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Original Research

Influence of *Azadirachta indica* and *Cnidoscolus angustidens* Dietary Extracts on Equine Fecal Greenhouse Gas Emissions

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ABSTRACT

The present study was conducted to investigate the aqueous extracts of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA), and their combination (MIX) at dosages of 0-, 0.6-, 1.2-, and 1.8- mL for their ability to reduce greenhouse gases and fermentation profiles in an *in vitro* study using horse feces and a nutrient-dense diet (as substrate). The quantity of greenhouse gas and fermentation profiles were determined in *in vitro* incubation for 48 h. Extracts of AZN, CNA, and MIX reduced total gas production of the incubated and degraded substrates in a dose-dependent and time-dependent manner. Production of CH_4 was reduced ($P < .05$) by 4.41% to 54.54% with the incubated substrates and by 1.16% to 61.82% with the degraded substrates. However, AZN and MIX reduced ($P < .05$) CO by 4.43% to 12.85% with the incubated substrates and by 0.70% to 16.78% with the degraded substrates. In like manner, the plant extracts and combination reduced ($P < .05$) H_2S production in a dose-dependent and time-dependent manner by 18.37% to 67.35% with the incubated substrates and by 8.51% to 67.23% with the degraded substrates. Extracts maintained pH within the normal range, reduced dry matter digestibility and metabolizable energy, and improved ($P < .05$) concentration of short chain fatty acids. Overall, aqueous extracts of AZN and CNA and their combinations had a positive effect on reducing the greenhouse gas production with no deleterious effect on fecal horses' fermentation activities.

1. Introduction

Greenhouse gases are that trap heat in the atmosphere [1], and this resulted in climate change by affecting the atmosphere chemically in the long term [2], leading to global warming. The three main greenhouse gases are methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O). Others are the fluorinated gases such as the hydrofluorocarbons, perfluorocarbons, sulfur hexafluoride, and nitrogen trifluoride [1]. Methane is a greenhouse gas that is formed when organic materials are decomposed in an anaerobic environment such as the rumen. It emanates both from natural and anthropogenic sources. Globally, 50% to 65% of total methane emissions come from anthropogenic sources [1]. Methane is produced mainly from enteric fermentation (39%) and manure storage (10%), [3]. In the agricultural sector, enteric methane accounts for the largest (40%) emission concentration [4,5], while in the livestock sub-sector, enteric methane accounts for 80% of the emissions [6]. Methane gas has a negative effect on the atmosphere than CO_2. This is because CH_4 gas has a global warming potentials of about 25 to 36 times higher than CO_2 over 100 years [1,7] and 80 times more potent over 10 to 20 years from release [7]. Methane emission is detrimental to animal productivity as it represents a significant gross energy loss of 2% to 12% of the gross energy (GE) intake by the animals [6–8].

Nitrous oxide (N_2O) is released from manure storage and the application of organic and inorganic fertilizers to soils. It is also very detrimental as its global warming potential is 265 times

☆ *Conflict of interest statement:* 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.

Ethical statement: Not applicable.

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Table 1
Chemical composition of the diet (substrate used during the *in vitro* evaluation) as well as the secondary metabolites (Total phenolics and flavonoids) of *Azadirachta indica* and *Cnidoscolus angustidens*.

Diet	g/kg DM	
Crude protein	130	
Nitrogen free extract	580	
Crude fiber	80	
Ether extract	30	
Organic matter	870	
Secondary metabolites:	*Azadirachta indica*	*Cnidoscolus angustidens*
Total phenolics (mg gallic acid /g)	72.6	65.3
Total flavonoid (mg quercetin /g)	122.2	5.62

Abbreviation: DM, dry matter.

higher than carbon dioxide [3]. The standard unit acceptable to account for the global warming potential is the carbon dioxide equivalent [3,9]. Mitigation strategies are therefore aimed at directly reducing the amount of these greenhouse gases emitted and by so doing, indirectly make more energy available to improve livestock productivity [3,8]. Many plants contain bioactive substances which make them quite useful in pharmacological studies and other life-cycle assessment. Neem **(***Azadirachta indica***)** is a medicinal plant that contains many biological active compounds. These compounds can be extracted from *A. indica* include alkaloids, flavonoids, saponins, tannins, reducing sugar, glycosidessteroids, triterpinoids, phenolic compounds, carotenoids, ketones, salannin, volatile oils, meliantriol, nimbin and *azadirachtin* [10,11]. In addition, Al-Marzooqi et al [12] reported the presence of 64% unsaturated fatty acids, 33% saturated fatty acids and 2.3% other fatty acids in neem seed oil.

The common name of *Cnidoscolus angustidens*is is mala mujer (in spanish) and it is an herbaceous perennial plant in the family Euphorbiaceae. *C. angustidens* is a wild flowering plant native to the Cape Region of Baja California Sur in north-west Mexico and it is known as "caribe" by the locals [13]. Mala mujer is rich in essential oils. Also, León et al [13] reported the following fatty acid composition in *C. angustidens*: 9.50% palmitic acid (C16), 5.00% stearic acid (C18), 32% oleic acid (C18:1), 52.00% linoleic acid (C18:2), 0.51% linolenic acid (C18:3), 0.70% eicosanoic acid (C20) and 0.73% arachidonic acid (C20:4).

Previous reviews on the different strategies to mitigate enteric CH_4 production and other greenhouse gases by ruminants have been published [3,8]. Grossi et al [3] in their review categorized the greenhouse gases mitigation strategies into enteric fermentation (including forage quality), manure storage and animal management, reporting variable (uncertain, low, medium and high) mitigation potentials. Black et al [8] among other things, reviewed the use of various feed supplements and the potential of anti-methanogenic forages such as Leucaena, Desmanthus, Australian Tar Bush shrub, *Eremophila glabra*, Biserrula, native Australian Melaleuca and Leptospermum plants and their extracts in reducing methane and other greenhouse gases. Most of these forages contain primary compounds such as essential oils and plant secondary metabolites such as condensed and hydrolysable tannins [14]. These biological active compounds possess both beneficial and detrimental effects [15,16]; increased bacterial proteins and overall nutrient flow and digestibility to the duodenum for subsequent absorption by the ruminant [17,18]; defaunation [19,20], bactericidal or bacteriostatic properties [17], antimethanogenic [21,22] and other antimicrobial activities. The objective of this study, therefore, was to assess the effect of the aqueous extract of *A. indica* (leaves) and *C. angustidens* (roots) inoculated with horse fecal contents on the mitigation potential of greenhouse gases, dry matter digestibility, short chain fatty acids, metabolizable energy and organic matter digestibility.

2. Materials and Methods

2.1. Vegetative Materials

Roots of the *C. angustidens* were taken manually from five plants in Puente de Ixtla, Morelos, Mexico, in the month of August, later they were washed and peeled for later use. Leaves samples of the *A. indica*, were taken from five trees in the municipal of Tecpan de Galeana, Guerrero, Mexico, in February, after which this vegetative material was dried at 40°C (BINDER Model FD115 Incubator, Germany) and ground (Willey Mill, Mexico with a 1 mm sieve) in the laboratory of bromatology at the Faculty of Veterinary Medicine and Animal Science of the Autonomous University of the State of Mexico. Finally, it was stored at room temperature (i.e., 25°C–30°C) for later use.

2.2. Preparation of Aqueous Extracts

Three aqueous extracts were prepared, the first was from the *C. angustidens* plant (CNA) using a ratio of one g of previously collected vegetative material and 8 mL of distilled water, which was crushed in a blender and left to macerate in amber bottles for 72 h at room temperature (i.e., between 22°C and 30°C), and , it was subsequently filtered for *in vitro* evaluation. The second aqueous extract of the *A. indica* plant (AZN) was obtained by mixing one g of dry matter/8 mL of distilled water and leaving it to rest in amber bottles for 72 h, and then it was filtered for use in *in vitro* gas production. Finally, an extract was made with the combination (MIX) of the two previous ones in a 1:1 vol/vol ratio (Table 1).

