and cinnamon extracts (500 mg/kg DM) were added to steers' diets.

Only after using aqueous and nano-aqueous extracts, did the pH parameter increase. The elevated fermentation pH caused by the extracts is a desired outcome, as ruminal pH primarily determines the activity of ruminal bacteria. With the higher fiber content in *M. oleifera* diets, salivation likely increased, which would have consequently lowered the pH of the rumen [43]. Salivation must have increased along with the higher fiber content of *M. oleifera* diets since this would have inevitably lowered the pH of the rumen [43]. Because they limit the growth of methanogens such as *Methanobrevibacter*, phytogenic feed additives can generally reduce the synthesis of CH_4. According to Soliva et al. [61], substituting moringa leaves for soybean or rapeseed meal led to a 17 % decrease in CH_4 generation. Furthermore, Dey et al. [62] observed a reduction *in vitro* CH_4 generation and an increase in total gas production and organic matter degradability by supplementing with *M. oleifera* leaves. The use of moringa leaves instead of soybean or rapeseed meal led to a 17 % reduction in CH_4 generation, as reported by Soliva et al. [61]. Additionally, Dey et al. [62] found that supplementing with *M. oleifera* leaves resulted in a decrease *in vitro* CH_4 generation and an increase in total gas production and organic matter degradability.

The amount of CH_4 produced per unit of short-chain fatty acids (SCFA), metabolizable energy (ME), and organic matter (OM) has decreased, suggesting that the efficiency of CH_4 conversion, which measures the amount of CH_4 produced per unit of rumen fermentation product, may have improved as a result of anaerobic fermentation. Propionate reduces the amount of H_2 available for producing CH_4. This is linked to the SCFA profile, especially the ratio of acetic to butyric acids (73]. The increased activity of fibrolytic bacteria and the production of propionate may explain the observed increases in SCFA and ME with methanolic and aqueous extracts [73]. Meanwhile, the decrease in other SCFA, such as acetate, is believed to be the reason for the overall decrease [37]. In the meanwhile, changes in feed carbohydrate content and degradability may have an impact on dry matter degradability (DMD) and SCFA, as indicated by the computed differences in CH_4 per unit of SCFA, ME, and organic matter [74]. The diversity and quantity of microorganisms in the rumen of each species are related to the differences in fermentation and methane conversion rates between sources of ruminal inoculum [75]. This, in turn, affects the microbial activity and fermentative potential of the rumen microbial community, as well as the fermentation outcomes [76].

5. Conclusions

It seems possible that the administration of the *M. oleifera* extract employed in this investigation could affect rumen fermentation, resulting in a more effective use of food protein and energy. *M. oleifera* supplementation enhanced rumen fermentation parameters, nutritional digestibility, and a commensurate reduction in methane generation. The

Table 9
Effect of methanolic and aqueous extracts of Moringa oleifera *(in nano and crude forms)* at different doses of each extract (0.0, 0.25, 0.5, and 1.0 mL of extract/g dietary DM) on rumen fermentation profile and CH_4 conversion efficiency of high concentrate diets compared with nanoparticles of chitosan (as positive control) using male bulls as a source of ruminal inoculum.

