Influence of aguamiel (*Agave atrovirens*) as a natural feed additive on cecal fermentation kinetics of some browse species in horse feeding

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Running head: Cecal fermentation of five plants species treated with aguamiel

Abstract

This study aimed to evaluate the effect of different dose levels of aguamiel (*Agave atrovirens*) on *in vitro* cecal gas, methane (CH_4) and carbon dioxide (CO_2) productions of five forage species (*Avena sativa* (hay)), *Moringa oleifera*, *Caesalpinia coriacea*, *Salix babylonica* and *Eichhornia crassipes*) using inocula from horse. The forage samples were incubated with three doses of aguamiel: 0, 34 and 68 µg of aguamiel/g dry matter (DM) of substrate. Cecal inocula were collected from 4 adult female Criolla horses (3 to 4 years of age and weighing 300 ± 15.0 kg) grazed on native grasses for about eight hours without supplementation. Forage type affected ($P < .001$) cecal asymptotic, rate and lag time of gas, CH_4 and CO_2 productions (mL/g DM) and pH and DM degradability. Aguamiel dose had linear and quadratic effects ($P < .05$) on the asymptotic and rate of CH_4 productions and rate and lag time of CO_2 productions (mL/g DM). Forage type x aguamiel dose interactions were significant ($P < .05$) for asymptotic, rate and lag time of gas, CH_4 and CO_2 productions (mL/g DM). Forage species effects were pronounced ($P < .05$) on CH_4 and CO_2 productions (mL/g incubated and degraded DM) and proportional CH_4 production at all hours of incubation, except for CO_2 production (mL/g incubated DM). Aguamiel dose affected ($P < .05$) CO_2 production (mL/g incubated DM) and proportional CO_2 production at the incubated hours. Forage type x aguamiel dose interactions were observed ($P < .05$) for CO_2 production (mL/g incubated DM) and proportional CO_2 production at the incubated hours but had no impact of CH_4 production. It is concluded that addition of aguamiel to five forage species affected fermentation kinetics of GP resulting in different *in vitro* cecal gas, methane and carbon dioxide productions from these substrates.

Keywords: aguamiel, cecal, forage, gas production, horse, methane, carbon dioxide.

1. Introduction

The ability of the horse to efficiently utilize fiber and roughages due to the presence of fermentative microorganisms in their hindgut, and the use of fibrous feeds as the main component of the mature horse diet has been documented [1,2].Forages are important primary natural component of horse diet needed

for normal function of their digestive system, and to suppress certain metabolic disorders like hindgut acidosis, laminitis and colic occasioned by feeding high-starch diets [3]. There is a renewed interest in utilizing fibrous ingredients as alternatives to starch-rich grains to horses as a way of covering their energy need and mitigating various diseases due to use of less fibrous and soluble carbohydrate sources. Forages of moderate to high nutritive value may meet the nutritional requirements of horses [4]. However, fibrous feeds such as forages are lignocellulosic, and poor in palatability, crude protein (CP) and digestibility [5,6]. Therefore, effective use of fibrous feeds requires some forms of treatment with feed additives to enhance their feeding value.

Feed additives, like exogenous enzymes, have been used to improve degradation of carbohydrate and cell wall in ruminant animals [7,8] and in equines [9], but little or nothing is known about the use of aguamiel, a natural feed additive, in horse nutrition. In recent years, supplementation of horse diet with feed additives has aroused the interest of livestock researchers [1,2,9,10]. Aguamiel (honey water) is the sap obtained from one of the agave species (*Agave atrovirens*) grown in the semi-desert areas of Mexico and used by Mexicans as a natural fortifying beverage. Multiple agave species including *Agave atrovirens, Agave salmiana, Agave mapisaga* and *Agave americana* are grown in the semi-desert areas of Mexico [11]. Aguamiel is a colorless, sweet sap-like juice from the core of the agave plant containing (w/w on dry matter basis) glucose, 26.5%; sucrose, 8.8%; fructose, 32.4%; water, gum, protein, minerals, vitamins and beneficial organisms such as *Kluyveromyces marcianus var. Bulgaricus* [12,13,14]. It is a rich source of fructans, such as inulin and fructooligosaccharides which have prebiotic property. Thus aguamiel has both prebiotic and probiotic properties. Aguamiel, used for the production of pulque (a drink with cultural importance in Mexico), contains fructooligosaccharides that are susceptible to fermentation in the colon by colonic microorganisms that produce short-chain fatty acids (SCFA), which reduce lipid and glucose levels in the blood and decrease the incidence of gastric lesions [11]. Besides, the anti-oxidant capacity and prebiotic effect of aguamiel during *in vitro* fermentation has been reported [11]. According to Tovar-Robles et al [15], aguamiel has been considered as a

neutraceutical product with nutritional value in animals' feeds and some other beneficial properties. In spite of these beneficial properties of aguamiel, there is a paucity of information on its nutritional roles as a natural feed additive in livestock. Romero-Lopez et al [11] observed a decreased pH and increased SCFA during the fermentation of aguamiel, with abundant acetate production indicating a good production of these compounds with possible beneficial effects of *in vivo* models.

The present experiment aimed to evaluate the cecal fermentative capacity of five plants species in presence of different levels of a natural feed additive of aguamiel in equine feeding.

2. Materials and methods

2.1. Substrate and Aguamiel

Five forage species were used as incubation substrates. The substrates, *Avena sativa* (hay), *Moringa oleifera*, *Caesalpinia coriacea*, *Salix babylonica* and *Eichhornia crassipes*, were incubated with aguamiel (*Agave atrovirens*) at 0, 34 and 68 µg of aguamiel/g DM of substrate. The chemical composition of the substrates used is shown in Table 1.

Aguamiel extracts were obtained from *A. atrovirens* grown in Toluca, Estado de México, México by draining the wound left in the plant after removing the shoot apex. Aguamiel extracts were collected with the help of agave growers who extracted the sap over 60 days; the extracts were kept in sterilized jars maintained at 4 ºC. The agave plants, which were under commercial exploitation, were selected at random by the agave growers. The macro - and micro-nutrients of the aguamiel are shown in Table 2.

2.2. In Vitro Incubations

Before starting incubation, cecal contents (the inoculum source, one kg from each horse) were collected from the local slaughterhouse of Toluca, Mexico State, Mexico from 4 adult female Criolla horses (3 to 4 years of age and weighing 300 ± 15 kg). Horses had about eight hours grazing and were given water twice a day without feed supplementation. They grazed predominantly on pasture containing two native

grasses (*Festuca arundinacea* and ryegrass). Individual cecal samples were equally collected from the cecum of each animal and then mixed and homogenized to obtain a homogenized sample of fecal contents which were mixed with the Goering and Van Soest [16] buffer solution without trypticase in the ratio of 1:4 v/v. The incubation media was subsequently mixed and strained through four layers of cheesecloth into a flask with an O_2-free headspace, and used to inoculate three identical runs of incubation in 120-mL serum bottles containing 1 g DM of substrate in presence of different doses of Aguamiel (i.e., 0, 34 and 68 µg/g DM).