2.3. Animals as Inoculum Donors

The fecal content was used as a source of inoculum from 3 horses with body weight of 400 to 500 kg; individual fecal samples (1/2 kg of each horse) were collected, from the rectum of each animal, before the first evacuation of the day. These samples were mixed and homogenized to obtain a homogeneous and representative sample of fecal content to be used as a source of microorganisms in *in vitro* incubation.

2.4. In Vitro Incubations

The fecal contents were mixed with the Goering and Van Soest [23] buffer solution without trypticase in the ratio of 1:4 vol/vol. The incubation medium was subsequently mixed and filtered through four layers of cheesecloth in a flask with an O_2-free

Table 2
In vitro fecal gas production kinetics and total production at 6, 24 and 48 h of the incubated and degraded diet with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

Plant Species Extract	Extract Dose (mL/g DM)	Gas Production Kinetics[a]			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
AZN	0	337.9	0.031	2.578	113.8	258.1	344.9	588.6	1333.8	1775.3
	0.6	205.3	0.231	2.710	69.9	152.8	214.2	325.9	723.3	1039.2
	1.2	358.6	0.033	1.933	119.8	273.9	368.1	652.2	1488.6	1994.0
	1.8	278.6	0.022	3.001	77.4	152.8	247.9	362.4	716.4	1166.0
	Linear	0.3957	0.9489	0.8007	0.2705	0.1816	0.2371	0.1856	0.138	0.1713
	Quadratic	0.4039	0.9546	0.5584	0.3903	0.3042	0.307	0.2277	0.1911	0.1744
CNA	0	337.9	0.031	2.578	113.8	258.1	344.9	588.6	1333.8	1775.3
	0.6	312.7	0.024	1.455	76.6	181.0	294.2	481.7	1089.7	1811.8
	1.2	280.8	0.035	1.953	96.1	229.0	291.2	444.5	1061.1	1343.3
	1.8	280	0.045	0.885	82.4	189.5	278.9	412.0	955.2	1422.3
	Linear	0.2738	0.3259	0.0394	0.0097	0.0409	0.1998	0.0717	0.0527	0.2701
	Quadratic	0.528	0.8306	0.7206	0.8159	0.8373	0.6256	0.4712	0.5789	0.3512
MIX	0	337.9	0.031	2.578	113.8	258.1	344.9	588.6	1333.8	1775.3
	0.6	303.5	0.030	1.266	84.5	199.2	294.2	477.0	1134.5	1611.4
	1.2	196.2	0.049	5.665	62.3	90.3	157.8	320.2	469.1	811.9
	1.8	303.2	0.028	0.635	78.6	187.0	293.4	371.5	882.5	1375.8
	Linear	0.5654	0.7655	0.165	0.0728	0.1016	0.392	0.0762	0.1075	0.171
	Quadratic	0.0377	0.065	0.0062	0.0506	0.0041	0.0113	0.1219	0.018	0.0106
SEM pooled [b]		35.65	0.0205	0.6342	12.11	30.06	38.71	75.71	174.35	210.32
P value:										
Extract		0.832	0.4026	0.3291	0.6007	0.4287	0.6127	0.7159	0.524	0.5055
Dose:		0.2275	0.4186	0.1149	0.0218	0.0294	0.1151	0.0381	0.0378	0.1086
Linear		0.1445	0.99	0.1518	0.0092	0.0091	0.0543	0.0064	0.006	0.0249
Quadratic		0.2527	0.8344	0.0811	0.7147	0.4401	0.2411	0.8306	0.5388	0.3243
Extract × Dose		0.1083	0.2919	0.0471	0.3503	0.0564	0.0652	0.1926	0.042	0.0235

Abbreviation: DM, dry matter.
[a] *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).
[b] SEM standard error of the mean.

Table 3
In vitro fecal methane production kinetics and total production at 6, 24 and 48 h of the incubated and degraded diet with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

Plant Species Extract	Extract Dose (mL/g DM)	CH_4 Production Kinetics[a]			CH_4 Production (mL CH_4/g DM incubated)			CH_4 Production (mL CH_4/g DM degraded)		
		b	c	Lag	6 h	24 h	48 h	6 h	24 h	48 h
AZN	0	7.06	0.063	13.591	0.11	0.68	2.46	0.55	3.45	12.76
	0.6	7.01	0.052	9.476	0.07	0.54	1.92	0.33	2.63	9.64
	1.2	11.61	0.102	19.310	0.13	0.74	4.52	0.72	4.02	24.37
	1.8	9.64	0.061	11.552	0.11	0.63	2.60	0.52	2.89	12.11
	Linear	0.4125	0.9553	0.7405	0.9109	0.8799	0.9195	0.8882	0.7249	0.9245
	Quadratic	0.2434	0.2868	0.2269	0.6159	0.7558	0.1129	0.3625	0.5386	0.0708
CNA	0	7.06	0.063	13.591	0.11	0.68	2.46	0.55	3.45	12.76
	0.6	8.85	0.095	12.501	0.10	0.65	2.72	0.63	3.78	15.63
	1.2	12.32	0.115	19.011	0.09	0.88	3.51	0.40	4.10	16.43
	1.8	21.95	0.095	18.378	0.16	1.57	6.41	0.78	7.97	32.76
	Linear	0.0016	0.6134	0.3633	0.0673	0.0281	0.0213	0.1762	0.0347	0.0274
	Quadratic	0.4511	0.5092	0.5016	0.0591	0.4215	0.4607	0.091	0.3275	0.354
MIX	0	7.06	0.063	13.591	0.11	0.68	2.46	0.55	3.45	12.76
	0.6	14.81	0.095	18.262	0.11	1.05	4.58	0.59	6.25	25.38
	1.2	9.45	0.891	17.468	0.09	0.75	2.54	0.49	4.01	13.03
	1.8	16.12	0.048	17.136	0.05	0.70	5.00	0.21	3.41	24.28
	Linear	0.0592	0.9797	0.386	0.1576	0.9697	0.0249	0.1457	0.9867	0.0676
	Quadratic	0.5647	0.1456	0.5472	0.6363	0.8725	0.1741	0.5587	0.8045	0.2786
SEM pooled [b]		1.999	0.0975	3.2451	0.0224	0.1969	0.7601	0.1195	1.0684	4.1153
P value:										
Extract		0.0952	0.4474	0.4369	0.4777	0.2809	0.293	0.4291	0.3063	0.3124
Dose:		0.002	0.3654	0.2669	0.9114	0.573	0.0298	0.9791	0.7397	0.0831
Linear		0.0002	0.9825	0.4743	0.9494	0.1844	0.0043	0.6972	0.2768	0.0119
Quadratic		0.837	0.0937	0.1265	0.8991	0.864	0.9426	0.917	0.9547	0.9912
Extract × Dose		0.07	0.5423	0.7089	0.1855	0.2748	0.0481	0.1018	0.2546	0.0342

Abbreviation: DM, dry matter.
[a] *b* is the asymptotic CH_4 production (mL/g DM); *c* is the rateof CH_4 production (/h); *Lag* is the initial delay before CH_4 production begins (h).
[b] SEM standard error of the mean.

