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Assessment of some browse tree leaves on gas production and sustainable mitigation of CH_4 and CO_2 emissions in dairy calves at different age

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ABSTRACT

The present context was aimed to determine the *in vitro* gas production (GP), mitigation of methane (CH_4) and carbon dioxide (CO_2) emission, and ruminal fermentation from nine different tree leaves as suitable alternatives for alfalfa hay. Tree leaves and alfalfa hay were incubated with rumen inoculum from calves at different ages (20, 40, and 60 d). The level of asymptomatic GP [mL/g dry matter (DM)] differed ($P < 0.05$) between different tree leaves compared with alfalfa hay. Most of the plant leaves showed differed ($P < 0.05$) asymptomatic CH_4 production (mL/g DM), fractional rate of GP and lag time. The asymptomatic CO_2 production and fractional rate of CO_2 production decreased effectively ($P < 0.01$) with the different tree leaves. *In vitro* rumen CH_4 and CO_2 productions (mL/g incubated DM) by species incubated in the rumen liquor of calves collected at 60 d of age increased effectively. Fermentation pH ($P < 0.001$) was found to be significant parameter but DM degradability ($P > 0.05$) was not significant by dose and substrate × dose interaction. Tree leaves showed lower production of CH_4 (mL/g incubated DM) and proportional CH_4 emission when compared to alfalfa hay after the required period of incubation at significant level ($P < 0.05$). However, different tree leaves had no influence ($P > 0.05$) on CO_2 production (mL/g incubated DM and mL/g degraded DM) and proportional CO_2 production. The incorporation of tested tree leaves in diet would be a valuable alternative of alfalfa hay with sustainable reduction properties of CH_4 and CO_2 productions. These potent tree leaves can be used as valuable cleaner product and feeding stuffs for the environment and ruminants respectively due to their *in vitro* fermentative properties.

1. Introduction

The ruminal microorganisms, residing the ruminants, digest the nutrients by fermentative process. The ruminal fermentation produces methane (CH_4), carbon dioxide (CO_2), and other detrimental gases. Methane production from ruminant livestock is one of the energetically wasteful processes responsible for the emission of greenhouse gas (GHG) that contribute about 18% of all GHG emissions, while CO_2 accounts for about 9% emission (FAO, 2006). The emissions of CH_4, CO_2, and other harmful gases into the environment are the major causative agents of global warming (Bunthoeun et al., 2007). Additionally, emissions of GHG from ruminant manures and urine to the environment affect not only the water quality but also the human health. At present, there is growing interest to reduce the emission of GHG by ruminants without affecting rumen function (Elghandour et al., 2016a).

Recently, researches focused on the use of safe strategies to mitigate the emission of GHG, including organic acid salts

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(Elghandour et al., 2016b), exogenous enzymes (Kholif et al., 2017), essential oils (Hernandez et al., 2017) and yeast (Elghandour et al., 2017), with enhancing the nutritive value of feeds. Such feed additives can reduce energy losses as CH_4, and nitrogen as ammonia, which reduce animal performance and contribute to the release of pollutants to the environment (Johnson and Johnson, 1995). The mechanisms where feed additives can affect CH_4 and CO_2 productions depend on altering the ruminal microbial populations, especially methanogenic bacteria. Some feed additives can compete and metabolize H_2 for other process than its utilization with methanogens, resulting in a reduced CH_4 formation and emissions (Reddish and Kung, 2007). Hydrogen in the rumen together with CO_2 is used to synthesize CH_4 by methanogenic microorganisms.

Tree leaves contain considerable amounts of secondary metabolites (Salem et al., 2014a,c) which may be used as a dual purpose as a feed ingredient and also as a feed additive to reduce GHG emission of animals. Tree leaves and plant secondary metabolites are generally considered as safe in order to modify rumen microbial fermentation process (Kholif et al., 2015). The phytoconstituents such as saponins, phenolics, terpenoids, tannins, phenolic glycosides, alkaloids, essential oils etc. have the potentiality to modify the rumen fermentation process (Salem et al., 2014a,c). Moreover, the effectiveness of phytogenic feed additives depends upon the type, source and level of active secondary metabolite (Elghandour et al., 2015a,b). Phytogenic feeds, rich in plant secondary metabolites, have improved the animal's nutrient digestion due to the effectiveness of their metabolites on ruminal activity (Kholif et al., 2015). The fermentation of dietary carbohydrates in the rumen produces gases which especially constitutes CO_2 and CH_4. However, there is significantly lower production of gases during the fermentation of dietary proteins (Makkar et al., 1995). Moreover, some tree leaves and plant extracts with high flavonoids and tannins contents decreased the CH_4 yield with high microbial biomass production (Broudiscou et al., 2002). Apart from those phytoconstituents, phenolics and saponins are crucial secondary metabolites. In fact, phenols and saponins enriched leaves have potential for increasing the efficiency of feed utilization, and decreasing methanogenesis. These two bioactive metabolites modify ruminal fermentation, and have been suggested to suppress rumen protozoa and bacteria, finally mitigating the gas production (Dohme et al., 1999). Revealing further the vast bioactivity and significant gas mitigation characteristics of phenols and saponins, the present study was undertaken to estimate mainly the total phenolics and saponins of nine different tree leaves.