Bottles with substrates plus three bottles without substrate and aguamiel as blanks were used. After filling all bottles, they were flushed with CO_2 and immediately closed with rubber stoppers, shaken and placed in an incubator set at 39 ºC. Gas and CO_2 productions were recorded at 6, 24 and 48 h using the Pressure Transducer Technique (Extech instruments, Waltham, USA) of Theodorou et al [17]. The production of CO_2 was recorded using Gas-Pro detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK).

As described in Rodriguez et al [18], at the end of incubation after 48 h, bottles were uncapped and the pH was measured using a digital pH meter (Conductronic pH15, Puebla, Mexico), and the residual of each bottle was filtered under vacuum through glass crucibles with a sintered filter, then fermentation residues dried at 65 °C for 72 h to estimate DM disappearance (DMD) [19].

2.3. Chemical Analyses and Calculations

Samples of the substrates were analyzed for DM, ash, N and EE according to AOAC [20]. The neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin analyses were carried out using an ANKOM200 Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA) according to AOAC [20]. The NDF was assayed without the use of an alpha amylase and sodium sulfite. Both NDF and ADF are expressed without residual ash. The minerals content of aguamiel was carried out using an atomic absorption spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI). Mesophilic

bacteria and yeast counts were enumerated by cultural methods using Heart Brain Infusion agar and Potato Dextrose Agar, respectively, and standard plate count agar for total counts.

Extracts of plant species leaves were prepared according to Salem et al [21]. Briefly, leaves were collected randomly from several young and mature trees during summer, chopped into 1 to 2 cm lengths and immediately extracted at 1 g leaf/8 mL of solvent mixture. The mixture of solvents contained 10 mL methanol, 10 mL ethanol and 80 mL distilled water. Plant materials were individually soaked and incubated in solvent in the laboratory at 25 to 30˚C for 48 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 and stored at 4˚C for analysis of secondary metabolites.

As described in Salem et al [21], secondary metabolites were determined in each plant extract. Extracts, 10 mL, were fractionated by funnel separation with a double volume of ethyl acetate to determine total phenolics by drying and quantifying total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol, was added to fractionate saponins.

To estimate the kinetic parameters of GP, results of GP (mL/g DM) were fitted using the NLIN option of SAS [22] according to the equation of France et al [23] as:

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

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

2.4. Statistical Analyses

Data of each of the three runs within the same sample of each of the three individual samples of substrates were averaged before statistical analysis. Mean values of each individual sample were used as the experimental unit. Results of *in vitro* GP and rumen fermentation parameters were analyzed as a factorial experiment using the PROC GLM option of SAS [22] as:

$$Y_{ijk} = \mu + R_i + A_j + (R \times A)_{ij} + E_{ijk}$$

Where: Y_{ijk} is every observation of the *i*th substrate (R_i) with *j*th aguamiel dose (A_j); μ is the general mean; $(R \times A)_{ij}$ is the interaction between substrate type and Aguamiel dose; E_{ijk} is the experimental error. Linear and quadratic polynomial contrasts were used to examine responses to increasing addition levels of Aguamiel. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Chemical Composition and Secondary Metabolites

The CP content of Moringa forage was higher than that of the other forage species while Avena had the lowest CP content. Whereas NDF and ADF were lowest in Moringa, both NDF and ADL, and ADF were highest in Avena and Eichornia respectively. Concentrations of total phenolics and saponins were lowest in Salix and highest in Caesalpinia.

3.2. In vitro cecal gas, methane and carbon dioxide productions and fermentation kinetics

Forage type linearly affected asymptotic GP ($P < .05$), fractional rate of GP and lag time (Table 3 and Figure 1). Moringa had the highest and lowest values for asymptotic GP and rate of GP respectively, while lag time was highest for Avena. Except for lag time which showed a linear trend ($P = .005$), asymptotic GP and fractional rate of GP were not ($P > .05$) affected by aguamiel dose. Lag time was, however, highest for 34 μg/g DM aguamiel dose rate. Forage type x aguamiel dose interactions had no effect ($P > .05$) on the asymptotic GP, fractional rate of GP and lag time. Asymptotic CH_4 and CO_2, fractional rate of CH_4 and CO_2, and lag time of CH_4 and CO_2 productions were linearly affected ($P < .001$) by forage type, with asymptotic CH_4 and lag time of CH_4 productions being highest and rate of CH_4 production lowest in Moringa forage. Salix reduced both the asymptotic and rate of CO_2 productions while Moringa increased the lag time of CO_2 production. Whereas aguamiel dose had no effect ($P > .05$) on asymptotic CO_2 production, it linearly ($P = .039$) and quadratically ($P = .006$) affected the rate of CO_2 production, with production being higher for the control dose relative to 34 and 68 μg/g DM aguamiel

dose levels. Lag time of CO_2 production showed linear (P = .003) and quadratic (P < .001) trends, with the control dose having a greater value than the 34 and 68 µg/g DM aguamiel doses. Effects of forage type x aguamiel dose interactions were pronounced (P < .05) for asymptotic CO_2, rate of CO_2 and lag time of CO_2 productions. Moringa increased (linear effect, P < 0.001) both the pH and DMD. Effects of aguamiel dose, and forage species x aguamiel dose interactions were marginal (P > .05) for pH and DMD.

3.3. Proportional in vitro methane and carbon dioxide productions

Methane production (mL/g incubated DM) was linearly increased (P = .05) at 6, 24 and 48 h incubations by Avena (Figure 2). Aguamiel dose, and forage type x aguamiel dose interaction did not (P > .05) affect CH_4 production (mL/g incubated DM and mL/g degraded DM) at all hours of incubation. Proportional CH_4 production was not (P > .05) affected by the treatments and their interaction at all hours. Effect of forage type on CO_2 production (mL/g incubated DM) was not (P > .05) significant at incubation hours. Aguamiel dose quadratically affected (P < .05) CO_2 production (mL/g incubated DM) at all hours, with 34 µ/g DM having the lowest values at all hours. Avena forage increased (linear effect, P < .05) CO_2 production (mL/g degraded DM) at all hours. Forage type x aguamiel dose interaction effects were not (P > .05) significant for CO_2 production (mL/g degraded DM) at all hours. Eichhornia forage increased (linear, effect P < .05) proportional CO_2 production at all hours. Proportional CO_2 production at all hours were linearly and quadratically affected (P < .05) by aguamiel dose, with 34 µ/g DM dose having the lowest production at all hours (Figure 3). Forage type x aguamiel dose interaction affected (P < .05) proportional CO_2 production.