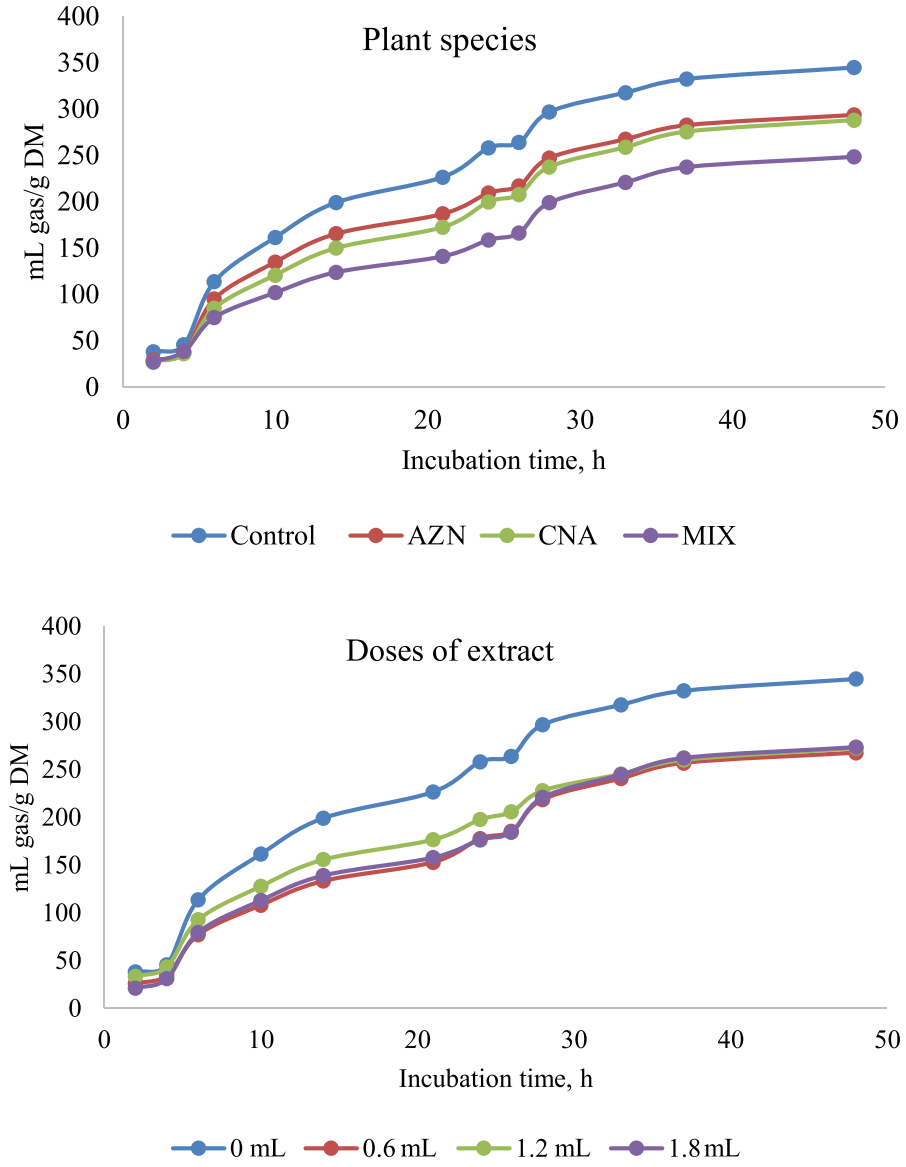

Fig. 1. Horse fecal total gas production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

headspace. Three identical incubation runs were inoculated into 120 mL serum bottles containing 0.5 g of substrate in the presence of different doses (i.e., 0, 0.6-, 1.2- and 1.8- mL) of the three extracts (i.e., 1.2, AZN, CNA and MIX), considering as blank the bottles with substrates but without the extracts. The dosage level of the extract was chosen based on previous studies, and as a result of reduced and sustainable mitigation of the production of equine fecal methane, carbon monoxide, and hydrogen sulfide [24,25].

However, after filling all the bottles with the substrate, extract, the buffer solution and fecal content, they immediately closed with rubber stoppers, sealed with aluminum on the bottle neck, shaken, and then they were placed in an incubator (BINDER Model FD115 Incubator, Germany) at 39°C.

Total gas production (PSI) was recorded from 2 to 48 h after incubation using the pressure transducer (Extech Instruments, Waltham, MA, USA) following the technique of Theodorou et al [26]. At the same incubation times, the concentrations of CH_4, CO and H_2S in the free space of the bottles were measured using a diffusion-based gas detector (Dräger Safety Monitor X-am 2500, Lübeck, Germany). To avoid gas accumulation, the gas was dispersed after each recording with a syringe needle.

2.5. Apparently Degraded Substrate

After sampling the supernatant for pH determination using electrodes on a pH meter (Conductronic pH15, Puebla, Mexico), the contents of each bottle were vacuum filtered through sintered glass crucibles (coarse porosity #1, pore size 100–160 mm; Pyrex, Stone, UK). Then, the incubation residues were dried at 70°C overnight to estimate the apparent disappearance of DM (DMD). The DM degradability at 48 h of incubation (i.e., substrate degraded, DMD; %) was calculated as the difference between the DM content of the substrate and its non-degradable DM.

2.6. Calculations

To estimate the kinetic parameters of gas production (GP), CH_4, CO and H_2S results of GP, CH_4, CO and H_2S (mL/g DM) were fitted

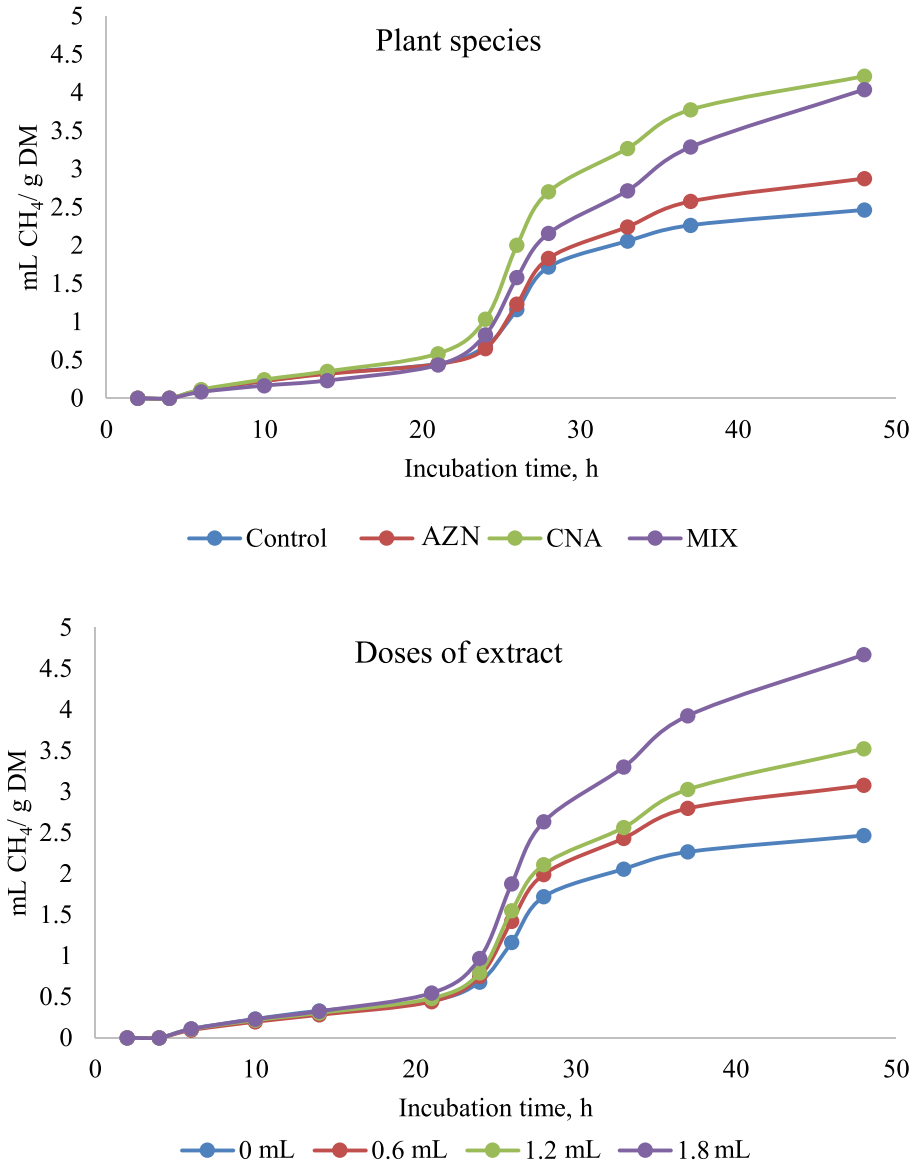

Fig. 2. Horse fecal Methane (CH_4) production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

using the NLIN option of SAS to the France et al [21] model as:

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

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 prior to GP, CH_4, CO and H_2S.