Recent investigations exploiting the tree leaves and plant extracts have become interested among researchers in evaluating a successful alternative to modulate rumen fermentation and the production of GHG during rumen fermentation (Elahi et al., 2016).

The *in vitro* gas production (GP) strategy is preferred to *in vivo* technique because of inexpensive and suitable tool for use in developing countries. The *in vitro* GP technique helps to study potential rumen degradation of ruminant feeds very effectively (Vallejo et al., 2016) and allows the estimation of substrate required to produce volatile fatty acids and the energetic value of feed. The application of tree leaves and plant extracts and their phytoconstituents seems to be a significant approach not only because of eco-friendly nature but also due to easy availability and non-toxicity properties.

Keeping in view of the potential role of phytoconstituents in reducing the production of harmful gases, the present context was conducted to study the fermentation kinetics and mitigation of GHG emission by nine types of plant leaves against alfalfa hay incubated with rumen inoculum of different ages (at 20, 40, and 60 d) as a method for a clean and environment friendly product and as a means of improving animal feed conversion efficiency.

2. Materials and methods

2.1. Substrates

Nine plants were used as incubation substrates against alfalfa hay. The substrates include: *Medicago sativa* (Alfalfa hay), *Pistacia vera*; *Dalbergia retusa*, *Crescentia alata*, *Azadirachta indica*, *Eichhornia crassipes*, *Cnidoscolus chayamansa*, *Guazuma ulmifolia*, *Vitex mollis*, *and Moringa oleifera*. Leaves of the plants species were randomly and manually harvested from different parts of plants to obtain three individual samples of young and mature leaves from each plant species. Leaf samples were dried at 40 °C for 72 h in an oven to achieve constant weight, ground in a hammer mill to pass through a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components, secondary metabolites, and *in vitro* GP.

2.2. In vitro incubations

Rumen inoculum was collected by stomach tube from 6 young calves (Holstein, with a live weight of 40–55 kg) at 20, 40 and 60 d of age using stomach tube. Calves were fed daily one time at 11:00 h on a total local formulated mixed ration containing (/kg dry matter (DM)): 200 g canola, 625 g sorghum grains, 150 g soybean meal, and 25 g mineral salts. The diet contained (/kg): 200 g crude protein, 230 g neutral detergent fiber, 50.3 g acid detergent fiber and 35.6 g either extract. The diet was formulated to cover their nutrient requirements. Calves were received 2 L of milk at 07:00 h and other 2 L at 16:00 h with a free grazing time from 8:00 to 16:00 h during the day on ryegrass and white clover *ad libitum* with free access to fresh water at all times during rumen contents collection phase.

Individual rumen content samples were equally collected from the rumen of each calf using a stomach tube and then mixed and homogenized, which were further mixed with the Goering and Van Soest (1970) buffer solution without trypticase in the ratio of 1:4 (v/v). The incubation media was then mixed and strained through four layers of cheese cloth into a flask with an O_2-free headspace, and used to inoculate three identical runs of incubation in 120 mL serum bottles containing 0.5 g DM of substrate.

Bottles with substrates, in addition to three bottles without substrate were used as blanks. After filling all bottles, they were flushed with CO_2 and immediately closed with rubber stoppers, shaken and placed in incubator at 39 °C. Gas production was recorded at 2, 4, 6, 8, 10, 12, 14, 24, 36, and 48 h using the Pressure Transducer Technique (Extech instruments, Waltham, USA) (Theodorou et al., 1994). The production of CO_2 and CH_4 was recorded using Gas-Pro detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK) at 2, 6, 10, 14, 24, 36, and 48 h of incubation.

Further, as described by Rodriguez et al. (2015), and at the end of incubation period, bottles were uncapped and the pH was measured using a digital pH meter (Conductronic pH15.0, Puebla, Mexico), and the residual of each bottle was filtered under vacuum through glass crucibles with a sintered filter. The fermentation residues were dried at 65 °C for 72 h in order to estimate DM digestibility (DMD) (Orskov and McDonald, 1979).

2.3. Chemical analyses, secondary metabolites assay, and calculations

Samples of the substrates were analyzed for DM (#934.01), ash (#942.05), nitrogen (#954.01), and ether extract (#920.39) according to AOAC (1997) official methods. Extracts of plant species leaves were prepared according to Salem et al. (2014a,c) with slight

modifications. In brief, leaves were collected randomly from several young and mature trees during summer, chopped into 1–2 cm lengths, and immediately extracted at 1 g leaf/8 mL of water. Plant materials were individually soaked and incubated in water at 25 to 30 °C for 72 h in closed jars of 20 L. After incubation, jars were heated at 39 °C for 1 h and then immediately filtered. Filtrates were collected further and stored at 4 °C for the analysis of secondary metabolites.

Plant secondary metabolites were determined in each plant leaves according to the methods of Salem et al. (2014a,c). About 20 mL of plant extract was fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont®, Monterrey, Mexico) to determine total phenolics (TP) by drying and quantifying the TP layer in the funnel. After TP separation, a double volume of *n*-butanol (99.9/100, analytical grade, Fermont®, Monterrey, Mexico) was added to fractionate the saponins. The chemical composition and secondary metabolites concentrations of the plants tested are shown in Table 1.

