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ORIGINAL ARTICLE

Effects of long-term supplementation of *Caesalpinia coriaria* fruit extract on ruminal methane, carbon monoxide, and hydrogen sulfide production in sheep

Pedro Enrique Hernández Ruiz[1] · Miguel Mellado[2] · Moyosore Joseph Adegbeye[3] · Abdelfattah Zeidan Mohamed Salem[1] · José Luis Ponce Covarrubias[4] · Mona Mohamed Mohamed Yasseen Elghandour[1] · Oluwatosin Bode Omotoso[3]

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Abstract

This study aimed to evaluate the long-term effect of oral administration of *Caesalpinia coriaria* Jacq (Cascalote) wild fruit extract to lambs on ruminal methane, carbon monoxide, and hydrogen sulfide production and in vitro fermentation profile. Rumen liquor (RL) was collected from lambs administered with 0- (RL0), 30- (RL30), and 60- (RL60) mL of aqueous extract of the *C. coriaria* fruit for 60 days per lamb, as well as during incubation, 0-, 0.6-, 1.2-, and 1.8- mL of *C. coriaria* fruit extract was added to each RL type. There was a dose-dependent increase in gas production with increasing levels of *C. coriaria* extract. The RL30 lambs had the lowest ($P=0.013$) methane output (g CH_4/kg DM) at 24 h, and RL30 with 1.8 mL/g DM had the lowest ($P=0.031$) CH_4 (g CH_4/kg DM) at 48 h. Furthermore, diets fermented with RL0 produced the highest ($P=0.001$) short-chain fatty acid (SCFA, mmol/g DM) and metabolizable energy (ME, MJ/kg DM 24 h), while diets incubated with RL30 produced the lowest. Without plant extract addition, 30 mL oral supplementation of *C. coriaria* fruit extract/day/lamb was optimal for digestion, ME, and SCFA. Therefore, 60 mL/d/lamb containing 1.2–1.8 mL fruit extract/g DM seemed to be a feasible means of decreasing emissions of gas production, ME, SCFA, CO, H_2S, and CH_4.

Keywords Hydrogen sulfide · Secondary metabolites · Methane gas · Ruminants · Greenhouse gas

1 Introduction

Rumen fermentation is important for the digestion and absorption of nutrients ingested by ruminants. During this process, several by-products including hydrogen sulfide (H_2S) and carbon monoxide (CO) are absorbed through the intestinal wall. The quantity of sulfide produced by rumen microbes is influenced by the diet sulfate content [1]. CO and H_2S can also serve as therapeutic purposes and could be alternative means of hydrogen sinking [2, 3].

Meanwhile, increasing greenhouse gas emissions, such as methane (CH_4) continues to be a great concern due to their potential adverse effects on global warming [4]. This gas is produced in both the foregut and hindgut during anaerobic fermentation by methanogens. The reduction of enteric methane production enhances nutrient utilization and productivity and reduces environmental pollution. While there have been efforts to mitigate CH_4 production both in vivo and in vitro [5], additional research on plants with the capacity to reduce methane synthesis in needed. This would ensure that each country could adopt feeding practices to mitigate CH_4 production regardless of the livestock reared.

C. coriaria, a tanniniferous tropical tree, has the potential to mitigate the enteric CH_4 production for optimum ruminant output. Pods of this tree are rich in tannin,

✉ Mona Mohamed Mohamed Yasseen Elghandour
mmohamede@uaemex.mx

[1] Facultad de Medicina Veterinaria Y Zootecnia, Universidad Autónoma del Estado de México, Toluca, Estado de México, México

[2] Department of Animal Nutrition, Autonomous Agrarian University Antonio Narro, Saltillo, Mexico

[3] Department of Animal Production and Health, Federal University of Technology, P.M.B. 704, Akure, Nigeria

[4] Escuela Superior de Medicina Veterinaria Y Zootecnia número 3, Universidad Autónoma de Guerrero, Tecpan de Galeana, Guerrero, México

phenols, and flavonoids. Previous studies have shown that *C. coriaria* extract reduces methane production in vitro [6, 7] apparently by creating alternative sink for H_2 and by competing and metabolizing H_2 for other uses, thereby preventing methanogens from using them for methane production [8], which improves performance and rumen fermentation in goats [9].

Despite all the above benefits, additional research on methane inhibitor additives is needed. Administration of unconventional feed additives to livestock often results in contrasting growth performance, gut manipulation, health, and greenhouse gases production. Thus, this study aims to evaluate the long-term effect of oral administration of *C. coriaria* fruit extract to lambs on reduction of ruminal CH_4, CO, and H_2S production.

2 Materials and methods

2.1 Cascalote fruit collection

For the preparation of the extract, fruits of *C. coriaria* (Jacq) wild were randomly and manually harvested from 10 trees in different zones of the State of Guerrero, Mexico, during March and April. Leaf samples were also collected and stored at room temperature (i.e., 30–35 °C) in the dark for subsequent chemical components determination, secondary metabolites, and preparation of extract for the in vitro gas production trials.

2.2 Preparation of the aqueous extract

Fruits were ground to a particle size of 1 mm using a blender and extracted at 1 g fruits/8 mL of plain water. The ground fruits were immersed in water at room temperature for 72 h in closed 5-L jars. After incubation, jars were filtered through 4–5 layers of gauze, and the extract was collected. Extracts were prepared weekly (stock volume of 8 L each); this mixture was stored at 4 °C before daily oral administration to lambs. The extract chemical composition is shown in Table 1.

2.3 Ruminal liquor

The rumen liquor (RL) was collected from lambs at the end of the experiment (800 g from two lambs per group) of three groups of lambs, each composed of 8 mixed-breed (crosses of Pelibuey, Black belly, and Criollo) male lambs (22 to 28 kg live weight). Lambs were offered a balanced diet (Table 2) twice a day, and feed intake was registered daily. The control group was fed the balanced diet; a second group received the balanced diet plus 30 mL aqueous extract of the *C. coriaria* fruit; group three was offered the balanced diet plus 60 mL of the aqueous extract of the *C. coriaria* fruit. Lambs received the aqueous extract for 60 consecutive days.

Table 1 Ingredients and chemical composition of experimental diets

	%
Diet ingredients	
Corn stubble	50
Soybean meal	10
Coconut paste	10
Wheat bran	10
Corn meal	19.7
Vit-mineral mixture[1]	0.33
Chemical composition	
Crude protein	16
Ether extract	2.6
Neutral detergent fiber	38
Acid detergent fiber	15.9
Organic matter	98.12
Secondary metabolites	mg/g
Total phenolics	770.4
Total flavonoid	149.1
Total condensed tannins	261.6

[1]Vit-mineral mixture: sodium chloride, calcium carbonate, magnesium sulfate, iron sulfate, zinc sulfate, sodium selenium, vitamin A, vitamin D3, vitamin E, vitamin B1, iodine 130 mg/kg, cereal by-products

2.4 In vitro incubations

Two lambs from each animal group (i.e., 0-, 30-, or 60- mL) were slaughtered at the end of the experimental period (60 days), and immediately the rumen contents were used as inoculum for the in vitro incubation with the same diet fed to lambs during the experiment. Rumen contents of each group were mixed with the Goering and Van Soest [10] buffer solution without trypticase at 1:4 vol/vol ratio.