Metabolizable energy (ME, MJ/kg DM) was estimated according to Menke et al [22] as:

$$ME = 2.20 + 0.136\ GP + 0.0057\ CP\left(g/kg DM\right)$$

where GP is net gas production in mL from 200 mg dry sample after 24 h of incubation.

Short chain fatty acids concentration (SCFA) was calculated according to Getachew et al. [33] as:

$$SCFA\left(mmol/200\ mg\ DM\right) = 0.0222\ GP - 0.00425$$

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

2.7. Statistical Analysis

Experiments were completely randomized with repeated measures in time. Data from each of the three runs within the same sample of each of the three individual samples (*A. indica, C. angustidens* or their combination (MIX)) were averaged prior before statistical analysis, and mean values of each individual sample were used as the experimental unit. Results of fecal fermentation parameters, methane conversion efficiency were analyzed as a factorial experiment using the PROC GLM option of SAS [24] as:

$$Y_{ijk} = \mu + P_i + E_j + (P \times D)_{ij} + E_{ijk}$$

here: Y_{ijk}= is every observation of the *i*th plant species (Pi) with *j*th extract dose (E_j); μ is the general mean; $(P \times E)_{ij}$ is the interaction between plant species and extract dose; E_{ijk} represents the experimental error, normally distributed with the average 0 and the constant variance. Linear and quadratic polynomial contrasts were used to examine responses of greenhouses gas production of the fermented diet fermentation with the increasing addition lev-

Table 4
In vitro fecal methane proportions at 6, 24 and 48 h of the incubated and degraded diet with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

Plant Species Extract	Extract Dose (mL/g DM)	CH_4 (mL / 100 mL gas)			CH_4 (mg /mL gas)			CH_4 (g /kg DM)		
		6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
AZN	0	0.94	2.69	7.08	0.28	0.81	2.12	0.50	3.16	11.46
	0.6	0.94	3.02	7.13	0.28	0.91	2.14	0.32	2.52	8.94
	1.2	1.11	2.75	12.08	0.33	0.83	3.63	0.61	3.46	21.00
	1.8	1.33	3.50	9.64	0.40	1.05	2.89	0.53	2.93	12.09
	Linear	0.3119	0.4894	0.4202	0.3116	0.4898	0.4206	0.903	0.8803	0.9194
	Quadratic	0.9323	0.7327	0.1916	0.9312	0.7318	0.1918	0.6158	0.755	0.113
CNA	0	0.94	2.69	7.08	0.28	0.81	2.12	0.50	3.16	11.46
	0.6	1.28	3.50	8.90	0.38	1.05	2.67	0.45	3.02	12.67
	1.2	0.89	3.83	12.17	0.27	1.15	3.65	0.40	4.10	16.32
	1.8	1.94	8.15	22.69	0.58	2.44	6.81	0.74	7.31	29.81
	Linear	0.0089	0.0028	0.0008	0.0089	0.0028	0.0008	0.0661	0.0281	0.0213
	Quadratic	0.059	0.1915	0.3269	0.0588	0.1912	0.3268	0.0593	0.4221	0.4607
MIX	0	0.94	2.69	7.08	0.28	0.81	2.12	0.50	3.16	11.46
	0.6	1.28	5.31	15.63	0.38	1.59	4.69	0.51	4.89	21.30
	1.2	1.39	6.35	14.17	0.42	1.91	4.25	0.43	3.48	11.82
	1.8	0.56	3.96	18.28	0.17	1.19	5.48	0.21	3.24	23.25
	Linear	0.2406	0.5628	0.0186	0.2406	0.5631	0.0187	0.1596	0.9696	0.0249
	Quadratic	0.0429	0.135	0.662	0.0429	0.135	0.6619	0.6331	0.8722	0.1741
SEM pooled[a]		0.1804	0.8720	1.9683	0.0541	0.2616	0.5905	0.1045	0.9157	3.5347
P value:										
Extract		0.3538	0.0882	0.0192	0.3539	0.0882	0.0192	0.4751	0.2809	0.2931
Dose:		0.361	0.0704	0.0003	0.3606	0.0705	0.0003	0.9095	0.573	0.0298
Linear		0.0846	0.0103	<0.0001	0.0844	0.0103	<0.0001	0.9598	0.1842	0.0044
Quadratic		0.9081	0.642	0.6169	0.909	0.6429	0.617	0.9035	0.8651	0.9424
Extract × Dose		0.0139	0.076	0.0535	0.0138	0.0759	0.0535	0.186	0.2747	0.0481

Abbreviation: DM, dry matter.
[a] SEM standard error of the mean.

els (doses) of the plant species extracts. Statistical significance was declared at $P < .05$.

3. Results and Discussion

3.1. Total Gas Production

Total gas production (mL gas/g DM incubated or degraded) was affected by plant species extract and dose dependent. However, apart from the incubated and degraded diet, with the AZN extract at 1.2 mL dosage, produced more gas than their respective control substrate diets at 6, 24 and 48 h. Diet characteristics, such as contents of crude protein, degradable nitrogen and dietary fiber influence the efficacy of plant extracts to produce gas (Table 2, Fig. 1). Since the plant species were high degradable nitrogen, and this must have been responsible for the lower gas production *versus* control diets, corroborating similar findings by Akanmu et al [27] with lucerne substrate. When Garcia-Montes de Oca et al [28] incubated seven different legumes and wild arboreal pods of Mexican calabash (*Crescentia alata*), esculent lead tree (*Leucaena esculenta*), guamuchil (*Phitecellobium dulce*), bastard cedar (*Guazuma ulmifolia*), needle bush (*Acacia farnesiana*), mimosa (*Mimosa* sp.) and elephant ear tree (*Enterolobium cyclocarpum*), They reported that *Leucaena esculenta* produced less gas *versus other plant species* due to the fact that it has a lower effective degradability and slower degradation speed. These characteristics allowed some of the protein in *L. esculenta* to escape during fermentation. Lower effective degradability and slower degradation speed may account for the low gas production of the leaf extracts and mixtures utilized in this study (Table 3, Fig. 2).

3.2. Methane Production

The potential of methane reduction, with the dietary inclusion of plants aqueous extracts and their combinations, was dose-dependent and time-dependent and did not follow any pattern. There was reduced methane production with the AZN leaf extract at 0.6 mL infusion irrespective of time of sampling for the incubated and degraded substrates. Reduced methane production was also recorded for AZN aqueous extract incubated at 1.8 mL dosage for 6 h. Moreover, AZN extracts substrate degraded at 1.8 mL reduced methane production at 6, 24 and 48 h. However, for the incubated AZN extracts substrate, the reduction of methane was of 7.35% (1.8 mL at 24 h) to 36.36% (at 0.6 mL at 6 h), while the range of methane reduction, for the AZN degraded substrate, was of 5.08% (1.8 mL at 48 h) to 40.00% (0.6 mL at 6 h). For the CNA extract incubated substrates, reduced methane production compared to the control were recorded at 0.6 mL at 6 h (9.09%), 0.6 mL at 24 h (4.41%) and 1.2 mL at 6 h (18.18%). The only low methane production for the degraded CNA extract substrate was at the 1.2 mL dosage at 6 h (27.27%).