To estimate the kinetic parameters of GP, CH_4 and CO_2, data of GP, CH_4 and CO_2 (mL/g DM) were fitted using the NLIN option of SAS (2002) according to the equation of France et al. (2000) as follows:

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

where: A is the volume of GP, CH_4 or CO_2 at time t; *b* is the asymptotic GP, CH_4 or CO_2 (mL/g DM); *c* is the rate of GP, CH_4 or CO_2 (/h), and *Lag* (h) is the discrete lag time prior to GP, CH_4 or CO_2.

2.4. Statistical analyses

Data of each of the three runs within the same sample of each plant leaves species (substrates) were averaged before statistical analysis. Mean values of each individual sample were used as the experimental unit. The experimental design was completely randomized with repeated measures in time, where individual sample was the experimental units. The statistical model used for the analysis was:

$$y_{ijkl} = \mu + d_i + a(d)_{j(i)} + p_k + (dp)_{ik} + e_{ijkl}$$

where y_{ijk} is the value measured at period k (day of rumen collection) on the j^{th} calf assigned to the i^{th} plant, μ the overall mean effect, d_i is the i^{th} fixed plant effect, $a(d)_{j(i)}$ is the random effect of the j^{th} calf within the i^{th} extract, p_k is the fixed k^{th} period (age time) effect when the measurement was taken, $(dp)_{ik}$ is the fixed interaction effect between plant and period, and $\varepsilon_{ijk}$is the random error associated with the j^{th} calf assigned to the i^{th} diet at period k.

Data were analyzed using the MIXED procedure of SAS (2002) for repeated measures. The structure of the variance–covariance error matrix employed was unstructured, based on Bayesian criteria observed with several alternative structures. Terms in the model were plant, experimental day and plant × period, with calf included as a random effect. The repeated term was day (age time), with calf within each treatment of the subject. Results reported in tables and in text were least square means of fixed effects with their corresponding standard errors. Test of simple effects were used to partition (slice) interaction effects by diet in order to test effects of period separately for each diet (SAS, 2002).

3. Results

3.1. In vitro gas production (GP) and fermentation kinetics

In vitro rumen GP (mL/g incubated DM) of plant species incubated in the rumen liquor of calves collected at 20, 40 and 60 d of ages are shown in Fig. 1. The inclusion of *Cnidoscolus chayamansa* depicted the maximum amount of GP when compared to other plant leaves and alfalfa hay in the rumen liquor of calves collected at 20 and 60 d of ages. In the contrary to this, *Azadirachta indica* showed the maximum production of gas after fermentative process in the rumen liquor of calves collected at 40 d of age. The level of asymptomatic GP (mL/g DM) was affected ($P < 0.05$) with different tree leaves against alfalfa hay. The fractional rate of GP and lag period also showed significant effect ($P < 0.05$) but the lag period was not affected by substrate × dose interaction (Table 2).

3.2. In vitro CH_4 production

Fig. 2 depicts *in vitro* rumen CH_4 production (mL/g incubated DM) of plant extracts incubated in the rumen liquor of calves collected at 60 d of ages. There was no CH_4 production estimated at 20 and 40 d for all the tree leaves incubated. Most of the tree leaves reduced significantly the production of CH_4. The tested tree leaves showed significant effect ($P < 0.05$) on the level of asymptomatic GP (mL/g DM), fractional rate of GP and lag time (Table 2).

3.3. In vitro CO_2 production

In vitro rumen CO_2 production (mL/g incubated DM) of tree leaves incubated in the rumen liquor of calves collected at 20, 40, and 60 d of ages are shown in Fig. 3. *Crescentia alata, Guazuma ulmifolia*, and *Moringa oleifera* when compared to alfalfa hay showed drastic reduction in the CO_2 production (mL/g incubated DM) of rumen liquor of calves collected at 20, 40, and 60 d of ages, respectively. The asymptomatic CO_2 production and fractional rate of CO_2 production decreased effectively ($P < 0.01$) with tree leaves compared with alfalfa hay. The asymptomatic CO_2 production was also affected by substrate and substrate × dose interaction. But the

Table 1
Chemical composition and secondary metabolites concentrations (g/kg) of the substrates used.

	OM	EE	CP	NSC	NDF	ADF	ADL	Total phenolics	Saponins
Alfalfa hay	850	19	219	161	451	302	298	6	10
Pistacia vera	849	54	121	350	324	292	106	60	41
Dalbergia retusa	859	16	239	105	499	459	181	31	26
Crescentia alata	770	14	88	199	469	423	92	35	43
Azadirachta indica	824	20	176	318	310	284	156	69	44
Eichhornia crassipes	785	22	195	60	508	481	76	23	25
Cnidoscolus chayamansa	880	59	341	328	152	139	34	25	48
Guazuma ulmifolia	833	27	130	215	461	400	165	52	20
Vitex mollis	830	15	86	272	457	439	143	68	26
Moringa oleifera	839	42	276	298	223	195	79	23	43

ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein; DM, dry matter; EE, ether extract; NDF, neutral detergent fiber; NSC, non-structural carbohydrates; OM, organic matter.