4. Discussion

Except for Avena which is a grass fodder, the other forage species are non-grass fodders. The studied forage species had a good nutrient profile except for Avena, which had the lowest CP content of < 90

g/kg DM and the highest NDF and ADL contents. With the exception of Avena, the high CP content of the other forage species shows their potential to provide degradable N when used as supplements to a low quality roughage or grass such as Avena [24,25]. Low CP and high fiber contents generally have some implications on the nutritive value of a diet. All the non-grass fodders, especially Caesalpinia with highest levels of total phenolics and saponins, contained secondary metabolites which are known to affect feed utilization in livestock. The high content of total phenolics and saponins in Caesalpinia may have some negative impacts like depression of feed intake and digestibility and /or toxic effect on hindgut microorganisms in the horse.

The *in vitro* fermentation technique has been widely used to evaluate fermentation of feed as well as test the efficacy of feed additives in livestock due to its simplicity, sensitivity and efficiency. It has been used in ruminants and horses to evaluate nutritive value and utilization of feeds. The technique has proved a reliable and successful tool to evaluate the nutritive value of diets of equine using inoculum either from feces or cecal contents [10,26]. In the present study, the *in vitro* incubation period was extended to 48 h to ensure complete fermentation of the substrates, though the average transit time for ingesta passing through the gastrointestinal tract of the horse ranges between 36 and 38 h [27]. Based on the available information at our disposal, there are no studies on *in vitro* fermentation in horses using aguamiel-treated forage species incubated with cecal contents. Therefore, our explanations will borrow from studies with horses using fecal inocula and other additives like exogenous fibrolytic enzymes, commercial *Saccharomyces cerevisiae* and live yeast additive. Also, because the fermentation in cecum of the horse is similar to the rumen [26], our discussion would be based on studies with ruminant animals.

Lower asymptotic GP and higher rate of GP of Caesalpinia versus other forage species may be related to its relatively high contents of total phenolics and saponins which are secondary metabolites capable of inhibiting fermentation. The increased rate of GP of the forage is indicative of an enhanced cecal fermentation. Ahmed et al [1] attributed increased rate of GP due to addition of 3 µl/g DM of exogenous enzymes to fibrous feeds incubated with fecal inocula of horse to stimulated fecal

fermentation. However, the higher rate of GP of Caesalpinia was unexpected because secondary metabolites have been reported to depress degradability and hence GP [24, 28]. Moringa forage had the highest asymptotic GP which suggests that the forage promoted an increasing availability of carbohydrate fractions to the microbial population, in consonance with previous studies in ruminants [19,29,30]. Nutrients availability from the inocula for microbes' activity and growth has been reported to promote degradability of different nutrients [10]. The pronounced effect of forage type x aguamiel dose interaction on rate of GP suggests that rate of GP depends on forage type and aguamiel dose. Based on this, treatment of Caesalpinia forage with 34 µg/g aguamiel dose improved the fermentability of the forage, and may likely enhance feed intake, since intake has been said to be mostly explained by rate of GP [31]. Higher lag time or delay in the onset of GP of Avena relative to other forage species can be explained by its low CP and high NDF and ADL contents Generally, fiber, especially lignin, is resistant to microbial degradation, and this coupled with low CP content could have delayed microbial adaptation and activities. Diets with low CP are usually less palatable, consumed and digestible, though CP content per se should not be the sole criteria for evaluating the relative importance and nutritive value of a particular diet [28]. Lower lag time of Salix indicates that the forage facilitates the access of microorganisms and promotes faster microbial adaptation, in consonance with previous reports [29,32]. Whereas the low dose of aguamiel (34 µg/g DM) increased the lag time relative to the control dose, the high dose (68 µg/g DM) reduced it implying that higher dose of aguamiel induced microbial adaptation [32], and has the tendency to make a greater proportion of nutrients available [33]. Caesalpinia forage decreased the asymptotic CH_4 and lag time of CH_4 productions but increased the rate of CH_4 production, but the reverse was the case for Moringa forage. Production of methane is affected by the diet's quality. Feeding fiber-rich diets has been reported to increase CH_4 production relative to better quality diets [34]. However, contrary to this expectation, Avena with high fiber content did not increase rate of CH_4 production. In the current study, it appears that secondary metabolites have a more pronounced effect on rate of CH_4 production than fiber. This is obviously due to the fact that Caesalpinia with highest

concentrations of total phenolics and saponins produced the least CH_4. These two secondary metabolites are anti-methogens and have been used to suppress methanogenesis in ruminants [25,28] However, Avena increased CH_4 production (mL/g incubated DM) and mL/g degraded DM) at all hours of incubation while Caesalpinia decreased the proportional CH_4 production at all hours. The high fiber of Avena and high secondary metabolite concentrations of Caesalpinia are likely responsible for the results, in agreement with earlier reports [1,28]. The reduced CH_4 production by Caesalpinia has some implications on the availability of dietary energy to the horse. Methane production in horses is between that of swine and ruminant animals, and accounts for 3-4% and 2-3% of the digestible energy and the gross energy intake respectively [35]. Methane production in ruminants and equine is predominantly by methanogenic archaea, which represents the main hydrogenotrophic community [36]. Lack of aguamiel dose effect on CH_4 production (mL/g incubated DM and mL/g degraded DM) and proportional CH_4 production at all hours shows the inefficacy or impotency of the natural additive in reducing CH_4 production. Similarly, the insignificant forage species x aguamiel dose interaction on CH_4 production at all hours indicates the independency of the two factors. The decreased lag of time of CH_4 production by Caesalpinia forage suggests faster adaptation of methanogenic archaea and bacteria to the forage. Aguamiel sap, being a secondary-metabolite containing substance was expected to reduce asymptotic CH_4 production contrary to the obtained result. The reason for this is unknown and may require further investigations. However, the lower rate of CH_4 production by 34 µl/g DM aguamiel dose could be related to the activities of the secondary metabolites of the substance on methanogenic organisms.

As earlier opined, higher asymptotic CO_2 production of Avena could due to its relatively fibrous nature while lower rate and lag time of CO_2 productions of Caesalpinia may be attributed to its high secondary metabolite contents relative to other forage species. The pronounced effects of forage type and aguamiel dose interactions on asymptotic CH_4 and CO_2, rate of CH_4 and CO_2 and lag time of CH_4 and CO_2 productions suggest that responses were affected by both sources of variation. The results indicate that treatment of the forage species with aguamiel dose can either mitigate or increase the

kinetics of CH_4 and CO_2 productions in the horse. Aguamiel dose at 34 µL/g DM reduced CO_2 production (mL/g incubated DM) and proportional CO_2 production at all hours, unlike CH_4 production which was unaffected. Similarly, forage type x aguamiel dose interaction reduced CO_2 production (mL/g incubated DM) and proportional CO_2 production at all hours.