A varying range in methane reduction was similarly observed in the substrates that had the AZN: CNA extract mixture (MIX). The lowest percentage (1.16%) of methane production was recorded for the substrate degraded at 1.8 mL extract dose for 24 h, while the highest percentage (61.8%) of methane reduction was for the substrate infused with 1.8 mL extract dose mixture for 6 h. The substrate with the extract mixture also reduced methane production up to 10.9% at the 1.2 mL extract dose degraded for 6 h. There was also an reduced, of methane production, by18.18% (1.2 mL at 6 h) and 54.54% (1.8 mL at 6 h) of the substrates incubated with the MIX. Thus, there was a 4.4% to 54.5% methane reduction with the incubated substrates and a 1.16% to 61.82% reduction with the degraded substrates. The anti-methanogenic activities of these aqueous extracts were likely due to the rich contents of the diets as well as the presence of plant secondary metabolites [29,30] and essential oils [12,21]. Chaturvedi et al [31] also reported that a combination of *Emblica officinalis: A. indica* and *A. indica: Clerodendrum phlomidis* at 0.5% supplementation reduced *in vitro* methane production by 30.93 and 28.17%, respectively. For the substrates

Table 5
In vitro fecal carbon monoxide (CO) production kinetics and total production at 6, 24 and 48 h of the incubated and degraded diet with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

Plant Species Extract	Extract Dose (mL/g DM)	CO Production Kinetics[a]			CO Production (mL /g DM incubated)			CO Production (mL /g DM degraded)		
		b	c	Lag	6 h	24 h	48 h	6 h	24 h	48 h
AZN	0	3475.0	0.813	5.019	0.054	0.316	1.214	0.275	1.572	5.940
	0.6	4066.5	0.068	4.859	0.086	0.393	1.058	0.382	1.790	4.943
	1.2	6594.2	0.028	6.059	0.141	0.701	2.374	0.768	3.823	12.751
	1.8	4861.9	0.068	6.876	0.074	0.475	1.414	0.343	2.214	6.594
	Linear	0.6058	0.1884	0.4412	0.7473	0.5732	0.812	0.8067	0.6309	0.8705
	Quadratic	0.3097	0.3848	0.9566	0.1652	0.2302	0.1714	0.0843	0.1216	0.09
CNA	0	3475.0	0.813	5.019	0.054	0.316	1.214	0.275	1.572	5.940
	0.6	6101.5	0.027	6.206	0.085	0.510	1.829	0.466	2.749	9.997
	1.2	8640.3	0.031	10.080	0.106	0.753	2.449	0.490	3.498	11.342
	1.8	4139.9	0.029	7.162	0.144	0.896	2.919	0.720	4.499	14.862
	Linear	0.7963	0.1677	0.1838	0.0443	0.0221	0.0593	0.0329	0.0143	0.0446
	Quadratic	0.0554	0.4085	0.0141	0.8436	0.4295	0.5844	0.9596	0.5854	0.7793
MIX	0	3475.0	0.813	5.019	0.054	0.316	1.214	0.275	1.572	5.940
	0.6	5549.2	0.034	10.694	0.095	0.600	2.492	0.504	3.343	13.394
	1.2	3936.3	0.025	5.581	0.071	0.302	1.098	0.359	1.561	5.556
	1.8	7065.6	0.095	10.462	0.056	0.540	2.239	0.248	2.482	10.566
	Linear	0.1657	0.2027	0.0375	0.9656	0.2998	0.1519	0.8993	0.3813	0.1336
	Quadratic	0.5312	0.3661	0.2869	0.6981	0.4921	0.2949	0.6034	0.599	0.2937
SEM pooled [b]		1604.00	0.1931	1.3593	0.0294	0.1448	0.4988	0.1347	0.7155	2.2635
P value:										
Extract		0.7875	0.9967	0.1018	0.4933	0.266	0.3072	0.435	0.2596	0.2538
Dose:		0.2585	0.0371	0.072	0.3101	0.1048	0.1611	0.2404	0.0935	0.1213
Linear		0.201	0.0193	0.0124	0.1899	0.0228	0.0334	0.2139	0.0249	0.0279
Quadratic		0.124	0.1255	0.5271	0.1787	0.3495	0.4755	0.1097	0.2464	0.3771
Extract × Dose		0.5134	1.000	0.0634	0.6066	0.3895	0.2033	0.326	0.1852	0.0765

Abbreviation: DM, dry matter.
[a] *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).
[b] SEM standard error of the mean.

incubated with the AZN extracts, lowered fecal methane proportions were emitted at the 6 h , 24 h and at the 48 h from the 0.6 mL dosage compared with their respective controls. Moreover, the CNA and MIX extracts incubated substrates emitted also lower CH_4 proportions *versus* controls at al the three incubation times (Table 4)This may likely be due to the high content of essential oils present in the aqueous extract mixture at that dosage [12]. They reported that neem seed oil affected ruminal fermentation in a dose-dependent manner which was dependent on the composition of the feed and the controlled conditions of the *in vitro* environment. They explained that the potency and interactions of the fatty acids, especially the unsaturated fatty acids, were responsible for the reduction in methane proportion. The lowest methane proportions obtained in this study with the highest dose (dose-dependent) mixture of the extracts at the 6 h (time-dependent) is in line with the findings of Al-Marzooqi et al [12].

3.3. Carbon Monoxide Production

The plant extracts and their combinations affected CO reduction in a dose-dependent and time-dependent manner compared to the respective controls. The AZN extract reduced CO at the rate of 12.85% at 0.6 mL incubated for 48 h and by 16.78% at 0.6 mL degraded for 48 h. The CNA extract did not reduce CO production at any dosage and time frame. The MIX reduced CO production *versus* control sample was 4.43% (at 1.2 mL incubated for 24 h), 9.55% (at 1.2 mL incubated for 48 h), 9.82% (at 1.8 mL degraded for 24 h), 0.7% (at 1.2 mL degraded for 24 h) and 6.46% (at 1.2 mL degraded for 48 h). Thus, in the incubated substrates, aqueous species extracts reduced CO production at the range of 4.43% (1.2 mL/24 h) to 12.85% (0.6 mL/48 h) and in the degraded diets, 0.7% (1.2 mL/24 h) to 16.78% (0.6 mL/48 h; Table 5, Fig. 3). The species-dependent, dose-dependent and time-dependent reduction of CO in this study may be due to the characteristics of the substrate diet, plant secondary metabolites and essential oils present in the plant species [12,27].

3.4. Hydrogen Sulfide Production

The H_2S production of the plant extracts were dose-dependent and time-dependent. For the AZN extract, there was a 34.69% (0.6 mL at 48 h) reduction in H_2S production in the incubated substrates and 12.50% (0.6 mL at 24 h) to 32.77% (0.6 mL at 48 h) H_2S reduction in the degraded substrates. The CNA extract substrates reduced H_2S production by 28.57% (1.2 mL at 48 h), 33.33% (1.2 mL at 24 h and 1.8 mL at 24 h) and 38.77% (1.8 mL at 48 h) in the incubated substrates, and by 29.36% (1.2 mL at 48 h), 33.62% (1.8 mL at 48 h) and 37.50% (1.2 mL at 24 h and 1.8 mL at 24 h) in the degraded substrates. There were significant reductions in H_2S production especially in the MIX at different doses and time of incubation and degradation. The incubated substrate including the MIX reduced H_2S by 18.37% (0.6 mL for 48 h) to 67.35% (1.2 mL for 48 h). In the same vein, the degraded substrate including the MIX reduced H_2S production by 8.51% (0.6 mL for 48 h) to 67.23% (1.2 mL for 48 h; Table 6, Fig. 4). The substrates with the aqueous extracts mixture produced less H_2S due to its lower effective degradability and slower degradation speed, as it was degraded in the 48 h. These findings corroborated the report of Garcia-Montes de Oca et al [28] for the high-quality legume *Leucaena esculenta*.