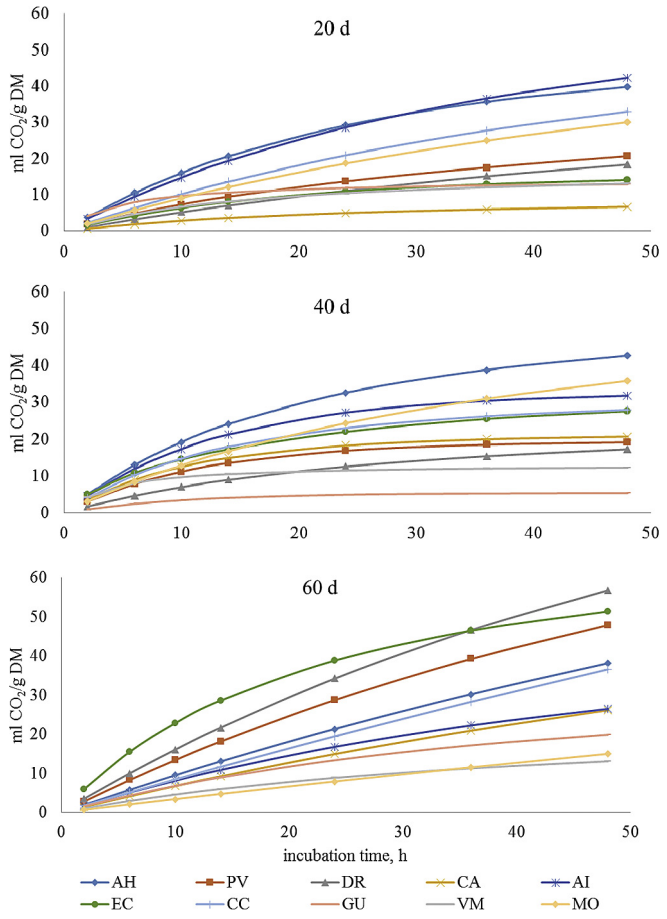

Fig. 1. *In vitro* rumen gas production (mL/g incubated DM) of 10 plant species incubated in the rumen liquor of calves collected at 20, 40 and 60 d of ages. AH, Alfalfa hay; PV, *Pistacia vera*; DR, *Dalbergia retusa*; CA, *Crescentia alata*; AI, *Azadirachta indica*; EC, *Eichhornia crassipes*; CC, *Cnidoscolus chayamansa*; GU, *Guazuma ulmifolia*; VM, *Vitex mollis*; MO, *Moringa oleifera*.

fractional rate of CO_2 production was unaffected ($P > 0.05$) by substrate × dose interaction. In the same manner, the lag time of CO_2 production was unaffected by substrate and substrate × dose interaction. However, the rumen liquor of calves collected at 40 d of age increased the lag time of CO_2 production (Table 2).

3.4. *In vitro* rumen fermentation profile

The effects of tree leaves and alfalfa hay on *in vitro* rumen fermentation profile are depicted in Fig. 4 and Table 2. Maximum rumen GP was observed in the rumen liquor of calves collected at 20 d of age. *In vitro* rumen CH_4 (mL/g incubated DM) and CO_2 production (mL/g incubated DM) of tree leaves incubated in the rumen liquor of calves collected at 60 d of age increased effectively. Fermentation pH was found to be significant ($P < 0.001$) parameter by substrate, dose, and substrate × dose interaction. On the other hand, DM degradability ($P > 0.05$) was not significant by dose and substrate × dose interaction.

3.5. *Proportional in vitro CH_4 and CO_2 production*

Plant leaves and alfalfa hay showed increased CH_4 production (mL/g incubated DM) after 6, 24, and 48 h of incubation. But the tree leaves showed lower production of CH_4 (mL/g incubated DM) when compared to alfalfa hay after the required period of incubation at significant level ($P < 0.05$). Moreover, CH_4 production (mL/g degraded DM) was increased ($P < 0.001$) after 6, 24, and 48 h of incubation with the inclusion of extracts, and data were more or less effective to the CH_4 production (mL/g degraded DM) using alfalfa hay. Increasing the incubation time from 6 h to 48 h resulted in the increased proportional CH_4 production by the inclusion of tree leaves as well as alfalfa hay. But the proportional CH_4 production at different time period by the plant leaves was found to be less when compared to the alfalfa hay. The proportional CH_4 production was unaffected ($P > 0.05$) by substrate and substrate × dose interaction (Table 3). In contrary to this, different tree leaves and alfalfa hay had no influence ($P > 0.05$) on CO_2 production (mL/g

Table 2
In vitro rumen gas, methane (CH_4) and carbon dioxide (CO_2) and fermentation kinetics of nine types of plant leaves against alfalfa hay incubated with rumen inoculum of different ages.