The newly mixed RL was managed according to the method of Salem et al. [11] with different doses (0-, 0.6-, 1.2-, and 1.8- mL) of the *C. coriaria* extract, considering the bottles without the extracts as blanks. After filling all bottles with the substrate, extract, Goering, and Van Soest buffer solution, they were flushed with CO_2, rubber stoppered, shaken, and placed in an incubator at 39 °C.

Total gas production (psi) was recorded at different hours after incubation, starting at 2 h until 48 h following the technique of Theodorou et al. [12]. In addition, CH_4, CO, and H_2S production was measured at the same hours of incubation using a diffusion-based gas detector

Table 2 In vitro ruminal fluid gas production kinetics and total production of the incubated and degraded diets at 48 h of incubation

Ruminal liquor (RL; oral dose per day per lamb (mL)	Extract dose (mL/g DM)	Gas production kinetics[1]			Gas production (mL gas/g DM incubated)			Gas production (mL gas/g DM degraded)		
		b	c	*Lag*	6 h	24 h	48 h	6 h	24 h	48 h
RL0	0	155.8	0.025	9.913	66.3	116.3	154.1	692.9	1243.7	1687.1
	0.6	194.2	0.043	1.688	62.5	173.5	197.1	445.5	1270.0	1524.9
	1.2	216.1	0.045	0.304	63.4	196.0	218.0	316.8	980.5	1099.2
	1.8	226.3	0.044	0.376	61.6	202.7	231.7	260.8	856.0	977.0
	Linear	0.016	0.002	< .0001	0.3759	0.0002	0.0122	0.1506	0.593	0.4684
	Quadratic	0.2476	0.0176	< .0001	0.9024	0.0157	0.2646	0.5157	0.9113	0.7805
RL30	0	186.3	0.039	3.799	44.9	72.7	163.0	188.8	323.5	714.3
	0.6	236.5	0.030	2.770	62.5	120.3	188.5	322.3	622.6	968.7
	1.2	218.6	0.033	1.783	59.1	170.1	215.9	271.5	777.0	993.2
	1.8	214.1	0.030	1.510	66.0	161.7	208.1	338.3	808.4	1060.1
	Linear	0.7549	0.6289	0.241	0.0592	0.0065	0.4315	0.0023	0.0006	0.1896
	Quadratic	0.811	0.9254	0.5928	0.6718	0.0366	0.5374	0.7943	0.0245	0.6255
RL60	0	141.4	0.034	4.188	34.8	63.7	74.8	196.6	365.1	428.4
	0.6	164.5	0.047	3.572	71.0	154.2	169.5	466.3	859.1	943.0
	1.2	252.4	0.088	2.748	65.6	150.7	377.5	417.1	959.2	2404.8
	1.8	254.7	0.062	2.837	73.4	173.0	394.9	299.3	707.1	1615.2
	Linear	0.002	0.1817	0.3603	0.026	0.005	< .0001	0.406	0.0975	0.0015
	Quadratic	0.038	0.040	0.543	0.375	0.2318	0.0053	0.134	0.028	0.0002
Pooled SEM [2]		23.94	0.0081	0.8075	5.44	14.81	24.91	78.01	180.47	271.01
P-value										
Ruminal fluid										
Linear		0.56	0.4525	0.3935	0.3116	0.0017	0.7691	0.0966	0.04	0.190
Quadratic		0.91	0.0032	0.3549	0.9308	0.1196	0.0049	0.8967	0.4554	0.386
Extract dose										
Linear		0.031	0.1608	< .0001	0.0052	< .0001	< .0001	0.5518	0.5505	0.417
Quadratic		0.234	0.0386	0.0049	0.353	0.0019	0.005	0.9479	0.3775	0.158
Ruminal fluid × extract dose		0.753	0.1849	0.002	0.124	0.757	0.000	0.1708	0.7106	0.084

[1] *b* is the asymptotic gas production (mL/g DM); *c* is the rate of gas production (/h); *Lag* is the initial delay before gas production begins (h)

[2] *SEM* standard error of the mean. *DM* dry matter

(MONITOR de Dräger Safety X-am 20,500, Lübeck, Germany) using a 5 mL sample. After each recording, the gas was dispersed using a syringe needle to avoid gas accumulation.

2.5 Degraded substrate dry matter

At the end of the incubation period (48 h), the pH was measured according to Rodriguez et al. [13], and the residual of each bottle was filtered and rinsed. Fermentation residues were dried at 45 °C for 72 h to estimate DM degradability [10], according to [11].

2.6 Diet chemical analyses

Proximate analysis of diet samples (3 subsamples) was performed according to AOAC [14]. The fiber fractions were determined out using an ANKOM200 Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY) according to AOAC [14], with the acid detergent fiber (ADF) and neutral detergent fiber (NDF) determined according to Rodriquez et al. [13].

2.7 Secondary metabolites of the Cascalote fruit extract

2.7.1 Determination of the total phenolic content and total condensed tannins

Total phenolic content of the extracts was determined by a colorimetric method utilizing Folin-Ciocalteu reagent [15], and the absorbance was measured at 765 nm against a reagent blank. The total phenolic content was expressed as mg of gallic acid equivalent per g. The tannin content was determined using tannic acid as a reference compound, following the method of Ayalew and Emire [16].

2.7.2 Determination of total flavonoid content

Modified $AlCl_3$ colorimetric method was used according to the technique of Sembiring et al. [17], and the absorbance was measured against methanol blank at 510 nm. The flavonoid was expressed as µg of quercetin equivalent per 1 g of dry extract.

2.8 Calculations

To estimate the kinetic variables of gas production (GP), CH_4, CO, and H_2S (mL/g DM) were fitted using the NLIN option of SAS [21] using the following model [18]:

$$A = b \times \left(1 - e^{-c(t-lag)}\right)$$

where A is the volume of GP, CH_4, CO, and H_2S at time t; b the asymptotic GP, CH_4, CO, and H_2S (mL/g DM); c is the rate of GP, CH_4, CO, and H_2S (/h); and *lag* (h) is the discrete lag time before GP, CH_4, CO, and H_2S.