The high pH of the cecal inocula is due to the nature of the substrates. pH is generally high in forage-fed animals, since they are fibrous feeds. Highest pH level of inocula incubated with Eichhornia suggests low level of non-fibrous carbohydrate in this forage. Increased DMD of Moringa demonstrates its superior nutritive value which can be attributed to its relatively high CP, low NDF and ADF contents [37, 38]. Okunade et al [24] previously attributed higher *in vitro* DMD of *Afzelia africana* fodder relative to other browse fodders to its lower NDF and ADF contents.

5. Conclusions

Forage type affected cecal gas, methane and carbon dioxide productions, pH and dry matter degradability with the results not following a particular trend. *Avena sativa* had lowest CP and highest fiber levels resulting in the highest methane production (mL/g incubated and degraded DM) at all hours of incubation. *Caesalpinia coriacea* had highest concentrations of secondary metabolites and reduced the asymptotic and lag time of CH_4 productions, lag time of CO_2 production and proportional CO_2 production. The effects of forage species on these parameters were more pronounced than that of aguamiel dose. Addition of aguamiel to five forage species affected fermentation kinetics of GP resulting in different *in vitro* gas, methane and carbon dioxide productions from these substrates. Aguamiel at 32 µg/g DM reduced CO_2 production (mL/g incubated DM) and proportional CO_2 production but increased asymptotic CH_4 and CO_2 production. These results have important implications on plane of nutrition and energy availability assuming the same situation occurs *in vivo* trials with equines. Additional studies, involving *in vitro* and *in vivo* experiments, are recommended to investigate the inclusion of the studied forages and aguamiel at varying concentrations on horses' performance.

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Table 1

Chemical composition (g/kg DM) of plant leaves species as the substrates used.

	Avena sativa (Oat hay)	*Moringa oleifera*	*Salix babylonica*	*Eichhornia crassipes*	*Caesalpinia coriacea*
Chemical composition					
Organic matter	940	866.1	945.1	850.7	933.1
Crude protein	83	276.3	166.7	195.1	136.3
Ether extract	18.3	42.2	11.7	21.6	52.5
Neutral detergent fiber	530	223.0	364.1	507.7	247.7
Acid detergent fiber	361	194.6	205.9	481.2	201.2
Acid detergent lignin	309	78.6	148.5	75.7	101.2
Cellulose	52.0	116.0	57.4	405.5	100.0
Hemicellulose	169.0	28.4	158.2	26.5	46.5
Secondary metabolites					
Total phenolics	Not determined	22.3	12.8	16.4	73.36
Total saponins	Not determined	43.4	4.8	24.8	55.2

Table 2

Composition of the aguamiel (*Agave atrovirens*) used as a natural feed additive.

	g/kg DM
Crude protein	6.5
Ether extract	7.1
Ash	40
Mineral composition	mg/L
Mg	385
Ca	6274
Na	66
P	4329
K	1867
Fe	1314
Mesophilic bacterial count	8×10^6
Yeast count	4×10^6
Secondary metabolites	g/kg
Total phenolics	178.0
Total saponins	314.4

Table 3

In vitro cecal gas, methane (CH_4) and carbon dioxide (CO_2) productions and fermentation kinetics of different plant leaves species as affected by different levels of aguamiel.

Substrate	Dose (µg/g DM)	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[4]
Avena sativa	0	179.6	0.079	1.92	22.51	0.005	5.53	111.0	0.004	1.75	6.60	609.7
	34	230.3	0.075	1.93	20.91	0.006	3.50	130.3	0.001	2.42	6.44	623.3
	68	200.7	0.109	3.08	11.48	0.014	4.53	162.8	0.015	6.05	6.56	546.3
Moringa oleifera	0	249.1	0.038	0.85	16.04	0.008	6.43	131.3	0.007	4.05	6.58	850.3
	34	245.3	0.034	1.33	116.05	0.001	5.05	144.4	0.013	7.39	6.72	835.7
	68	269.8	0.033	1.62	188.7	0.000	5.32	116.5	0.006	8.42	6.63	875.0
Caesalpinia coriacea	0	104.9	0.105	1.72	3.32	0.014	1.68	79.3	0.006	1.82	6.63	450.0
	34	127.9	0.113	1.87	4.12	0.013	0.71	96.1	0.008	1.54	6.64	454.0
	68	106.4	0.061	1.97	4.24	0.009	1.79	87.0	0.003	1.73	6.62	474.7
Salix babylonica	0	189.5	0.061	0.40	19.40	0.006	1.99	127.0	0.013	8.52	6.54	548.0
	34	168.2	0.050	1.57	126.50	0.000	3.60	115.4	0.003	7.05	6.50	531.7

	68	269.8	0.044	1.12	8.37	0.019	7.77	108.1	0.006	8.56	6.56	541.7
Eichhornia crassipes	0	101.6	0.049	1.30	13.32	0.002	2.62	85.5	0.037	9.24	6.87	482.0
	34	97.9	0.085	1.21	3.11	0.012	1.44	92.5	0.003	1.77	6.86	500.3
	68	91.5	0.119	1.69	3.91	0.012	0.62	52.3	0.013	6.95	6.89	446.7
Pooled SEM[5]		28.80	0.0125	0.344	6.940	0.0020	0.978	9.50	0.0035	0.492	0.040	23.59
Substrate effect		<0.001	<0.001	0.006	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
Dose effect												
Linear		0.222	0.410	0.005	<0.001	<0.001	0.572	0.808	0.039	0.003	0.853	0.461
Quadratic		0.881	0.798	0.939	<0.001	0.005	0.081	0.073	0.006	<0.001	0.475	0.615
Substrate × Dose		0.476	0.003	0.441	<0.001	0.028	0.026	0.004	<0.001	<0.001	0.089	0.292

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

[2]*b*, asymptotic methane production (mL/g DM); *c*, rate of methane production (/h); *Lag*, initial delay before methane production begins (h).

[3]*b*, asymptotic carbon dioxide production (mL/g DM); *c*, rate of carbon dioxide production (/h); *Lag*, initial delay before carbon dioxide production begins (h).

[4]DMD, the DM degradability.

[5]SEM, the standard error of the mean.