	Gas production (mL/g DM)[2]			CH_4 production (mL/g DM)[3]			CO_2 production (mL/g DM)[4]			Fermentation kinetics	
	b	*c*	*Lag*	*b*	*c*	*Lag*	*b*	*c*	*Lag*	pH	DMD
Substrate (S)											
Alfalfa hay	202^{b}	0.056^{de}	1.39^{ab}	1.94^{bc}	0.005^{cde}	1.31^{ab}	77^{ab}	0.041	9.72	6.42^{e}	588^{b}
P. vera	180^{bc}	0.072^{cde}	2.27^{ab}	1.43^{cde}	0.007^{cd}	1.26^{ab}	78^{ab}	0.045	7.71	6.66^{ab}	599^{b}
D. Retusa	150^{cd}	0.073^{cde}	1.38^{ab}	1.25^{cde}	0.007^{bc}	1.25^{ab}	53^{bc}	0.029	5.00	6.71^{a}	477^{c}
C. alata	173^{bc}	0.080^{cd}	1.65^{ab}	1.63^{ac}	0.006^{cd}	1.62^{a}	59^{bc}	0.042	7.40	6.51^{bcde}	444^{c}
A. indica	186^{bc}	0.084^{c}	1.74^{ab}	0.94^{cde}	0.011^{ab}	1.50^{ab}	56^{bc}	0.039	6.57	6.59^{abcd}	506^{c}
E. crassipes	117^{de}	0.142^{b}	2.13^{ab}	0.77^{de}	0.015^{a}	1.19^{ab}	43^{bc}	0.077	5.48	6.73^{a}	470^{c}
C. chayamansa	245^{a}	0.050^{e}	1.74^{ab}	2.98^{b}	0.003^{de}	1.31^{ab}	111^{a}	0.034	9.86	6.50^{cde}	838^{a}
G. ulmifolia	90^{e}	0.192^{a}	2.63^{a}	0.51^{e}	0.014^{a}	0.71^{b}	15^{c}	0.097	5.38	6.63^{abc}	246^{d}
V. mollis	89^{e}	0.142^{b}	2.18^{ab}	0.44^{e}	0.013^{a}	0.96^{ab}	17^{c}	0.099	7.12	6.54^{bcde}	457^{c}
M. oleifera	196^{b}	0.051^{e}	1.17^{b}	4.30^{a}	0.002^{e}	1.33^{ab}	86^{ab}	0.024	6.46	6.44^{de}	766^{a}
SEM	8.4	0.0054	0.285	0.228	0.0008	0.179	10.7	0.0189	1.298	0.035	16.7
Sampling day (D)											
d 20	166^{b}	0.112^{a}	1.79^{ab}	ND^{b}	ND^{b}	ND^{b}	55^{b}	0.044^{b}	6.29	6.53^{b}	558^{a}
d 40	149^{b}	0.094^{b}	2.18^{a}	ND^{b}	ND^{b}	ND^{b}	28^{c}	0.095^{a}	8.60	6.53^{b}	534^{ab}
d 60	173^{b}	0.076^{c}	1.52^{b}	4.87^{a}	0.025^{a}	3.73^{a}	96^{a}	0.019^{b}	6.32	6.66^{a}	526^{b}
SEM	4.6	0.0030	0.156	0.125	0.0005	0.098	5.9	0.0100	0.711	0.019	9.1
F test probabilities											
S	<0.001	<0.001	0.012	<0.001	<0.001	0.046	<0.001	0.037	0.111	<0.001	<0.001
D	0.001	<0.001	0.014	<0.001	<0.001	<0.001	<0.001	<0.001	0.038	<0.001	0.040
S x D	0.005	0.010	0.082	<0.001	<0.001	0.018	0.035	0.603	0.649	<0.001	0.018

[a,b,c,d,e]Mean values within for substrates (S) or sampling days (D) with different letters differ ($P < 0.05$).
[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]*b* is the asymptotic methane production (mL/g DM); *c* is the rate of methane production (/h); *Lag* is the initial delay before methane production begins (h).
[3]*b* is the asymptotic carbon dioxide production (mL/g DM); *c* is the rate of carbon dioxide production (/h); *Lag* is the initial delay before carbon dioxide production begins (h).
DMD is the DM degradability; ND is not detected; SEM is the standard error of the mean.

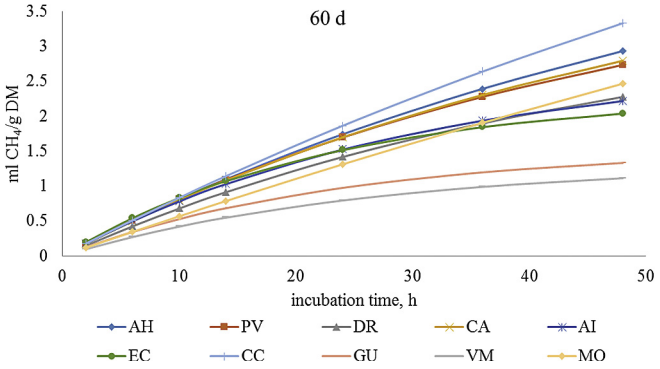

Fig. 2. *In vitro* rumen CH_4 production (mL/g incubated DM) of 10 plant species incubated in the rumen liquor of calves collected at 60 d of ages. Methane production at 20 and 40 d for all the plant species incubated was not detected. AH, Alfalfa hay; PV, *Pistacia vera*; DR, *Dalbergia retusa*; CA, *Crescentia alata*; AI, *Azadirachta indica*; EC, *Eichhornia crassipes*; CC, *Cnidoscolus chayamansa*; GU, *Guazuma ulmifolia*; VM, *Vitex mollis*; MO, *Moringa oleifera*.

incubated DM and mL/g degraded DM) and proportional CO_2 production (Table 4).