Metabolizable energy (ME, MJ/kg DM) and in vitro organic matter digestibility (IVOMD, g/kg OM) were estimated according to Menke et al. [19] as:

$$ME = 2.20 + 0.136GP + 0.0057CP\left({}^{g}/_{kg}\,DM\right)$$

SCFA was calculated according to [20] as:

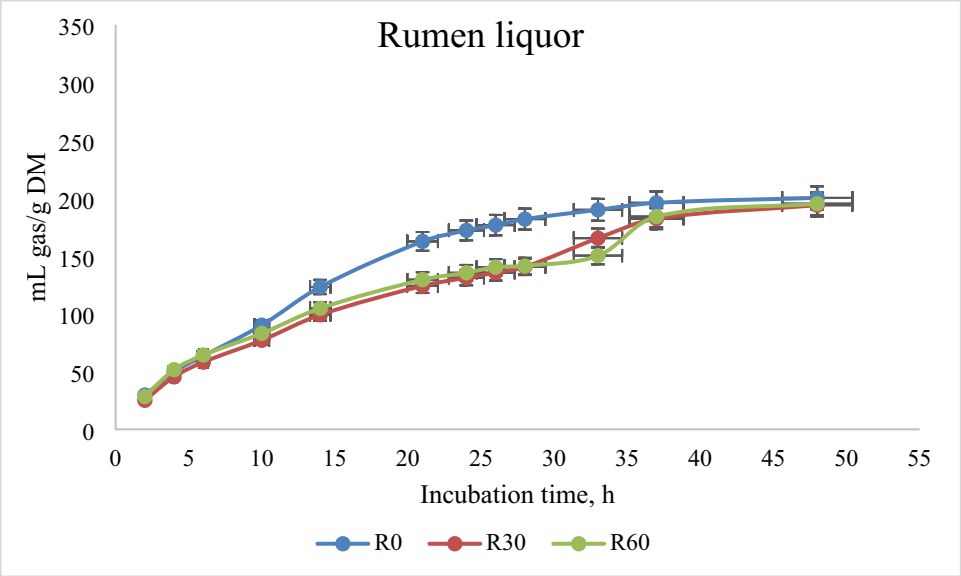

Fig. 1 Rumen total gas production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Caesalpinia coriaria* (Jacq.) wild fruit

$$SCFA\left({}^{mmol}/_{200mgDM}\right) = 0.0222GP - 0.00425$$

where GP is the 24-h net gas production (mL/200 mg DM).

3 Statistical analyses

Data of in vitro ruminal gas production variables were analyzed as a 3×4 factorial experiment (i.e., *C. coriaria* 3 ruminal fluids (fixed effect) and 4 extract doses (random effect)), according to a randomized block design using the PROC MIXED procedure of SAS [21] using the following statistical model:

$$Y_{ijk} = \mu + S_i + R_j + S_i * R_j + \varepsilon_{ijk}$$

where Y_{ijk} represents every observation of the dose when incubated in the *j*th rumen type, S_i = the dose effect (0, 0.6, 1.2, and 1.8 mL), R_j (j = 0-, 30-, or 60-mL aqueous extract fed to lambs) is the rumen liquor type effect, S_i*R_j is the interaction rumen liquor type and *C. coriaria* extract dose, and ε_{ijk} is the experimental error.

4 Results

4.1 Total gas production

GP (mL gas/g DM incubated and degraded) linearly increased (P=0.002; P=0.04) with increasing RL type at 24 h of incubation (Table 2, Fig. 1). In addition, doses of *C. coriaria* extract

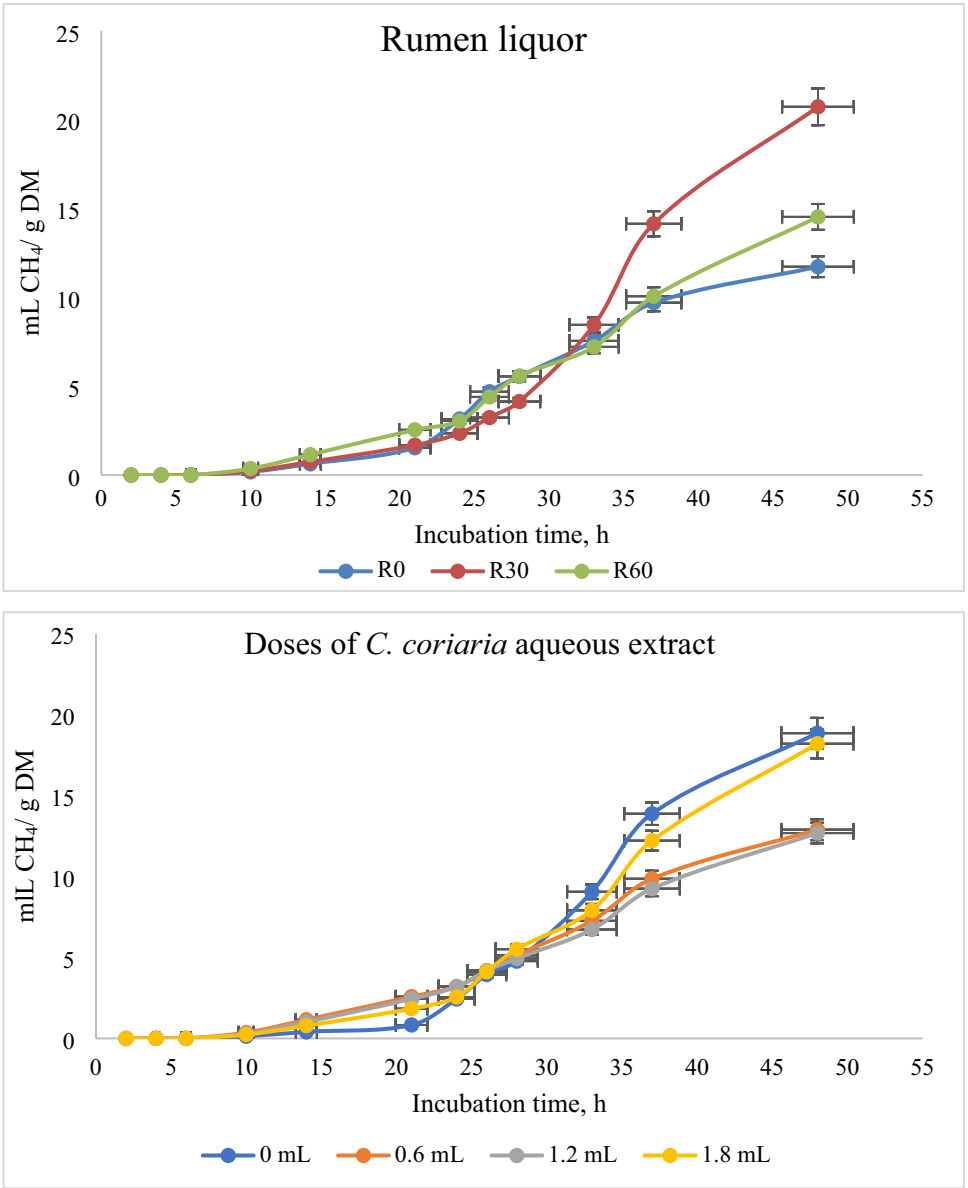

Fig. 2 Rumen methane (CH_4) production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Caesalpinia coriaria* (Jacq.) wild fruit

used during incubation linearly improved ($P=0.031$) asymptotic gas production kinetics and gas production rate (/h) ($P<0.001$).

4.2 Methane production

No trend was observed for CH_4 production during the incubation period. RL30 produced the highest CH_4, while RL0 lambs produced the least. In contrast, 0 and 1.8 mL/ g DM extract of *C. coriaria* had the highest CH_4 production, while 0.6 and 1.2 ml/g DM extract had the lowest CH_4 output (Fig. 2).