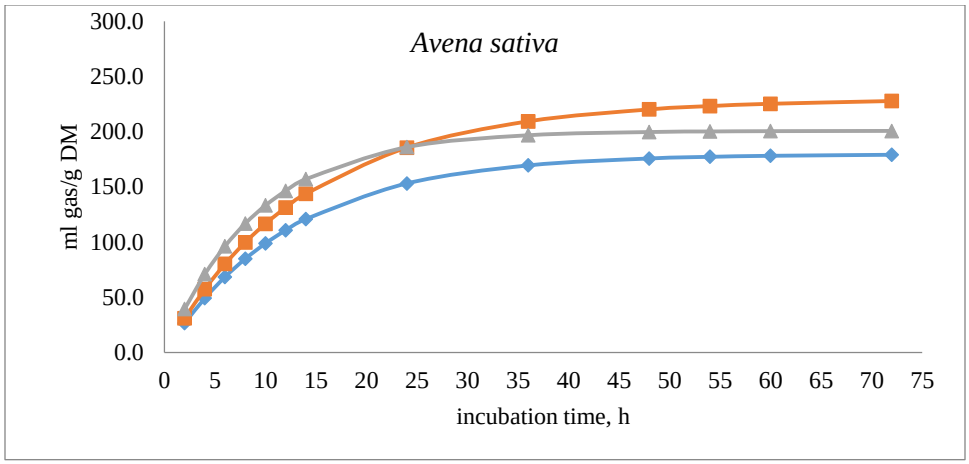
Table 4

Proportional *in vitro* methane (CH_4) and carbon dioxide (CO_2) productions as a percent of total gas production of different plant leaves species as affected by different levels of aguamiel.

		CH_4 production										CO_2 production									
		mL/g incubated DM			mL/g degraded DM			Proportional CH_4 production			mL/g incubated DM			mL/g degraded DM			Proportional CO_2 production			
Substrate	Dose (µg/g DM)	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	
Avena sativa	0	0.65	2.46	4.55	0.46	2.79	7.41	0.90	1.52	2.46	2.90	11.15	21.16	19.07	105.44	179.10	4.61	7.82	12.86	
	34	0.65	2.48	4.62	0.52	3.63	6.34	0.81	1.35	2.16	0.59	2.32	4.56	13.09	90.81	144.90	3.71	5.32	7.31	
	68	0.93	3.26	5.57	0.70	4.99	9.06	0.96	1.74	2.76	14.54	49.97	82.98	18.36	112.36	194.42	15.24	27.00	41.77	
Moringa oleifera	0	0.67	2.49	4.57	0.24	1.98	4.09	1.33	1.69	2.21	5.36	19.68	35.36	1.49	41.24	112.80	10.13	12.81	16.57	
	34	0.43	1.69	3.35	0.22	1.66	3.61	0.95	1.25	1.71	10.35	37.12	64.62	3.33	36.40	120.44	23.32	27.67	33.41	
	68	0.29	1.17	2.32	0.22	1.67	4.86	0.61	0.79	1.07	4.23	15.74	28.67	1.96	23.35	104.41	8.71	10.71	13.52	
Caesalpinia coriacea	0	0.26	0.93	1.58	0.43	2.13	3.24	0.54	0.96	1.52	2.57	8.94	15.03	6.53	22.95	50.21	5.35	9.74	15.30	
	34	0.31	1.10	1.90	0.55	2.62	3.92	0.49	0.93	1.50	3.93	13.19	21.35	10.58	40.57	72.82	6.71	12.01	18.25	
	68	0.22	0.83	1.50	0.27	1.68	2.91	0.72	1.04	1.51	1.67	6.29	11.63	5.50	20.92	38.35	6.05	8.22	11.42	
Salix babylonica	0	0.47	1.79	3.36	0.40	2.45	4.83	1.10	1.49	2.07	10.02	35.48	60.79	3.65	26.17	96.90	20.24	27.08	36.20	
	34	0.24	0.97	1.92	0.33	2.16	4.16	0.78	1.04	1.46	1.82	6.95	13.03	4.54	37.03	101.46	4.22	6.06	8.87	
	68	0.93	3.14	5.06	0.30	2.12	6.25	2.44	2.61	2.84	3.79	13.94	25.04	7.77	56.99	173.72	8.89	12.86	18.32	
Eichhornia crassipes	0	0.19	0.74	1.43	0.21	1.44	2.64	0.75	1.07	1.58	16.99	49.99	70.48	1.78	8.36	24.93	28.28	42.83	47.74	
	34	0.22	0.79	1.37	0.32	1.72	2.72	0.57	0.93	1.44	1.74	6.75	12.99	3.97	24.50	50.20	4.64	8.30	14.15	
	68	0.22	0.78	1.37	0.40	1.88	2.85	0.50	0.94	1.52	4.11	14.33	24.21	2.43	21.15	61.57	9.05	17.16	26.97	
Pooled SEM[2]		0.140	0.481	0.802	0.055	0.429	1.078	0.107	0.109	0.356	2.062	6.772	10.792	1.519	21.512	18.838	4.814	5.948	8.235	

Substrate effect		0.004	0.001	<0.001		<0.001	<0.001	<0.001		0.012	0.020	0.008		0.070	0.078	0.055		<0.001	0.003	0.002		<0.001	0.005	0.010
Dose effect																								
Linear		0.441	0.618	0.897		0.403	0.261	0.283		0.530	0.697	0.897		0.156	0.253	0.382		0.824	0.625	0.342		0.004	0.007	0.083
Quadratic		0.147	0.190	0.268		0.417	0.850	0.270		0.127	0.103	0.136		0.014	0.018	0.023		0.778	0.848	0.827		0.006	0.006	0.015
Substrate × Dose		0.077	0.123	0.222		0.003	0.086	0.855		0.064	0.083	0.210		<0.001	<0.001	<0.001		0.756	0.949	0.880		<0.001	<0.001	0.002

[1]SEM, standard error of the mean.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150.0
100.0
50.0
0.0
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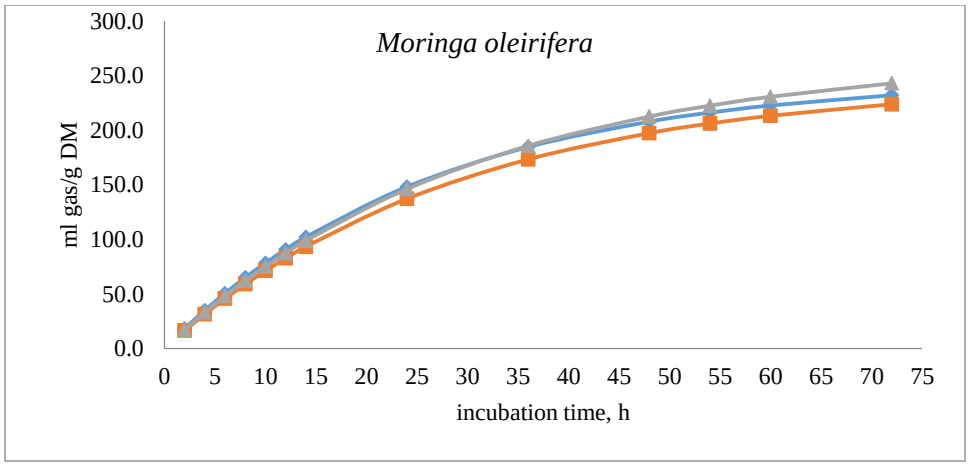
incubation time, h