4. Discussion

At present, there is an emerging concern to reduce feed antibiotics due to the increment in the total counts of antibiotics resistant bacteria. Hence, new inexpensive and easily available additives are required which have the characteristics of modifying the rumen fermentation process, with no residues in the animal products. Plant leaves and their extracts are suitable alternatives to commercially available antibiotics that could be incorporated to modulate the rumen fermentation (Salem et al., 2014a,c). In the present experiment, the asymptomatic GP differed between the different types of tree leaves compared with alfalfa hay, revealing the ability of the tested tree leaves to modify and improve ruminal fermentation (Salem et al., 2014a,c). For example, *C. chayamansa* and *A. indica* leaves showed the maximum GP compared with other plant leaves or alfalfa hay. It is well documented that the *in vitro* higher GP is an efficient indicator of the ruminal fermentation of feeds, representing the good fermentability, digestibility and a better nutrient availability for ruminants (Salem et al., 2014b), revealing that both of *C. chayamansa* and *A. indica* leaves succeeded to provide the ruminal bacteria with more nutrients compared with the other leave species resulting in better degradability or fermentability of feed. In general, the higher digestion of diets or feed is estimated based on the high fractional rate of GP because the rate of digestion of feed or its constituents resembles the extent of digestion. The growth of the microorganisms and accessibility of the diet or feed to the respective enzymes indicates the rate of the fermentation of the constituents (Getachew et al., 2004). In the line with this research, several findings have demonstrated that plant secondary metabolites at lower and moderate concentrations have the capability to enhance rumen fermentation and GP (Cedillo et al., 2014). The improvement in the GP with the utilization of

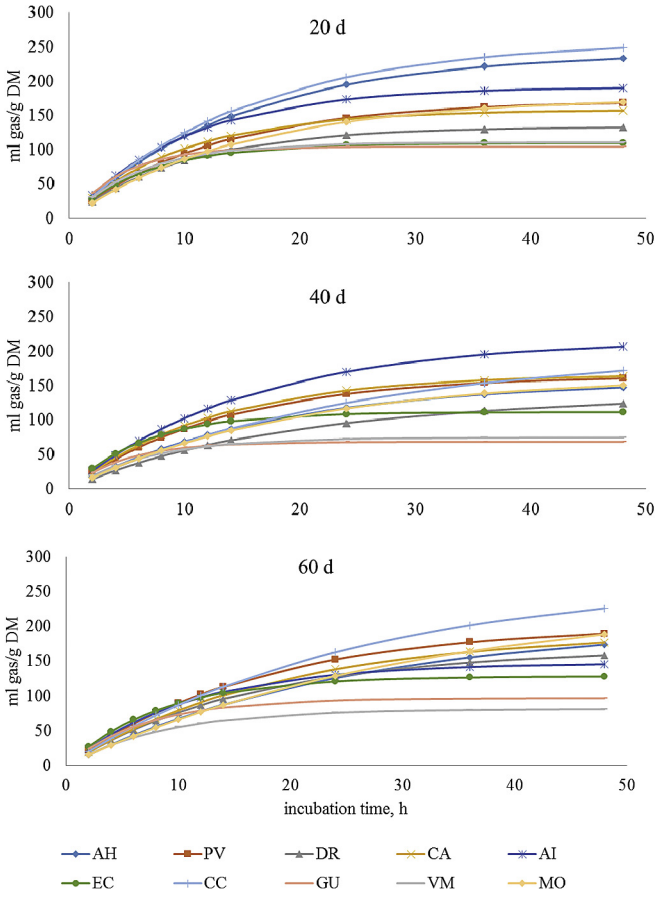

Fig. 3. *In vitro* rumen CO_2 dioxide production (mL/g incubated DM) of 10 plant species incubated in the rumen liquor of calves collected at 20, 40, and 60 d of ages. AH, Alfalfa hay; PV, *Pistacia vera*; DR, *Dalbergia retusa*; CA, *Crescentia alata*; AI, *Azadirachta indica*; EC, *Eichhornia crassipes*; CC, *Cnidoscolus chayamansa*; GU, *Guazuma ulmifolia*; VM, *Vitex mollis*; MO, *Moringa oleifera*.