Lambs of RL30 had the shortest delay of CH_4, while RL0 had the most extended delay (Table 3). In Table 4, RL type had a linear effect on g CH_4/kg DM at 24 h ($P=0.013$), whereas RL30 had the lowest, while control lambs (i.e., RL0) had the highest at 24 h. Furthermore, there was a dose-dependent linear ($P<0.05$) decrease in CH_4 production (mL CH_4/100 mL gas; mg CH_4/mL gas) at 24 and 48 h of incubation (Table 4). The RL x *C. coriaria* extract dose showed that when measured as CH_4 (g CH_4/kg DM), RL0, 1.8 mL/g DM had the lowest CH_4 production at 24 and 48 h; RL30, 0 mL/g DM had the lowest ($P=0.0002$) CH_4 production at 24 h, while 1.8 mL/g DM had the lowest ($P=0.031$) at 48 h of incubation (Table 4, Fig. 2).

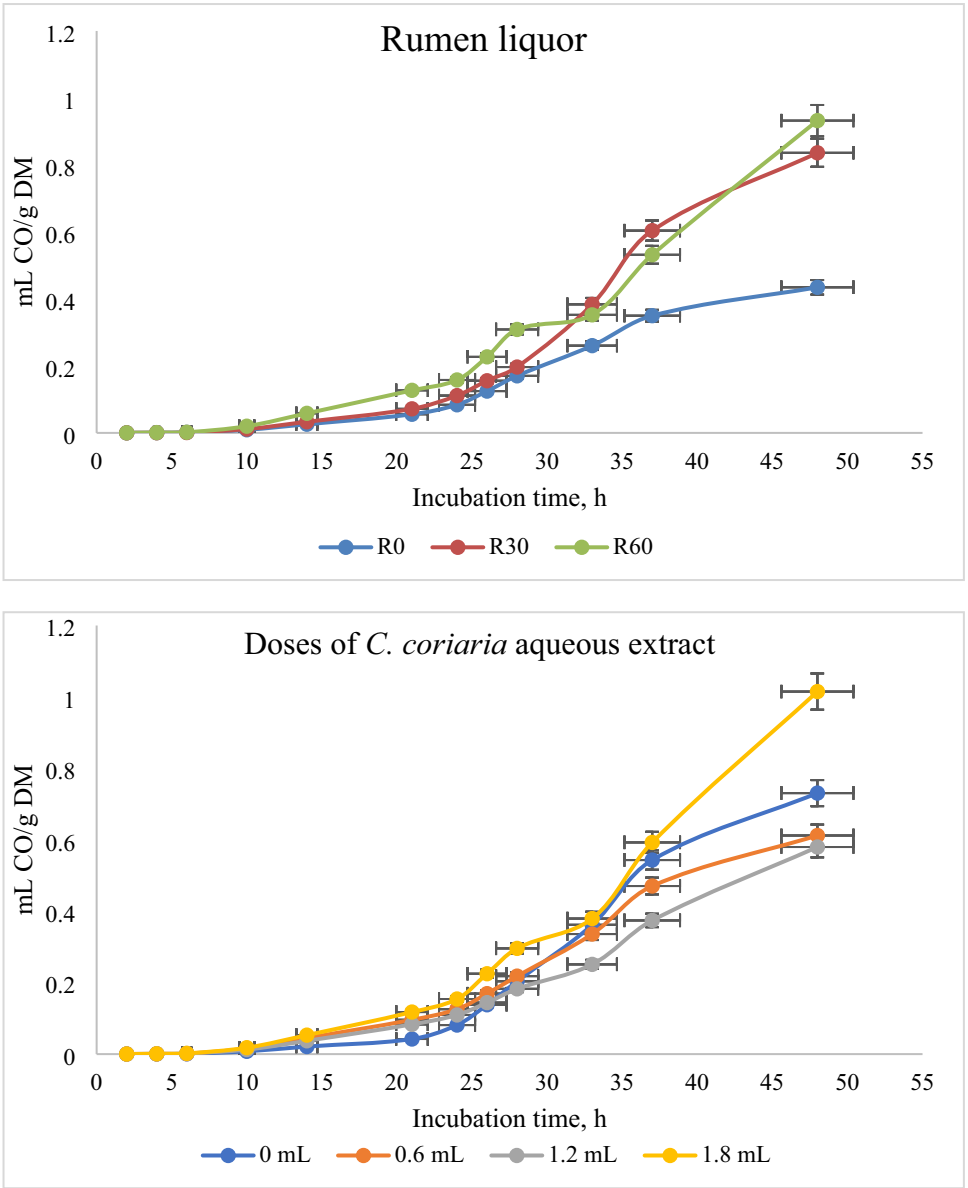

Fig. 3 Rumen carbon monoxide (CO) production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Caesalpinia coriaria* (Jacq.) wild fruit

4.3 Carbon monoxide (CO) and hydrogen sulfide (H_2S)

Table 5 and Fig. 3 showed that RL type and doses had no linear effect on CO production kinetics (mL/g DM incubated) and CO production (mL/g DM degraded) at 24 and 48 h of incubation. However, RL type × dosage affected CO production (mL/g DM incubated) at 24 ($P=0.028$) and 48 h ($P=0.018$) of incubation.

Table 6 and Fig. 4 showed that extract dose ($P=0.004$), rumen type × extract dose ($P<0.001$) affected the H_2S production rate. Dose 1.8 mL/g DM had the highest H_2S production, while 0.6 mL/g DM produced the lowest. RL0 and RL30, in combination with 1.2 and 1.8 mL/g DM, produced the lowest H_2S, while RL60, 0.6 mL/g/DM, had the slowest gas production rate. Lambs of RL0 produced the lowest H_2S (mL/g degraded DM) at 24 h, and RL30 lambs produced the highest. Dose level of *C. coriaria* extract had a linear effect where H_2S decreased with the increasing dose of the extract in 24 h ($P=0.039$); the reverse occurred in 48 h ($P<0.0001$). The ruminal fluid × dose of *C. coriaria* extract showed that at 48 h, 1.8 mL/g DM had the lowest ($P=0.0001$) H_2S production in RL0 and RL30, whereas, in RL60 lambs, the inverse occurred, and 0 mL/g DM produced the lowest.