Table 6
In vitro fecal hydrogen sulfide (H_2S) production kinetics and total production at 6, 24 and 48 h of the incubated and degraded diet with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

Plant Species Extract	Extract Dose (mL/g DM)	H_2S Production Kinetics[a]			H_2S Production (mL/g DM incubated) [b]		H_2S Production (mL/g DM degraded)	
		b	c	Lag	24 h	48 h	24 h	48 h
AZN	0	398.3	0.033	5.022	0.003	0.049	0.016	0.235
	0.6	255.0	0.028	6.708	0.003	0.032	0.014	0.158
	1.2	396.7	0.028	7.014	0.004	0.079	0.021	0.433
	1.8	531.8	0.027	6.554	0.015	0.107	0.072	0.502
	Linear	0.6454	0.485	0.623	0.064	0.104	0.078	0.160
	Quadratic	0.7848	0.7799	0.649	0.276	0.962	0.373	0.678
CNA	0	398.3	0.033	5.022	0.003	0.049	0.016	0.235
	0.6	651.8	0.030	5.880	0.003	0.066	0.018	0.389
	1.2	638.0	0.032	2.931	0.002	0.035	0.010	0.166
	1.8	104.1	0.042	2.542	0.002	0.030	0.010	0.156
	Linear	0.5623	0.3364	0.2282	0.307	0.408	0.472	0.413
	Quadratic	0.3856	0.4373	0.6193	0.715	0.825	0.675	0.716
MIX	0	398.3	0.033	5.022	0.003	0.049	0.016	0.235
	0.6	170.8	0.039	2.746	0.002	0.040	0.011	0.215
	1.2	69.3	0.029	6.010	0.001	0.016	0.008	0.077
	1.8	255.8	0.125	5.275	0.002	0.034	0.008	0.164
	Linear	0.5424	0.2403	0.8825	0.195	0.430	0.225	0.412
	Quadratic	0.2205	0.4431	0.5647	0.286	0.132	0.470	0.126
SEM pooled [c]		193.83	0.0141	1.3682	0.0016	0.0151	0.0082	0.0727
P value:								
Extract		0.4174	0.4014	0.1503	0.0357	0.041	0.0536	0.0488
Dose:		0.9656	0.4489	0.982	0.2152	0.7814	0.3071	0.9045
Linear		0.6204	0.2118	0.8603	0.1608	0.556	0.1662	0.5867
Quadratic		0.9086	0.3647	0.7184	0.1636	0.4396	0.2556	0.6369
Extract × Dose		0.6057	0.6255	0.4331	0.0562	0.0539	0.0901	0.0342

Abbreviation: DM, dry matter.
[a] *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).
[b] Production of H_2S at 6 hours (mL/g DM incubated and degraded) was zero.
[c] SEM standard error of the mean.

Table 7
In vitro fecal fermentation profile[a] and methane conversion efficiency to short chain fatty acids (CH_4: SCFA at 24 h, mmol/mmol), metabolizable energy (CH_4: ME (g/MJ)), and organic matter (CH_4:OM (mL/g)) of the dietary inclusion with different doses of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

Plant Species Extract	Extract Dose (mL/g DM)	Fecal Fermentation Profile				Methane Conversion Efficiency		
		pH	DMD%	SCFA mmol/g DM	ME, MJ/kg DM 24 h	CH_4: ME (g/MJ)	CH_4:OM (mL/g)	CH_4: SCFA at 24 h (mmol/mmol
AZN	0	6.43	5.71	39.32	6.91	3.52	0.46	0.75
	0.6	6.24	3.38	40.66	5.71	4.92	0.38	0.60
	1.2	6.05	6.06	37.06	7.09	3.60	0.49	0.83
	1.8	6.13	3.3716667	42.644	5.71	4.60	0.47	0.70
	Linear	0.4518	0.181	0.3569	0.1816	0.4668	0.9606	0.8804
	Quadratic	0.5036	0.3035	0.2199	0.3041	0.7174	0.9099	0.7548
CNA	0	6.43	5.71	39.32	6.91	3.52	0.46	0.75
	0.6	6.91	4.00	33.98	6.03	4.59	0.49	0.72
	1.2	6.84	5.06	43.38	6.58	5.02	0.62	0.98
	1.8	6.80	4.18	40.26	6.13	10.68	1.18	1.75
	Linear	0.1496	0.0409	0.8707	0.0408	0.0027	0.0128	0.028
	Quadratic	0.2889	0.8373	0.4784	0.8374	0.1905	0.341	0.4215
MIX	0	6.43	5.71	39.32	6.91	3.52	0.46	0.75
	0.6	6.89	4.40	37.02	6.24	6.97	0.78	1.17
	1.2	7.05	1.98	39.31	5.00	8.40	0.62	0.83
	1.8	6.96	4.13	42.05	6.10	5.20	0.54	0.77
	Linear	0.0532	0.1016	0.6154	0.1017	0.5598	0.819	0.9695
	Quadratic	0.1172	0.0041	0.7688	0.0041	0.1284	0.6733	0.8728
SEM pooled[b]		0.147	0.667	3.015	0.343	1.127	0.144	0.219
P value:								
Extract		0.0005	0.4276	0.958	0.4291	0.1374	0.2144	0.2807
Dose:		0.4562	0.0293	0.4755	0.0294	0.0658	0.3811	0.5727
Linear		0.246	0.009	0.4114	0.0091	0.0095	0.091	0.1841
Quadratic		0.4263	0.4399	0.8157	0.4404	0.6268	0.9011	0.8644
Extract × Dose		0.2867	0.0562	0.6961	0.0564	0.0637	0.2698	0.2745

Abbreviation: DM, dry matter.
[a] SCFA is the short chain fatty acids (mmol/g DM); DMD is the *in vitro* dry matter digestibility (%); ME is the metabolizable energy (MJ/kg DM).
[b] SEM standard error of the mean.