7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ml gas/g DM
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50.0
0.0
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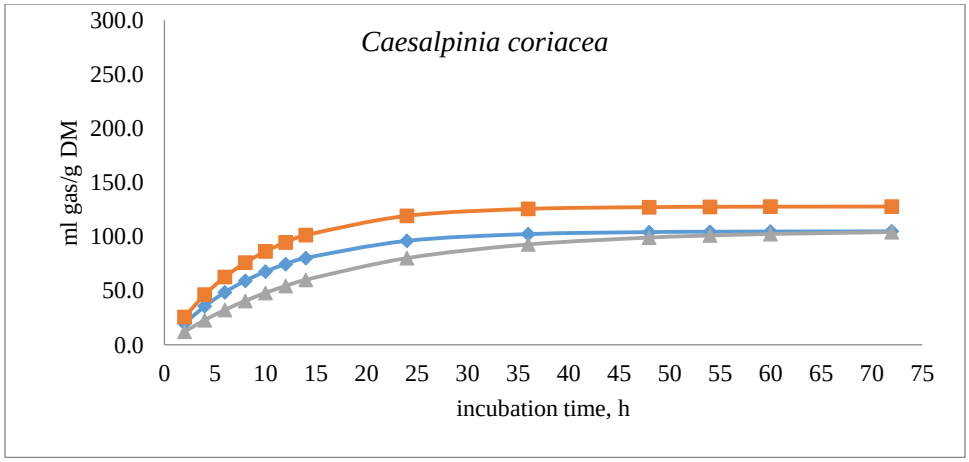
incubation time, h

8


Caesalpinia coriacea
ml gas/g DM
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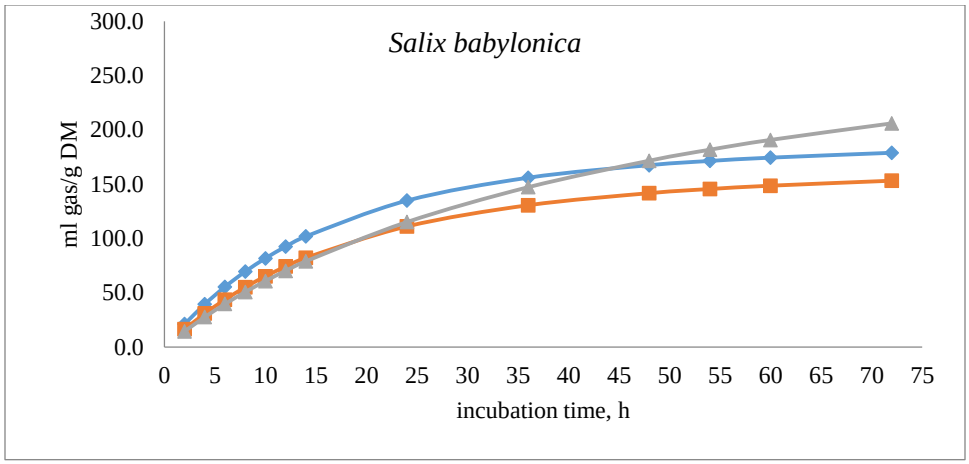
incubation time, h

9



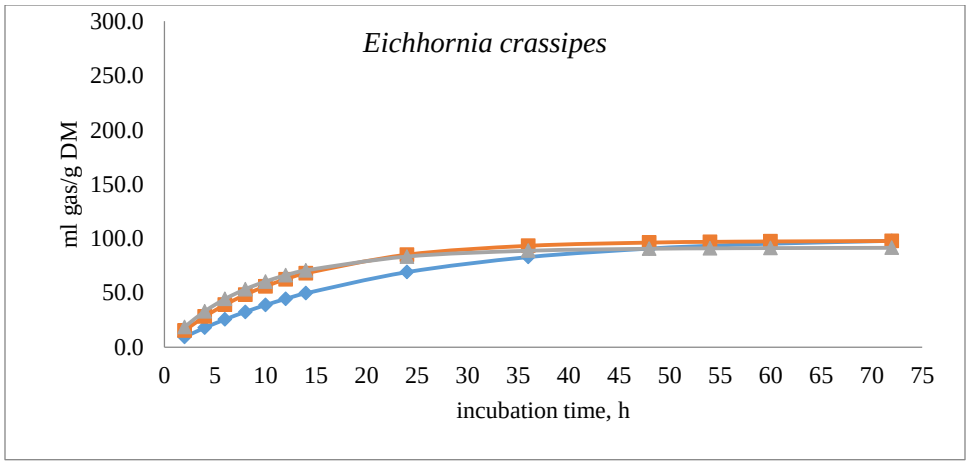
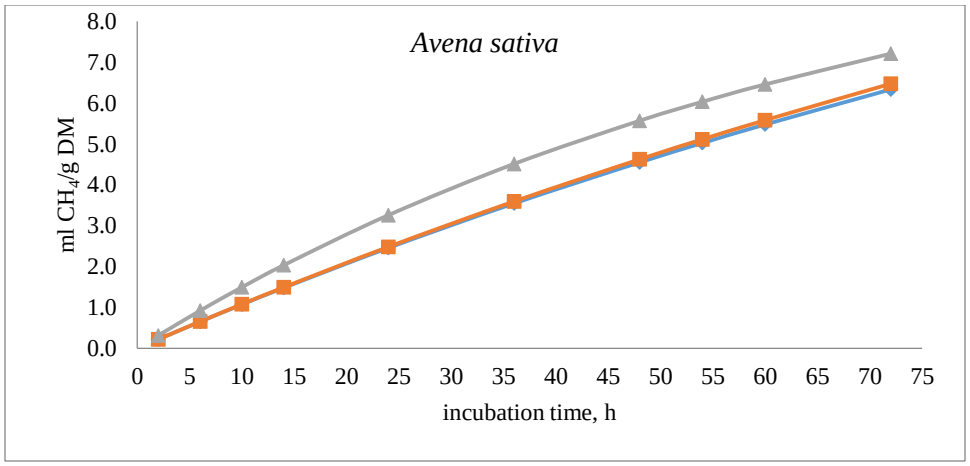


Fig. 1. *In vitro* cecal gas production (mL/g incubated DM) of plant species incubated in the inocula of horses in the presence of aguamiel at 0 (-♦-), 34 (-■-), and 68 (-▲-) µg/g DM of the substrate.