4.4 Fermentation profile

Table 7 shows the rumen fermentation profile and CH_4 conversion efficiency of diet after in vitro digestibility. Diets fermented with RL0 produced the highest SCFA

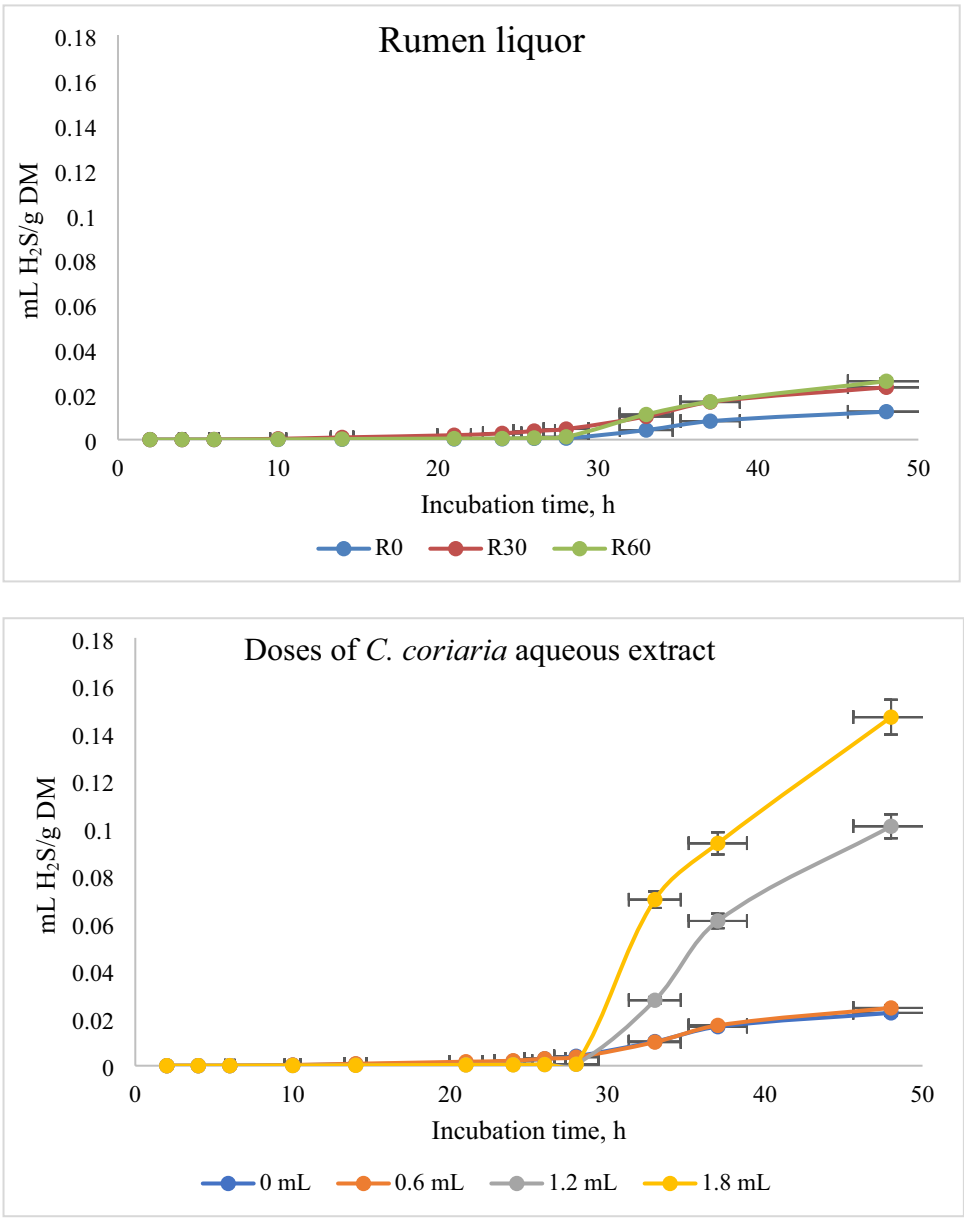

Fig. 4 Rumen hydrogen sulfide (H_2S) production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Caesalpinia coriaria* (Jacq.) wild fruit

($P = 0.001$) and ME (MJ/kg DM at 24 h) (P = 0.001), while diets incubated with RL30 produced the lowest. Furthermore, there was a linear ($P < 0.001$) and quadratic ($P = 0.002$) dose-dependent increase in SCFA and ME. The CH_4:ME and CH_4:OM showed that diets fermented with RL0 produced the highest CH_4 level for every unit of ME, while RL30 produced the lowest. The CH_4 to SCFA ratio showed that for every increase in *C. coriaria* extract, less ($P < 0.001$) CH_4 was produced per SCFA.

Rumen fluid type × dose of *C. coriaria* extract showed that with RL0, the decrease in CH_4 to ME ratio, OM, and SCFA ratio, there was a dose-dependent decrease in CH_4 with increasing *C. coriaria* extract. Moreover, RL60 and RL30 generated the lowest CH_4 output per ME and OM, while 0.6 mL/g DM generated more CH_4. Similarly, 1.8 mL/g DM produced the lowest ($P = 0.0003$) CH_4 per SCFA, while 0.6 mL/g DM produced the highest.

5 Discussion

5.1 Gas production

Phytogenic additives manipulate rumen fermentation due to their secondary metabolites which may improve nutrient digestion and availability [22]. It has been reported that some phenolic compounds stimulate the growth of microbial communities [23]. *C. coriaria* extract increased GP kinetics and

Table 3 In vitro ruminal fluid methane production kinetics and total production of the incubated and degraded diet at 48 h of incubation

Ruminal liquor (RL; oral dose per day per lamb (mL)	Extract dose (mL/g DM)	CH_4 production kinetics[1]			CH_4 production (mL gas/g DM incubated)[2]		CH_4 production (mL gas/g DM degraded)[2]	
		b	c	Lag	24 h	48 h	24 h	48 h
RL0	0	36.25	0.202	21.519	4.59	22.24	49.13	73.95
	0.6	43.16	0.035	12.732	2.86	8.74	20.96	38.67
	1.2	47.24	0.050	14.446	2.99	10.20	14.95	51.85
	1.8	23.26	0.084	17.163	2.18	5.73	9.20	24.19
	Linear	0.1304	0.1549	0.0091	0.0002	0.0654	0.0516	0.0145
	Quadratic	0.0307	0.1884	0.0022	0.25	0.5879	0.375	0.8461
RL30	0	23.92	0.034	22.980	1.20	29.95	5.80	28.72
	0.6	33.29	0.035	17.524	2.64	18.92	13.73	37.05
	1.2	34.07	0.034	14.755	3.42	12.91	15.59	60.22
	1.8	30.32	0.032	11.647	2.14	21.15	10.93	31.75
	Linear	0.5809	0.824	0.0276	0.1139	0.6298	0.1021	0.8133
	Quadratic	0.4914	0.8421	0.5032	0.0051	0.43	0.0171	0.0233
RL60	0	59.35	0.034	12.736	1.52	4.39	8.65	24.82
	0.6	64.66	0.037	11.470	4.13	11.04	22.37	60.09
	1.2	39.81	0.047	11.513	3.18	14.96	20.37	86.82
	1.8	69.59	0.080	18.804	3.36	27.74	13.71	80.67
	Linear	0.5544	0.0326	0.0055	0.0568	0.0047	0.3036	0.0013
	Quadratic	0.1249	0.5677	0.0159	0.3318	0.8367	0.0503	0.0093
SEM pooled[3]		7.894	0.0169	1.5237	0.370	6.032	4.626	7.695
P-value								
Ruminal fluid								
Linear		0.2637	0.0139	0.8484	0.0134	0.1341	0.0316	0.2621
Quadratic		0.0001	0.4877	0.0184	0.2731	0.7389	0.7849	0.0024
Extract dose								
Linear		0.8656	0.3528	0.0513	0.7318	0.9231	0.1171	0.6995
Quadratic		0.9902	0.1453	0.0082	0.0303	0.3242	0.8902	0.0029
Ruminal fluid × extract dose		0.1518	0.0772	0.0049	0.0002	0.3262	0.0308	0.0007