Type of extract	Extract dose (mL/g DM)	Rumen fermentation profile[a]								CH_4 conversion efficiency[b]					
		pH	±SD	DMD, %	±SD	SCFA mmol/g DM	±SD	ME, MJ/kg DM 24 h	±SD	CH4: ME (g/MJ)	±SD	CH4:OM (ml/g)	±SD	CH4: SCFA at 24 h (mmol/mmol)	±SD
Without extract	0	6.32	0.031	58.05	26.253	8.91	0.598	8.78	0.307	0.96	0.121	2.04	0.307	5.89	0.654
Nano-chitosan	0.25	6.32	0.017	42.99	32.129	9.47	0.149	9.07	0.076	1.06	0.058	2.32	0.117	6.32	0.378
	0.5	6.22	0.015	78.35	8.582	9.19	0.303	8.92	0.156	1.04	0.056	2.25	0.115	6.32	0.378
	1	6.20	0.070	61.81	34.413	9.39	0.059	9.02	0.030	1.02	0.062	2.22	0.132	6.10	0.378
Methanolic extract	0.25	6.27	0.042	80.45	6.920	10.56	0.961	9.63	0.494	1.11	0.086	2.57	0.085	6.32	0.756
	0.5	6.20	0.059	66.64	27.870	10.57	0.184	9.63	0.095	1.04	0.008	2.41	0.042	5.88	0.000
	1	6.27	0.090	47.73	40.972	10.68	0.165	9.69	0.085	0.96	0.068	2.26	0.166	5.45	0.377
Nano-methanolic extract	0.25	6.27	0.131	75.44	9.757	9.95	0.343	9.31	0.176	0.97	0.234	2.19	0.529	5.67	1.361
	0.5	6.27	0.040	73.86	4.584	9.97	0.371	9.32	0.190	1.19	0.217	2.69	0.444	6.97	1.362
	1	6.12	0.025	59.88	28.975	9.20	0.551	8.93	0.283	1.12	0.157	2.40	0.330	6.76	0.999
Aqueous extract	0.25	6.33	0.101	61.75	23.713	9.73	0.200	9.20	0.103	0.96	0.065	2.14	0.153	5.67	0.378
	0.5	6.55	0.193	71.71	2.699	9.83	0.548	9.25	0.281	1.08	0.080	2.41	0.237	6.32	0.377
	1	6.51	0.106	75.17	2.186	10.04	0.276	9.36	0.142	1.05	0.273	2.37	0.590	6.10	1.646
Nano-aqueous extract	0.25	6.51	0.215	71.46	19.469	9.44	0.401	9.05	0.206	0.87	0.095	1.91	0.176	5.23	0.654
	0.5	6.54	0.090	57.30	38.266	9.97	0.578	9.32	0.297	0.97	0.088	2.20	0.264	5.67	0.377
	1	6.46	0.05	81.72	2.985	8.37	1.965	8.51	1.009	0.96	0.224	2.02	0.671	6.11	0.752
SEM pooled [c]		0.046		11.191		0.2765		0.1420		0.0684		0.1574		0.3911	
P value:															
Type of extract (TE)		<0.0001		0.9028		0.0009		0.0009		0.2366		0.118		0.2776	
Extract dose (ED)		0.4767		0.8744		0.2706		0.2706		0.3954		0.3471		0.4085	
TE × ED		0.0835		0.3743		0.3027		0.3027		0.6787		0.7395		0.5116	
Nano vs. Crude		0.9163		0.7033		0.0072		0.0072		0.7449		0.3152		0.7181	

[a] pH = ruminal pH; DMD = dry matter degradability; SCFA = short-chain fatty acids; ME = metabolizable energy.
[b] CH_4:SCFA = methane:short-chain fatty acids ratio; CH_4:ME = methane:metabolizable energy ratio; CH_4:OM = methane:organic matter ratio.
[c] SEM = standard error of the mean; ±SD = standard deviation.

findings imply that optimal quantities of *M. oleifera* extract can simultaneously promote sustainable husbandry by lowering methane emissions, improving feed nutritional value, and partially substituting a perennial plant and an agricultural waste product for a staple crop. To obtain the best results without negatively impacting feed degradability, several concentrations of the extracts should be examined; consequently, research on rumen adaptability is necessary.

CRediT authorship contribution statement

Mona M.M.Y. Elghandour: Investigation, Methodology, Supervision. **Deli Nazmín Tirado-González:** Formal analysis, Writing – review & editing. **Paulina Vazquez-Mendoza:** Data curation, Software. **Moisés Cipriano-Salazar:** Data curation, Software. **Ofelia Márquez-Molina:** Data curation, Software. **Valiollah Palangi:** Writing – review & editing. **Ashkan Fekri:** Writing – review & editing. **Maximilian Lackner:** Validation, Writing – review & editing. **Abdelfattah Z.M. Salem:** Investigation, Methodology, Supervision.

Ethics approval

The ruminal contents of cattle were taken from the slaughterhouse of Toluca, Estado de Mexico, Mexico.

Consent for publication

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

Data availability

Data will be made available on request.

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