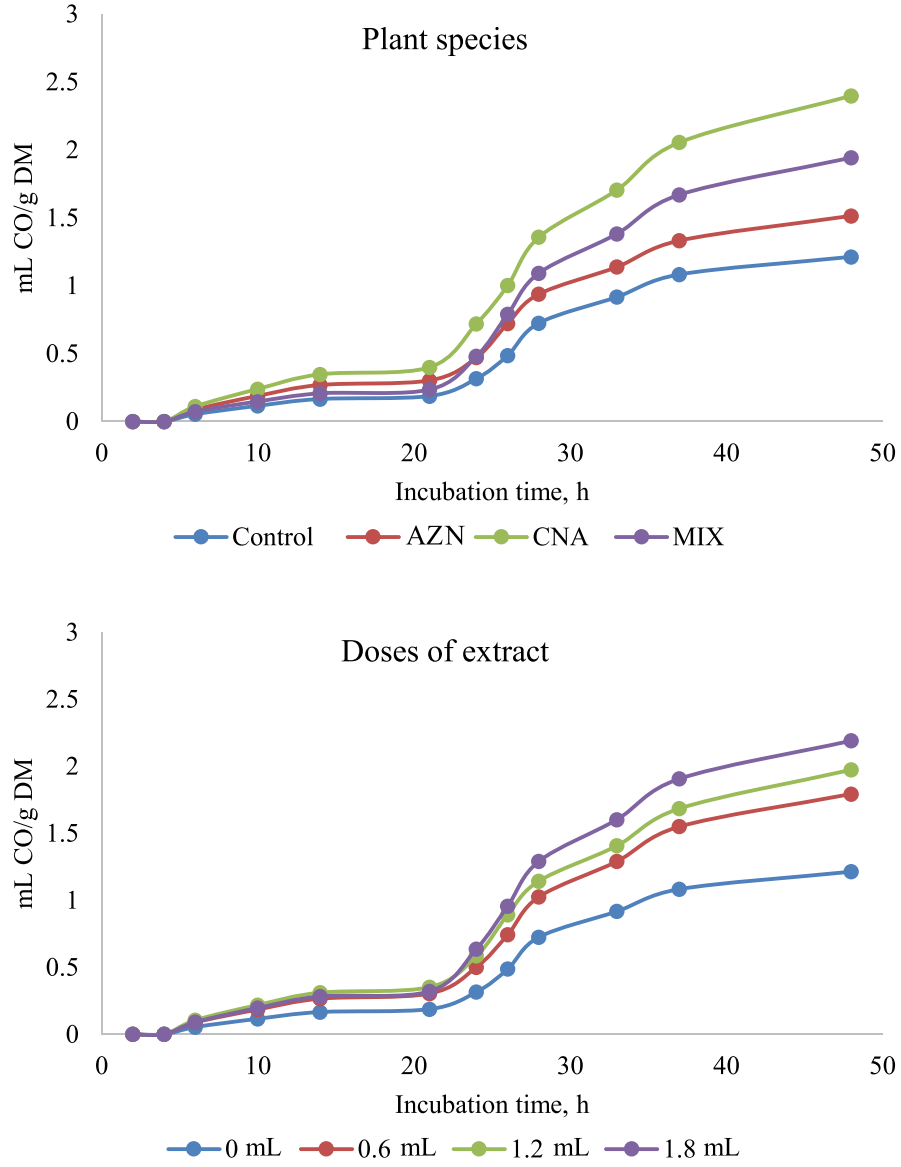

Fig. 3. Horse fecal carbon monoxide (CO) production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

3.5. Fermentation Profile

Fecal pH ranged from 6.05 (in the AZN extract at 1.2 mL dose) to 7.05 (in the MIX at 1.2 mL dose). However, the solution with the AZN extracts lowered the pH at all dosages of inclusion, whereas increased pH *versus* control, was recorded for incubated substrates with the CNA and AZN: CNA mixture. The fatty acid composition of AZN may likely be responsible for the pH reduction [12]. The pH range was within the normal values (6.05–7.05), however, the range of values from 6.44 to 6.69 were reported by Chaturvedi et al [31] except for the MIX at 1.2 mL dose .

The dry matter digestibility (DMD) ranged from 1.98% (MIX at the 1.2 mL dose) to 6.06% (AZN extract at the 1.2 mL dose). The highest DMD (6.06%) was obtained in the AZN extract substrate at the 1.2 mL dose, while the least DMD (1.98%) was recorded for the MIX at the 1.2 mL dose *versus* control. Reduced DMD was recorded for the plant extracts and their combination. Only the AZN substrate at 1.2 mL dose increased DMD *versus* control.

The contents of short chain fatty acids were also species-dependent and dose-dependent and ranged from 33.98 to 43.38 mmol/g DM in all the substrates. Metabolizable energy (ME) ranged from 5.00 MJ/kg in the 1.2 mL dose in the mixture to 7.09 MJ/kg in the AZN substrate with 1.2 mL doses. Lower ME (MJ/kg DM at 24 h) was released from the incubated substrates. Apart from the AZN, 1.2 mL dose substrate, which released higher energy than the control, all other diets, irrespective of extract type and dose produced lower energy. The varying results of the fermentation profile were likely due to the essential oil, fatty acids, and plant secondary metabolites in the leaf extracts [12,32].

For the methane conversion efficiency, the CH_4:ME (g/MJ) was lowest in the AZN extract at the 1.2 mL dose. The proportion of methane to *in vitro* organic matter (CH_4: OM (mL/g) was least in the AZN extract at 0.6 mL dose. The proportion of methane to SCFA (CH_4: SCFA at 24 h mmol/mmol) was lower in the AZN extract at 0.6 mL (0.60 mmol/mmol), AZN extract at 1.8 mL dose (0.70 mmol/mmol) and the CNA extract at 0.6 mL dose

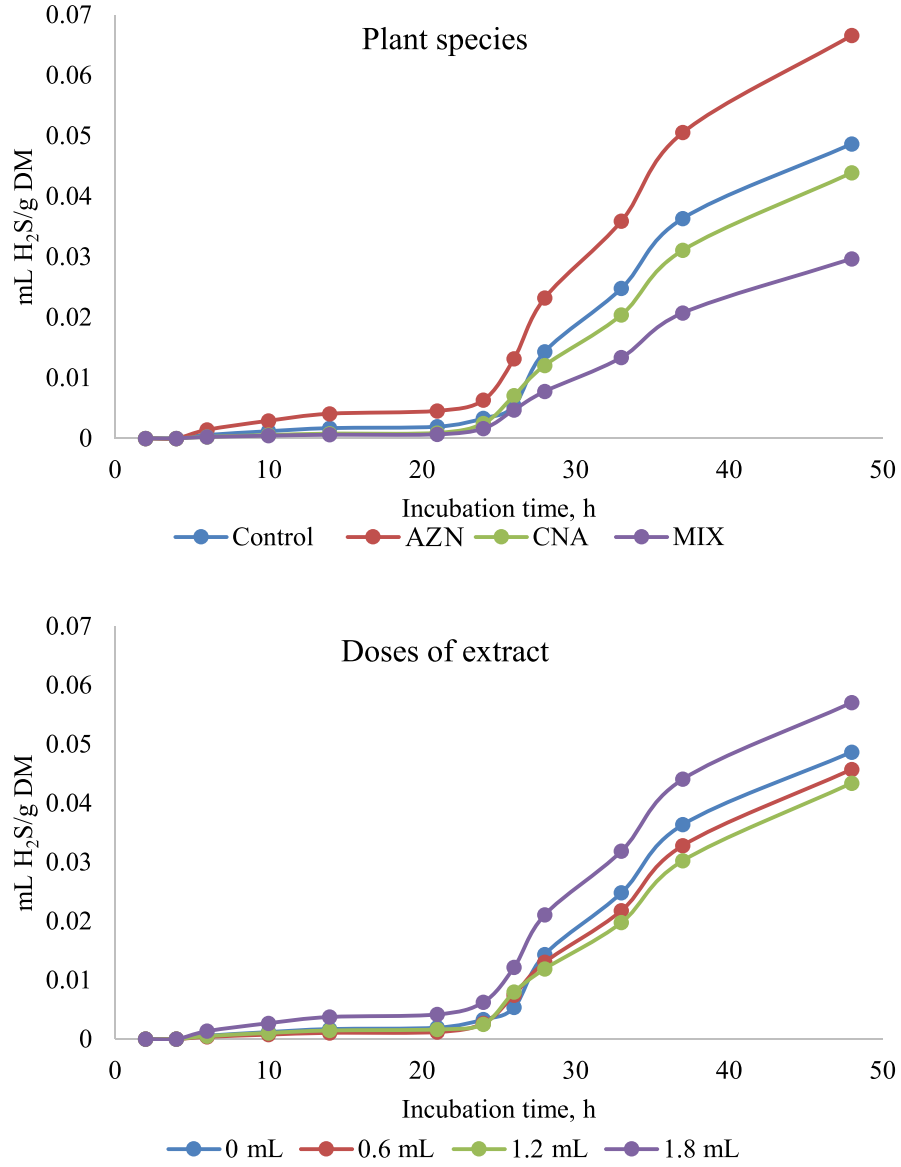

Fig. 4. Horse fecal Hydrogen sulfide (H_2S) production (mL/g dry matter (DM)) at different hours of incubation as affected by the dietary inclusion with the aqueous extract of *Azadirachta indica* (AZN), *Cnidoscolus angustidens* (CNA) or their combination (MIX, 1:1, vol/vol) at 0, 0.6, 1.2, and 1.8 mL/g DM incubated with fecal contents of horses.

(0.72 mmol/mmol) compared to the control (0.75 mmol/mmol) – (Table 7).