[1] *b* is the asymptotic CH_4 production (mL/g DM); *c* is the rate of CH_4 production (/h); *Lag* is the initial delay before CH_4 production begins (h)

[2] Values of CH_4 production at 6 h (mL CH_4/g DM incubated and mL gas/g DM degraded) were zero

[3] *SEM* standard error of the mean. *DM* dry matter

decreased the *lag* time. This outcome disagrees with Campos-Perez et al. [6], who observed that higher condensed tannin concentrations from *C. coriaria* decreased biogases production. Despite the condensed tannin present in the extract used in the present study, the increased gas production in the current study may be associated with a high level of phenols and flavonoids of the *C. coriaria* fruit, which may have stimulated the growth of a particular microbial community. Besides, the short *lag* time suggests that the phenol and flavonoid helped microbes to easily adapt to the diet, and fermentation rapidly began. This view is further supported by the interaction of RL type and dose of *C. coriaria* extract, where the groups with the highest dose of *C. coriaria* extract had the shortest *lag* time, while those with no extract had the longest *lag* time, indicating that the absence of *C. coriaria* extract prolonged the microbe adaptation to the diet. This suggests that the inclusion of *C. coriaria* extract enhances ruminal fermentation, benefiting ruminants receiving this diet.

However, when gas production was measured per dry matter incubated and digested, lambs fed *C. coriaria* had the lowest gas production. This indicates that long-term in vivo use of *C. coriaria* extract altered the rumen fermentation pattern. Furthermore, Manuel-Pablo et al. [6] showed that *C. coriaria* fruit offered to goats reduced the population of some rumen microbes. This suggests that prolonged use of *C. coriaria* fruit at higher dose affected the rumen microbial population without affecting goat growth. It is, however, interesting that when *C. coriaria* fruit extract was added in vitro, the gas production improved compared with RL without *C. coriaria* fruit extract at 24 and 48 h of incubation. This implies that the phenolic and flavonoid compounds of the *C. coriaria* fruit extract exerts a "restorative/booster"

Table 4 In vitro ruminal fluid methane proportions[1] of incubated and degraded diet at 48 h of incubation

Ruminal liquor (RL; oral dose per day per lamb (mL)	Extract dose (mL/g DM)	CH_4 (mL CH_4/100 mL gas)		CH_4 (mg CH_4/mL gas)		CH_4 (g CH_4/ kg DM)	
		24 h	48 h	24 h	48 h	24 h	48 h
RL0	0	39.50	47.83	1.98	6.59	21.35	70.08
	0.6	16.50	43.33	0.83	2.17	13.31	40.63
	1.2	15.25	46.42	0.76	2.32	13.90	47.44
	1.8	10.75	24.75	0.54	1.24	10.13	26.65
	Linear	< .0001	0.0102	< .0001	0.0109	0.0002	0.02
	Quadratic	< .0001	0.1293	< .0001	0.2896	0.25	0.945
RL30	0	17.33	124.67	0.87	4.57	5.57	72.60
	0.6	21.42	84.25	1.07	4.21	12.28	87.97
	1.2	20.08	59.92	1.00	3.00	15.88	60.02
	1.8	13.50	30.67	0.68	1.97	9.93	31.68
	Linear	0.2612	0.0957	0.2612	0.1086	0.1139	0.3343
	Quadratic	0.1276	0.6913	0.1276	0.8335	0.0051	0.825
RL60	0	21.75	59.58	1.09	2.98	7.06	20.43
	0.6	29.25	69.42	1.46	3.47	19.22	51.35
	1.2	21.42	39.41	1.07	1.97	14.77	69.55
	1.8	19.50	31.32	0.98	3.40	15.61	95.67
	Linear	0.7472	0.1154	0.7472	0.6228	0.0568	0.0002
	Quadratic	0.8955	0.6745	0.8955	0.1252	0.3318	0.2828
Pooled SEM[2]		2.078	13.930	0.104	0.725	1.721	12.376
P-value							
Ruminal fluid							
Linear		0.2723	0.0337	0.2723	0.5996	0.0134	0.1977
Quadratic		0.0593	0.5599	0.0593	0.607	0.273	0.6793
Extract dose							
Linear		< .0001	0.011	< .0001	0.0034	0.7318	0.8379
Quadratic		0.5002	0.7674	0.5002	0.1379	0.0303	0.6333
Ruminal fluid × Extract dose		0.0003	0.6346	0.0003	0.0943	0.0002	0.0306

[1]Values of CH_4 production at 6 h (mL CH_4/100 mL gas, mg CH_4/mL gas, and g CH_4/ kg DM) were zero

[2]*SEM* standard error of the mean. *DM* dry matter

activity on rumen microbes, increasing their fermentative activities.

5.2 Rumen methane production

Lambs with RL given *C. coriaria* had the highest CH_4 production. However, when CH_4 was measured per gram of DM incubated or digested, *C. coriaria* decreased CH_4 output for every gram of incubated and digested feed. The decreased CH_4 production can be attributed to the tannin content of this plant, which exhibited antimethanogenic activity. Campos-Perez et al. [6] reported that *C. coriaria* fruit can reduce CH_4 output by creating an alternative form of H_2 sink/utilization preventing methanogens from using H_2. In the present study, the alternative form of hydrogen sink could be the sulfide-reducing bacteria (SRB). This is because there is an interactive and competitive relationship between methanogens and sulfide-reducing bacteria. This bacterial speceies can also competitively attach to hydrogen ions since the energy provided by the sulfates is greater. Besides, the incubation temperature and rumen temperature (37 °C) favor the sulfur-reducing bacteria, which dominate methanogen for hydrogen use [24]. Therefore, if sulfate levels exceed a particular concentration in the rumen, the sulfate-reducing bacteria proliferates, creating alterative hydrogen sink and decreasing CH_4 production [25, 26].

Manuel-Pablo et al. [9] reported the reduction of rumen protozoa in goats fed *C. coriaria* fruit. This suggests that the *C. coriaria* antimethanogenic activity reduced CH_4 through creation of an alternative sink for H_2 or the decrease in

Table 5 In vitro ruminal fluid carbon monoxide (CO) production kinetics and total production of the incubated and degraded diet at 48 h of incubation