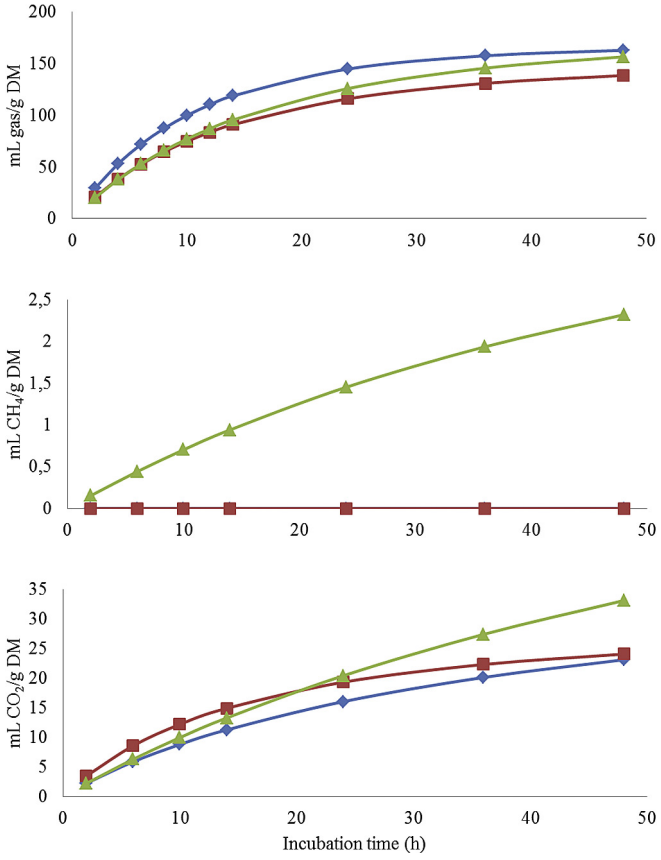

Fig. 4. Fermentation capacity of calves rumen gas, methane and carbon dioxide productions at 20 (- ◆ -), 40 (- ■ -), and 60 (- ▲ -) d of age. Values are average of plant species.

Table 3
Proportional *in vitro* methane (CH_4) production as a percent of total gas production of nine types of plant leaves against alfalfa hay incubated with rumen inoculum of different ages.

	CH_4 production								
	mL/g incubated DM			mL/g degraded DM			Proportional CH_4 production		
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Substrate (S)									
Alfalfa hay	0.17^{ab}	0.58^{a}	0.98^{ab}	0.10^{cde}	0.73^{cd}	1.42^{bcde}	0.38	0.46	0.56
P. vera	0.17^{a}	0.57^{a}	0.91^{ab}	0.14^{bcde}	0.91^{bc}	1.59^{abcd}	0.28	0.37	0.48
D. Retusa	0.14^{ab}	0.47^{abc}	0.76^{abc}	0.16^{bcd}	0.96^{bc}	1.67^{abc}	0.28	0.37	0.48
C. alata	0.16^{ab}	0.56^{a}	0.93^{ab}	0.17^{bcd}	1.17^{ab}	2.14^{a}	0.32	0.41	0.53
A. indica	0.17^{ab}	0.51^{ab}	0.74^{abcd}	0.17^{bc}	0.98^{bc}	1.57^{abcd}	0.28	0.40	0.52
E. crassipes	0.18^{a}	0.50^{abc}	0.68^{bcd}	0.19^{b}	0.98^{bc}	1.44^{bcd}	0.28	0.43	0.55
C. chayamansa	0.17^{a}	0.62^{a}	1.11^{a}	0.09^{de}	0.68^{cd}	1.32^{cde}	0.30	0.38	0.49
G. ulmifolia	0.11^{ab}	0.32^{bc}	0.44^{cd}	0.33^{a}	1.37^{a}	1.98^{ab}	0.20	0.35	0.46
V. mollis	0.09^{b}	0.27^{c}	0.37^{d}	0.12^{bcde}	0.58^{d}	0.86^{e}	0.23	0.35	0.46
M. oleifera	0.11^{ab}	0.44^{abc}	0.82^{ab}	0.07^{e}	0.53^{d}	1.09^{de}	0.27	0.34	0.43
SEM	0.017	0.052	0.081	0.017	0.068	0.124	0.038	0.043	0.047
Sampling day (D)									
d 20	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
d 40	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
d 60	0.44^{a}	1.45^{a}	2.32^{a}	0.46^{a}	2.67^{a}	4.52^{a}	0.85^{a}	1.16^{a}	1.49^{a}
SEM	0.009	0.028	0.044	0.009	0.037	0.068	0.021	0.024	0.026
F test probabilities									
S	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	0.143	0.583	0.632
D	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
S × D	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	0.096	0.649	0.710

[a,b,c,d,e]Mean values within for substrates (S) or sampling days (D) with different letters differ ($P < 0.05$).
ND is not detected; SEM is standard error of the mean.

different plant leaves suggest that the plant secondary metabolites contained fermentable and digestible carbohydrates (Elghandour et al., 2015a,b). The fractional rate of GP and lag time of GP were varied between the tested leaves types. The increment in the GP likely promoted higher nutrient availability to ruminants. In this study, the incubation of tree leaves delayed the adaptational strategy of microorganisms to the diets, showing longer lag period and delayed GP. In like manner, lower lag phase suggests its availability to provide a better proportional of nutrients (Ferraro et al., 2016).

It is very important to show that the varied responses of plant leaves to the GP parameters might be due to the nature, activity, concentration of its active constituents, and other factors that can affect the concentration of phytoconstituents (Salem et al., 2014a,c). Additionally, genotypic characteristics of plant metabolites could also be responsible for the variation in the GP among different incubated substrates (Elghandour et al., 2015a,b).

At 20 and 40 d of calves age, no CH_4 production was observed, and this was expected because at this age, the ruminal microflora are not complete and lacks time for the complete development

Table 4
Proportional *in vitro* carbon dioxide (CO_2) production as a percent of total gas production of nine types of plant leaves against alfalfa hay incubated with rumen inoculum of different ages.