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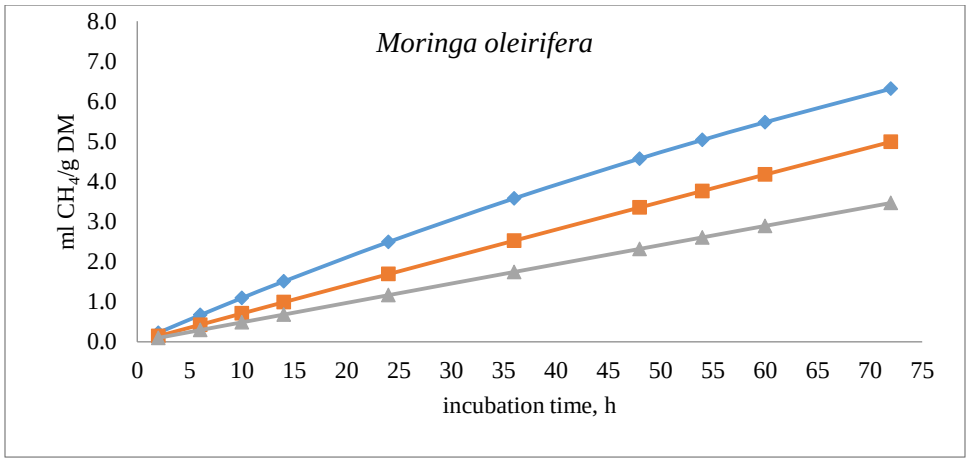

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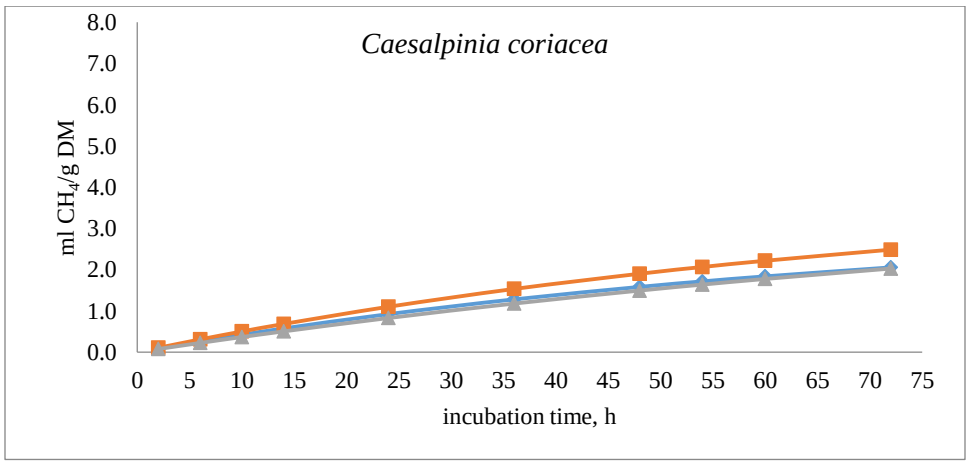

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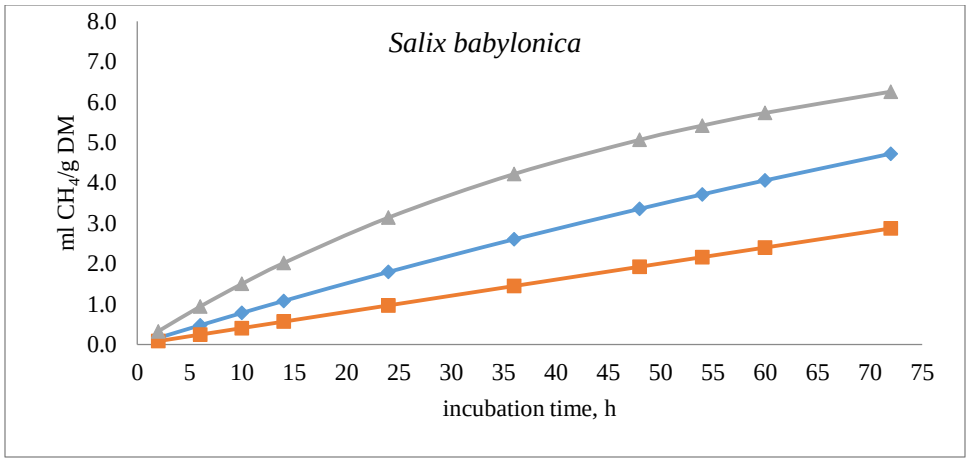


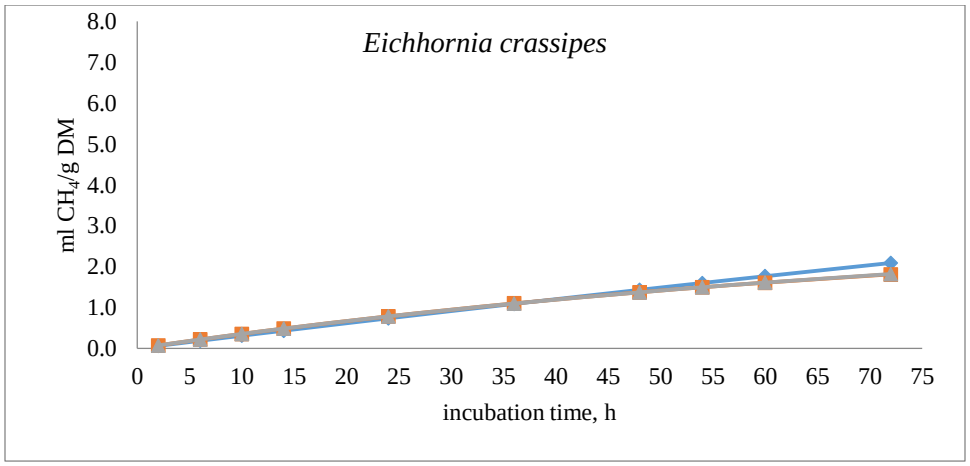
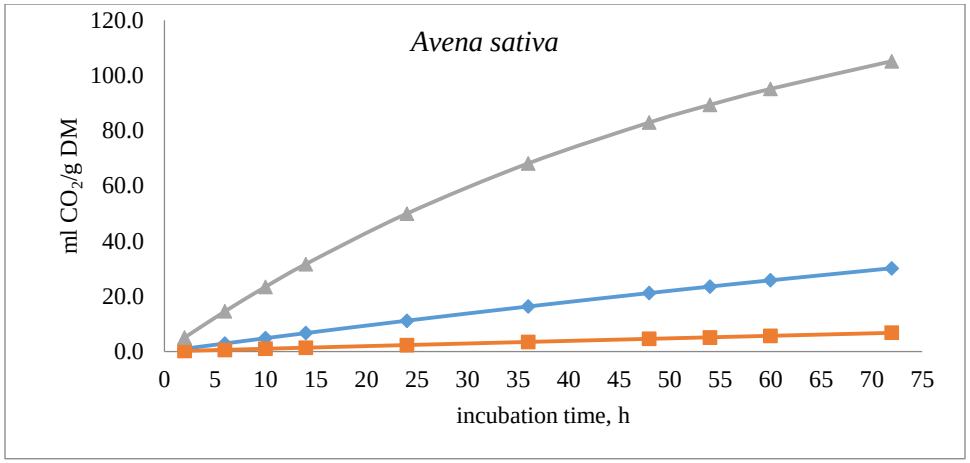


Fig. 2. *In vitro* cecal methane production (mL/g incubated DM) of plant species incubated in the inocula of horses in the presence of aguamiel at 0 (-♦-), 34 (-■-), and 68 (-▲-) µg/g DM of the substrate.