Ruminal liquor (RL; oral dose per day per lamb (mL)	Extract dose (mL/g DM)	CO production kinetics[1]			CO production (mL/g DM incubated)			CO production (mL/g DM degraded)		
		b (ppm)	c	Lag	6 h	24 h	48 h	6 h	24 h	48 h
RL0	0	4943.6	0.045	8.881	0.001	0.154	0.925	0.010	2.348	13.634
	0.6	9102.4	0.020	3.801	0.001	0.047	0.297	0.009	0.633	3.035
	1.2	2988.4	0.028	2.336	0.002	0.077	0.313	0.010	0.423	1.676
	1.8	938.0	0.040	2.696	0.002	0.058	0.203	0.009	0.245	0.842
	Linear	0.4372	0.6136	0.0006	0.0143	0.3707	0.203	0.8302	0.2215	0.1836
	Quadratic	0.9913	0.1338	0.0077	0.1542	0.7534	0.594	0.8526	0.5426	0.4858
RL30	0	4583	0.0338	5.0986	0.001	0.066	1.105	0.003	0.311	4.774
	0.6	5326	0.0359	7.4992	0.001	0.148	0.955	0.006	0.763	4.893
	1.2	2918	0.0271	6.7644	0.001	0.112	0.523	0.007	0.519	2.470
	1.8	1550	0.0337	3.5808	0.001	0.121	0.761	0.006	0.618	4.231
	Linear	0.2391	0.982	0.6115	0.1108	0.0469	0.5679	0.0607	0.0493	0.8517
	Quadratic	0.9443	0.213	0.3582	0.0362	0.3894	0.4356	0.0651	0.6472	0.428
RL60	0	3404.4	0.035	5.396	0.001	0.025	0.162	0.003	0.133	0.906
	0.6	3449.8	0.039	3.924	0.002	0.183	0.584	0.011	0.991	3.211
	1.2	2193.1	0.064	5.235	0.002	0.140	0.903	0.012	0.894	5.739
	1.8	5394.2	0.035	4.055	0.003	0.284	2.081	0.012	1.165	8.524
	Linear	0.0564	1	0.6201	0.0025	0.0003	0.0001	0.0321	0.0012	0.0002
	Quadratic	0.0213	0.1099	0.8269	0.7158	0.7024	0.3771	0.1445	0.2152	0.3602
Pooled SEM[2]		1582.99	0.0068	1.3791	0.0002	0.0331	0.2594	0.0021	0.3343	2.1540
P-value										
Ruminal fluid										
Linear		0.5779	0.947	0.2731	0.0314	0.4049	0.1038	0.033	0.4451	0.7953
Quadratic		0.7563	0.076	0.674	0.0034	0.0418	0.1618	0.1482	0.8757	0.9489
Extract dose										
Linear		0.3696	0.819	0.0346	< .0001	0.0633	0.3104	0.1154	0.6382	0.5448
Quadratic		0.6337	0.6309	0.8833	0.0585	0.8019	0.2292	0.1593	0.6832	0.423
Ruminal fluid × extract dose		0.5028	0.1239	0.213	0.0529	0.0283	0.0175	0.595	0.2906	0.2394

[1] *b* is the asymptotic carbon monoxide (CO) production (ppm); *c* is the rate of carbon monoxide (CO) production (/h); *Lag* is the initial delay before carbon monoxide (CO) production begins (h)

[2] *SEM* standard error of the mean. *DM* dry matter

protozoa which reduced the hydrogen exchange relationship with methanogens [6]. Despite the apparent tendency of *C. coriaria* to reduce CH_4 production, care must be taken to use the right combination.

Lambs of RL30 had the lowest CH_4 output, while a dose level of 1.8 mL/g DM had the lowest level with similar CH_4 production between RL0 and RL60 lambs. This condition suggests that the action of phenols and flavonoids of *C. coriaria* could have neutralized the antimethanogenic activity of tannins at a higher level to the extent that it would be not different or even produce more CH_4 than the unsupplemented groups. This indicates that a balance is needed to use the right combination of *C. coriaria* fruit to avoid an antimethanogenic neutralization effect. This earlier submission can be observed in Table 4, where RL60 with CC extract began to increase CH_4 output per DM incubated even higher than the control. This is similar to what was observed in the in vitro report of Jack [27] for the percentage of CH_4 per total gas volume, using RL from rams fed water washed neem, where RL with the highest water washed neem produced the highest CH_4 production, while the lower water washed neem produced the lowest. Nonetheless, the advantage of reducing CH_4 production per gram DM degraded is that, if fed to ruminants, it could reduce CH_4 eructed.

Table 6 In vitro ruminal fluid hydrogen sulfide (H_2S) production kinetics and total production of the incubated and degraded diet at 48 h of incubation

Ruminal liquor (RL; oral dose per day per lamb (mL)	Extract dose (mL/g DM)	H_2S production kinetics1			H_2S production (mL/g DM incubated)			H_2S production (mL/g DM degraded)		
		b (ppm)	c	Lag	6 h	24 h	48 h	6 h	24 h	48 h
RL0	0	668.3	0.041	5.390	0.00000	0.00037	0.01870	0.00000	0.00453	0.15080
	0.6	1014.5	0.020	4.681	0.00000	0.00027	0.02547	0.00003	0.00197	0.12117
	1.2	382.9	0.007	7.800	0.00000	0.00037	0.00320	0.00013	0.00177	0.01600
	1.8	343.4	0.006	6.509	0.00003	0.00033	0.00240	0.00013	0.00140	0.01013
	Linear	0.5175	0.0263	0.7337	0.195	0.7328	0.2023	0.0961	0.1283	0.0075
	Quadratic	0.7748	0.1615	0.5202	0.4379	0.8434	0.4901	0.308	0.4747	0.0968
RL30	0	387.0	0.052	5.886	0.00000	0.00487	0.03917	0.00013	0.02630	0.21277
	0.6	1159.0	0.034	6.609	0.00003	0.00520	0.03573	0.00020	0.02630	0.18453
	1.2	1951.9	0.011	9.714	0.00000	0.00067	0.01153	0.00007	0.00297	0.05283
	1.8	673.9	0.013	9.276	0.00000	0.00040	0.00653	0.00000	0.00193	0.03587
	Linear	0.8063	0.0286	0.1236	1.0000	0.0503	0.0339	0.0353	0.0947	0.0888
	Quadratic	0.1851	0.1295	0.2464	1.0000	0.2755	0.3362	1.0000	0.346	0.3926
RL60	0	271.2	0.0335	8.3293	0.00000	0.00050	0.00873	0.00010	0.00267	0.04963
	0.6	868.0	0.0169	5.2345	0.00003	0.00083	0.01173	0.00030	0.00453	0.06403
	1.2	748.1	0.1584	3.4743	0.00000	0.00027	0.28747	0.00007	0.00177	1.83070
	1.8	1817.3	0.1738	3.4687	0.00000	0.00037	0.73097	0.00003	0.00157	2.99663
	Linear	0.0123	< .0001	0.0064	1.0000	0.7308	0.0001	0.6811	0.5706	0.0003
	Quadratic	0.497	< .0001	0.0682	1.0000	0.6209	0.3993	1.0000	0.8335	0.4931
Pooled SEM [2]		395.18	0.00681	1.39322	0.0000083	0.0004332	0.02000	0.000053	0.0028	0.0101
P-value										
Ruminal fluid										
Linear		0.2589	0.1597	0.134	1.0000	0.0003	0.7327	0.6339	0.0043	0.7498
Quadratic		0.7564	< .0001	0.0735	1.0000	0.0413	< .0001	0.4116	0.0917	< .0001
Extract dose										
Linear		0.2645	0.0043	0.9301	0.4222	0.0278	< .0001	0.7136	0.0393	< .0001
Quadratic		0.3893	0.3804	0.6541	0.6416	0.2286	0.2918	0.6719	0.2748	0.6979
Ruminal fluid × extract dose		0.2979	< .0001	0.1364	0.5184	0.0174	< .0001	0.174	0.1199	< .0001