	CO_2 production								
	mL/g incubated DM			mL/g degraded DM			Proportional CO_2 production		
	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Substrate (S)									
Alfalfa hay	9.78	27.7	40.2	2.53^{bc}	40.7	77.9	18.1	19.4	21.9
P. vera	6.97	19.8	29.2	4.97^{abc}	38.0	75.7	11.6	13.7	16.8
D. Retusa	5.93	19.3	30.8	3.79^{abc}	32.4	62.4	12.4	16.1	20.6
C. alata	5.00	12.7	17.9	4.20^{abc}	38.5	73.0	7.9	8.7	10.2
A. indica	8.83	24.2	33.5	5.09^{abc}	43.1	72.8	11.4	14.4	17.8
E. crassipes	10.13	23.9	31.0	7.31^{ab}	47.1	72.0	15.7	20.8	25.5
C. chayamansa	7.23	21.1	32.5	2.41^{c}	26.0	57.2	13.9	14.0	15.5
G. ulmifolia	4.87	10.0	12.6	7.46^{a}	38.5	55.7	7.4	10.7	13.3
V. mollis	5.19	10.2	12.8	3.11^{abc}	19.4	29.5	10.6	12.3	14.8
M. oleifera	5.39	17.0	27.0	2.74^{abc}	22.7	46.4	11.8	13.3	16.1
SEM	1.599	4.34	6.73	1.050	6.03	11.67	2.55	2.82	3.56
Sampling day (D)									
d 20	5.89	16.0	23.1	4.39^{ab}	32.1^{ab}	53.8^{b}	8.1^{b}	10.8^{b}	13.7^{b}
d 40	8.60	19.3	24.1	2.51^{b}	29.3^{b}	44.1^{b}	16.6^{a}	16.1^{a}	16.7^{ab}
d 60	6.31	20.4	33.1	6.18^{a}	42.5^{a}	88.8^{a}	11.5^{b}	16.1^{a}	21.4^{a}
SEM	0.876	2.38	3.69	0.575	3.30	6.39	1.40	1.55	1.95
F test probabilities									
S	0.141	0.06	0.069	0.004	0.026	0.097	0.128	0.111	0.149
D	0.071	0.405	0.115	0.002	0.016	<0.001	0.003	0.025	0.024
S × D	0.165	0.312	0.511	0.115	0.057	0.127	0.111	0.161	0.248

[a,b,c,d,e]Mean values within for substrates (S) or sampling days (D) with different letters differ ($P < 0.05$).
ND is not detected; SEM is standard error of the mean.

(Kamra, 2005). A reduced CH_4 production was observed with the incubated tree leaves when being compared with alfalfa hay, where most of produced CH_4 was noted at late hours of incubation. This is normal in ruminant (Elghandour et al., 2016a) and also in non-ruminant animals (Kholif et al., 2016). Greenhouse gases especially CH_4 and CO_2 are produced in rumen during the feed fermentation and degradation. In the present context, replacing alfalfa hay with various plant leaves showed negative effect on *in vitro* CH_4 production, demonstrating the potentiality of plant secondary metabolites in reducing the global warming effects. The mitigation of CH_4 emission might be due to the reason that high cell wall content reduced the activity of microorganisms, causing decreased production of CH_4. Similar observations were reported by Broudiscou et al. (2002) who estimated the lowered production of CH_4 due to the plant extracts tested. Few of the plant leaves increased the lag period of CH_4 production, illustrating that the initial delay before CH_4 production begins is dependent upon the nature of plants and their metabolites. The delayed adaptation of methanogenic microorganisms to the few plants in rumen is the possible reason for the delayed onset of CH_4 production and hence a longer lag period. Similarly, the lack of *in vitro* rumen CH_4 emission at 20 and 40 d of ages of calves suggested that the high cell wall content reduced the activity of microorganisms, causing lack of CH_4 production at 20 and 40 d of ages.

Drastic reductions in the CO_2 production were observed with *C. alata, G. ulmifolia,* and *M. oleifera*. The reduction in CO_2 emission is very important from the perspective of global warming. Most of the plants showed significant mitigation in CO_2 production. Substrate × dose had no influence on the reduction of CO_2 production. It might be due to the non-significant effects of these parameters on GP. As suggested earlier, the reduction in the CO_2 production and lack of significance in the lag period might be due to the increased cell wall content, causing lowered activities of microorganisms.

The lower ruminal pH due to the inclusion of different plant leaves suggested the degradation of leaves to emit volatile fatty acids. The decline in the ruminal pH because of the incubation of various tree leaves was found to be more or less similar to the ruminal pH values compared with alfalfa hay. Previous reports have illustrated the correlation between fermentable carbohydrates, pH, and volatile fatty acids (Ramos et al., 2009). Similarly, few of the plant leaves tested here showed increased *in vitro* DMD values. The study appears to be promising due to the more degradation of plants leaves compared to alfalfa hay. Akinfemi et al. (2009) reported *in vitro* DMD to be positively correlated with the rate of GP.

5. Conclusion

Different plants studied in the context of this research appear to have a promising influence on rumen fermentation by emitting low amount of GHG, especially CH_4 and CO_2. Most of the studied plant leaves reduced CH_4 production with about 16–77%, and CO_2 production with about 45–80% compared with alfalfa hay. These plant leaves can be used as valuable cleaner product and feedstuff for the environment and ruminants respectively in order to avoid the toxicity of ruminal gas produced anaerobically at large quantity. The reduction in CH_4 and CO_2 is comparable with many previous *in vitro* studies. Therefore, further *in vitro* and *in vivo* elaborative studies are needed to be carried out to realize the obtained results, and also the results of previous studies.

Conflict of interest

None declared.

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