Avena sativa
ml CO2/g DM
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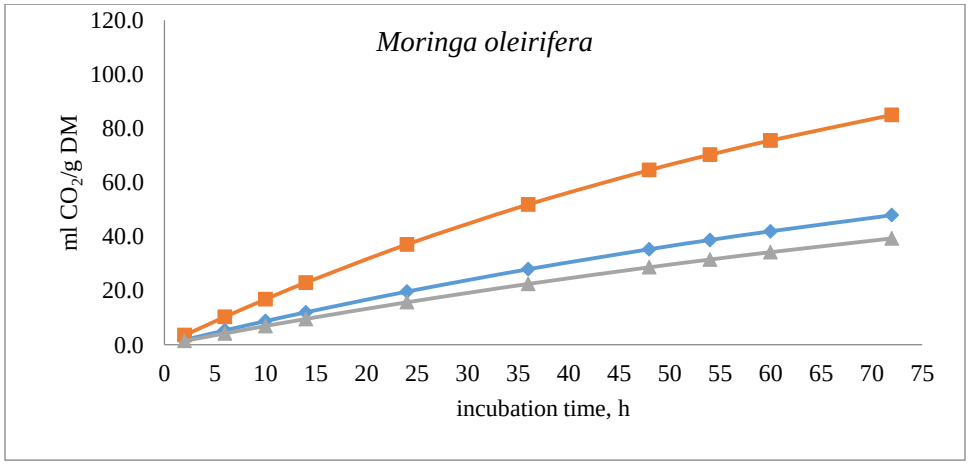
100.0
80.0
60.0
40.0
20.0
0.0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
incubation time, h


Moringa oleirifera
ml CO2/g DM
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100.0
80.0
60.0
40.0
20.0
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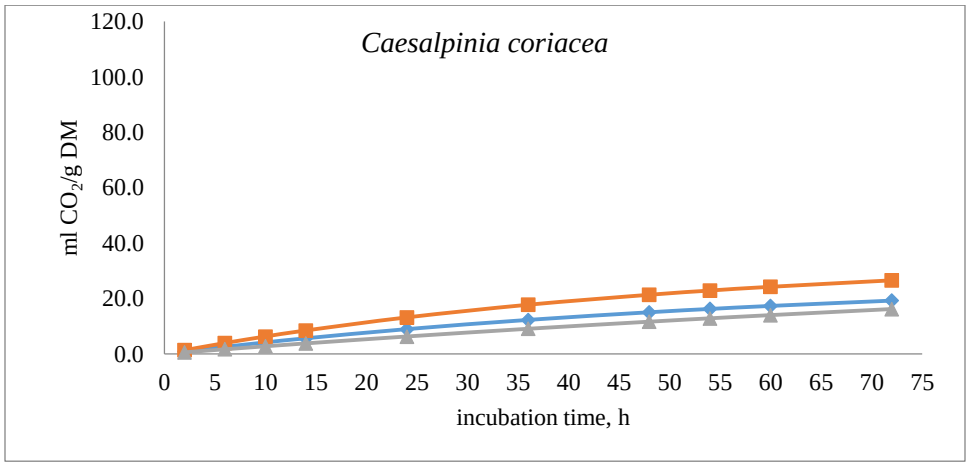
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
incubation time, h


Caesalpinia coriacea
ml CO2/g DM
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100.0
80.0
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40.0
20.0
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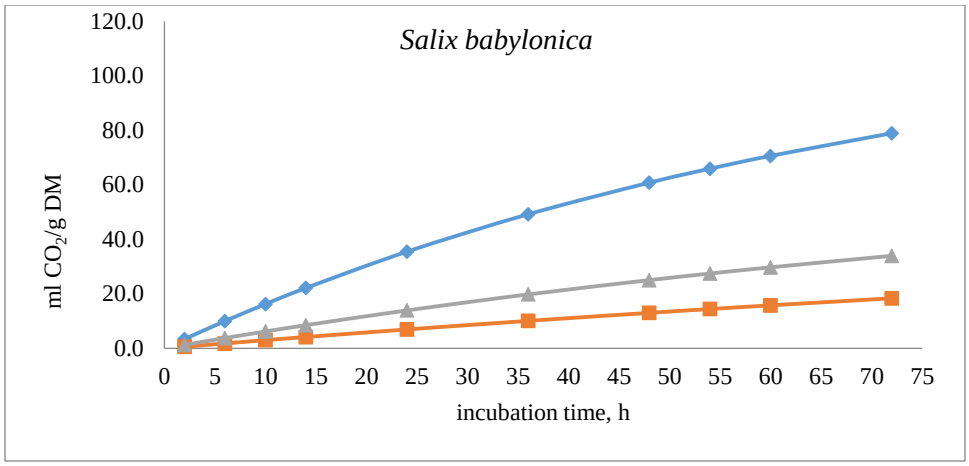
incubation time, h



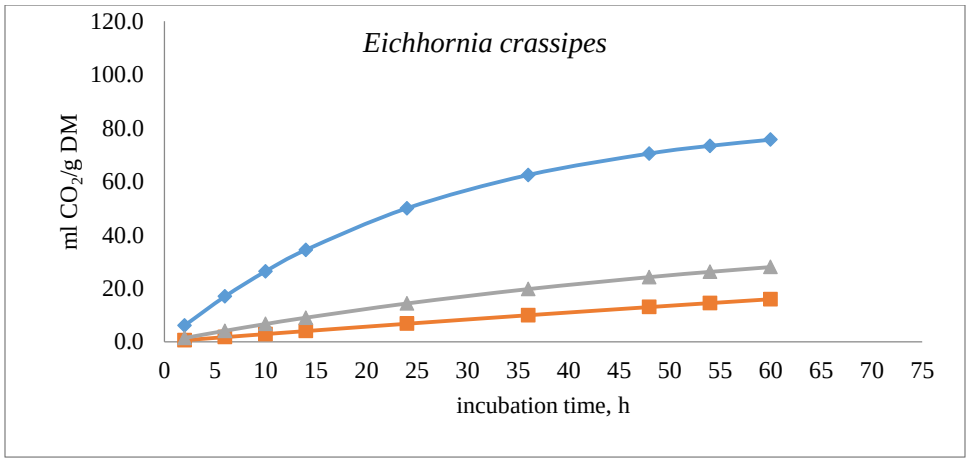


Fig. 3. *In vitro* cecal carbon dioxide production (mL/g incubated DM) of plant species incubated in the inocula of horses in the presence of aguamiel at 0 (-♦-), 34 (-■-), and 68 (-▲-) µg/g DM of the substrate.