[1] *b* is the asymptotic hydrogen sulfide (H_2S) production (ppm); *c* is the rate of hydrogen sulfide (H_2S) production (/h); *Lag* is the initial delay before hydrogen sulfide (H_2S) production begins (h)

[2] *SEM* standard error of the mean. *DM* dry matter

Table 7 In vitro ruminal fluid fermentation profile[1] and methane conversion efficiency of the incubated diet

Ruminal liquor (RL; oral dose per day per lamb (mL)	Extract dose (mL/g DM)	Ruminal fermentation profile				Methane conversion efficiency		
		ruminal pH	DMD (%)	SCFA[1] (mmol/g DM)	ME (MJ/kg DM 24 h)	CH4: ME (g/MJ)	CH_4:OM (ml/g)	CH_4: SCFA at 24 h (mmol/mmol)
RL0	0	6.517	24.463	2.560	5.293	4.019	5.102	51.981
	0.6	6.453	54.897	3.831	5.946	2.233	3.181	21.652
	1.2	6.470	40.468	4.329	6.202	2.239	3.320	19.998
	1.8	6.670	47.491	4.479	6.279	1.614	2.421	14.095
	Linear	0.171	0.1994	0.0002	0.0002	< .0001	0.0002	< .0001
	Quadratic	0.2	0.7608	0.0157	0.0157	0.0198	0.25	< .0001
RL30	0	6.587	46.572	1.592	4.797	1.160	1.332	22.964
	0.6	6.623	38.811	2.650	5.340	2.274	2.935	28.172
	1.2	6.637	43.599	3.755	5.907	2.675	3.795	26.359
	1.8	6.200	40.106	3.568	5.811	1.707	2.374	17.728
	Linear	0.07	0.4993	0.0065	0.0065	0.191	0.1139	0.2476
	Quadratic	0.1673	0.9747	0.0366	0.0366	0.0057	0.0051	0.1367
RL60	0	6.310	35.091	1.394	4.695	1.416	1.686	28.935
	0.6	6.170	36.608	3.403	5.726	3.420	4.593	38.437
	1.2	6.363	31.402	3.324	5.686	2.608	3.529	28.129
	1.8	6.067	49.064	3.820	5.941	2.624	3.729	25.590
	Linear	0.0614	< .0001	0.0053	0.0053	0.1391	0.0568	0.7154
	Quadratic	0.1087	0.0001	0.2318	0.2318	0.3827	0.3319	0.9128
Pooled SEM[2]		0.0912	4.8449	0.3288	0.1688	0.2857	0.4113	2.7359
P-value								
Ruminal fluid								
Linear		0.8205	0.9361	0.0017	0.0017	0.0302	0.0134	0.2801
Quadratic		< .0001	0.4049	0.1196	0.1196	0.2101	0.273	0.0567
Extract dose								
Linear		0.0577	0.1195	< .0001	< .0001	0.457	0.7318	< .0001
Quadratic		0.1671	0.7208	0.0019	0.0019	0.1059	0.0303	0.4749
Ruminal fluid × extract dose		0.0354	0.2298	0.7564	0.7565	< .0001	0.0002	0.0003

[1] *SCFA* short-chain fatty acids (mmol/g DM), *DMD* in vitro dry matter digestibility (%), *ME* metabolizable energy (MJ/kg DM)

[2] *SEM* standard error of the mean. *DM* dry matter

5.3 CO and H_2S production

An imbalance between oxidants and antioxidants causes oxidative stress. This stress could be induced by the constant contact with ingested materials and microbial pathogens. H_2S and CO are endogenous gaseous mediators implicated in gut function [2]. However, CO and H_2S protect the gut against inflammation and serve as antioxidant enzyme to adapt to stress. Endogenous CO can initiate a compensatory expression of antioxidant enzymes and other adaptations to oxidative stress [3]. The higher CO and H_2S in the rumen of *C. coriaria*-fed lambs suggests that the gut is protected against inflammation or irritation due to ingestion of unconventional feed ingredient or additives. It also suggests that the prevention of gastrointestinal inflammation will aid the absorption of nutrients by the rumen and intestine and limit the compromise of tight junctions of the gut. In addition, CH_4 formation is also driven by reactive oxygen species across all living organisms, and these respond to inducer of oxidative stress by enhanced CH_4 formation [28]. This suggests that CO and H_2S ability serve as antioxidant enzyme which could be a factor in reducing CH_4 in this study. Thus, supplementation of *C. coriaria* extract has the potential to protect the gut from oxidative stress, hence offering a therapeutic function and improving the antioxidative status of the gastric mucosa [29], as well as indirectly reducing CH_4 production.

5.4 Rumen fermentation profile

Adequate ruminal pH is required for rumen health and microbial proliferation. For optimal microbial stability, pH should range between 6.0 and 6.8 [30]. In the present study, pH after incubation was optimal for microbial function. The SCFA level indicates energy availability and can provide about 80% of livestock's daily energy requirement [31, 32]. The lower SCFA in RL types of lambs given *C. coriaria* fruit indicates the effect of tannin present in the fruit, which, when given for a prolonged time, might have affected the rumen microbes compared with the control lambs. Nevertheless, the *C. coriaria* extract had a dose-dependent increase in SCFA concentration. This suggests that phenols had a stimulatory effect on the microbes to aid feed fermentation and SCFA production. The predominance of SCFA could be ascribed to increased proportion of volatile fatty acids [22] and could enhance milk production [33]. The increased ME indicates the availability of energy which will be useful for microbial protein production [34], and the ME also followed the pattern of SCFA production.

The CH_4 conversion efficiency ratio showed that the RL type of lambs ingesting the higher *C. coriaria* fruit extract was more efficient in producing CH_4 as they had the lowest value. This attests to the anti-methanogenic properties of *C. coriaria* fruit extract.

6 Conclusion

Rumen liquor of lambs orally administrated with 60 mL of *C. coriaria* fruit extract and 1.2–1.8 mL/g DM resulted in the best antimethanogenic activity and reduced CO and H_2S, reduced total gas production, and had better ME and SCFA. However, without adding extra fruit extract, 30 mL/day/lamb oral supplementation is optimal for digestion, ME, and SCFA production. Therefore, oral supplementation with 60/mL/day/lamb of fruit extract is optimal for rumen fermentation and methane reduction. The results on antimethanogenic activity of *C. coriaria* secondary metabolites indicated their potential as feed additives for decreasing the enteric CH_4 emission, which is important for the sustainable development of ruminant production.

Author contribution PH, MM, MMMYG, and AZMS conceived and designed the experiment; PH, MMMYG, and AZMS conducted the experiment; PH, MM, MMMYG, AZMS, MJA, and OBO prepared the manuscript. All authors approved of the manuscript.

Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval Animal studies have been approved by the ethical committee. The research was performed in accordance with the ethical standard laid down in the 1996 Declaration of Helsinki and its later amendments.

Consent to participate All authors agree to participate in the current work.

Consent for publication All authors agree to publish the findings of the current research.

Competing interests The authors declare no competing interests.

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