4. Conclusions

Aqueous extracts of *A. indica, C. angustidens* and their combination (MIX) presented a detected reduction in total gas production as well as the greenhouse gases production and considered as a new strategy to mitigate the greenhouse gas emissions in horses toward a clean environmental agriculture. However, varying reduction results in total gas, methane, carbon monoxide (CO) and hydrogen sulfide (H_2S) emissions, which were plant species-dependent, dose-dependent and time-dependent was obtained. Future studies, however, are warranted to confirm the current results and to investigate the effects of dietary plant supplementation for *C. angustidens, A. indica* and their combination on digestion and enteric greenhouse gas emissions in horses.

References

[1] EPA (United States Environmental Protection Agency) (2022). Overview of Greenhouse Gases. https://www.epa.gov/ghgemissions/overview-greenhouse-gases#f-gases. Retrieved April 16, 2022.
[2] Dogan, S. (2007). IklimDe_gis¸ ikli_gi'ndeTürkiye'ninAdımları. Available at: http://www.bitem.gazi.edu.tr/pdf/iklimturkiye.pdf; 2007 (in Turkish). Accessed 10/01/2016.
[3] Grossi G, Goglio P, Vitali A, Williams AG. Livestock and climate change: impact of livestock on climate and mitigation strategies. Anim Front 2019;9:69–76.
[4] Braimoh AK, Hou X, Heumesser C, Zhao YG. Greenhouse gas mitigation opportunities in agricultural landscapes: a practitioner's guide to agricultural and land resources management. Washington, DC, USA: Office of the Publisher, The World Bank; 2016.
[5] Statistical yearbook 2014: Latin America and the Caribbean food and agriculture. Vitacure, Santiago de Chile: Food and Agriculture Organization of the United Nations (FAO); 2014.
[6] Gerber PJ, Hristov AN, Henderson B, Makkar H, Oh J, Lee C, et al. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. Animal 2013;7:220–34.
[7] Fouts JQ, Honan MC, Roque BM, Tricarico JM, Kebreab E. Board invited review: enteric methane mitigation interventions. Transl Anim Sci 2022;6:txac041.

[8] Black JL, Davison TM, Box I. Methane emissions from ruminants in Australia: mitigation potential and applicability of mitigation strategies. Animals 2021;11:951.
[9] IPCC Climate change 2013: the physical science basis. Contribution of working group i to the fifth assessment report of the intergovernmental panel on climate change. NY, USA: Cambridge University United Kingdom and New York; 2013.
[10] Deshpande PK, Gothalwal R, Pathak AK. Phytochemical analysis and evaluation of antimalarial activity of *Azadirachta indica*. Pharm Innov 2014;3:12.
[11] Vinoth B, Manivasagaperumal R, Rajaravindran M. Phytochemical analysis and antibacterial activity of *Azadirachta indica* A. Juss. Int Res J Plant Sci. 2012;2:50–5.
[12] Al-Marzooqi W, Sallam SM, Alqaisi O, El-Zaiat HM. Potential of graded doses of neem (*Azadirachta indica*) seed oil on ruminal fermentation characteristics, degradability, and methane formation *in vitro*. Ann Anim Sci 2021.
[13] León-De La Luz JL, Troyo-Diéguez E, Ortega-Nieblas MM, López-Gutiérrez F. 'Caribe'(*Cnidoscolus angustidens* Torr.), a promising oilseed geophyte from north-west Mexico. J Arid Environ 1999;41:299–308.
[14] Paengkoum S, Petlum A, Purba RAP, Paengkoum P. Protein-binding affinity of various condensed tannin molecular weights from tropical leaf peel. J Appl Pharm Sci 2021;11:114–20.
[15] Purba RAP, Yuangklang C, Paengkoum P. Enhanced conjugated linoleic acid and biogas production after ruminal fermentation with *Piper betle* L. supplementation. Ciênc Rural 2020;50:e20191001.
[16] Ekanem NJ, Inyang UA, Ikwunze K. Chemical composition, secondary metabolites and nutritive value of elephant-ear tree (*Enterolobium cyclocarpum* (Jacq.) Griseb): a review. Niger J Anim Prod 2022;49:277–86.
[17] Purba RAP, Yuangklang C, Paengkoum S, Paengkoum P. Milk fatty acid composition, rumen microbial population and animal performance in response to diets rich in linoleic acid supplemented with *Piper betle* leaves in Saanen goats. Anim Prod Sci 2020. doi:10.1071/AN20182.
[18] Purba RAP, Paengkoum S, Yuangklang C, Paengkoum P, Salem AZM, Liang JB. Mammary gene expressions and oxidative indicators in ruminal fluid, blood, milk, and mammary tissue of dairy goats fed a total mixed ration containing piper meal (*Piper betle* L.). Ital J Anim Sci 2022;21:129–41.
[19] Koenig KM, Ivan M, Teferedegne BT, Morgavi DP, Rode LM, Ibrahim IM, et al. Effect of dietary *Enterolobium cyclocarpum* on microbial protein flow and nutrient digestibility in sheep maintained fauna-free, with total mixed fauna or with *Entodinium caudatum* monofauna. Brit J Nutr 2007;98:504–16.
[20] Ban C, Paengkoum S, Yang S, Tian XZ, Thongpea S, Purba RAP, et al. Feeding meat goats mangosteen (*Garcinia mangostana* L.) peel rich in condensed tannins, flavonoids, and cinnamic acid improves growth performance and plasma antioxidant activity under tropical conditions. J Appl Anim Res 2022;50:307–15.
[21] France J, Dijkstra J, Dhanoa MJ, López S, Bannink A. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: derivation of models and other mathematical considerations. Br J Nutr 2000;83:143–50.
[22] Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Schneider W. The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor *in vitro*. J Agr Sci 1979;93:217–22.
[23] Goering HK, Van Soest PJ. Forage fiber analyses (apparatus, reagents, procedures, and some applications). Vitacure, Santiago de Chile: US Agricultural Research Service; 1970.
[24] PROC GLM option SAS, et al. User's guide: statistics, version 9.0. Cary, NC. SAS Institute 2002;114:104021.
[25] Elghandour MMMY, Adegbeye MJ, Barbabosa-Pilego A, Perez NR, Hernández SR, Zaragoza-Bastida A, et al. Equine contribution in methane emission and its mitigation strategies. J Equine Vet Sci 2019;72:56–63.
[26] Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Tech 1994;48:185–97.
[27] Akanmu AM, Hassen A, Adejoro FA. Gas production, digestibility and efficacy of stored or fresh plant extracts to reduce methane production on different substrates. Animals 2020;10:146.
[28] Garcia-Montes de Oca CA, Gonzalez-Ronquillo M, Salem AZM, Romero-Bernal J, Pedraza JF, Estrada JG. Chemical composition and *in vitro* gas production of some legume browse species in subtropical areas of Mexico. Trop Subtrop Agroecosystems 2011;14:589–95.
[29] Suong NTM, Paengkoum S, Salem AZM, Paengkoum P, Purba RAP. Silage fermentation quality, anthocyanin stability, and *in vitro* rumen fermentation characteristic of ferrous sulfate heptahydrate-treated black cane (*Saccharum sinensis* R.). Front Vet Sci. 2022;9:896270.
[30] Suong NTM, Paengkoum S, Purba RAP, Paengkoum P. Optimizing anthocyanin-rich black cane (*Saccharum sinensis* Robx.) silage for ruminants using molasses and iron sulphate: A sustainable alternative. Fermentation 2022;8:248.
[31] Chaturvedi I, Dutta TK, Singh PK, Sharma A. Effect of combined herbal feed additives on methane, total gas production and rumen fermentation. Bioinformation 2015;11:261–6.
[32] Purba RAP, Paengkoum S, Yuangklang C, Paengkoum P. Flavonoids and their aromatic derivatives in *Piper betle* powder promote *in vitro* methane mitigation in a variety of diets. Cienc Agrotec 2020;44:e012420.
[33] Getachew G, DePeters EJ, Robinson PH. In vitro gas production provides effective method for assessing ruminant feeds. Calif Agric 2004;58:1–12.