View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Repositorio Institucional de la Universidad Autónoma del Estado de México

Journal of Equine Veterinary Science 48 (2017) 103–112

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Influence of Aguamiel (*Agave atrovirens*) as a Natural Feed Additive on Cecal Fermentation Kinetics of Some Forage Species in Horse Feeding

Abdelfattah Z.M. Salem [a,*], Nestor Torres Valdez [a], Olurotimi A. Olafadehan [b], Mona M.Y. Elghandour [a], Alberto Barbabosa Pliego [a], Rosalía Lugo Coyote [a]

[a] *Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, México*
[b] *Department of Animal Science, University of Abuja, Abuja, Nigeria*

ARTICLE INFO

Article history:
Received 15 August 2016
Received in revised form 27 August 2016
Accepted 31 August 2016
Available online 9 September 2016

Keywords:
Aguamiel
Cecal fermentation
Forage
Gas production
Methane
Carbon dioxide

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 the 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 four adult female Criolla horses (3–4 years of age and weighing 300 ± 15.0 kg) grazed on native grasses for about 8 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), 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 × aguamiel dose interactions were significant ($P < .05$) for asymptotic, rate and lag time of gas, and 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 × 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 on CH_4 production. It is concluded that addition of aguamiel to five forage species affected fermentation kinetics of gas production resulting in different *in vitro* cecal gas, CH_4 and CO_2 productions from these substrates.

© 2016 Elsevier Inc. All rights reserved.

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 have 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

* Corresponding author at: Abdelfattah Z.M. Salem, Universidad Autónoma del Estado de México, 50000 Toluca, Mexico.
E-mail address: asalem70@yahoo.com (A.Z.M. Salem).

0737-0806/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2016.08.012

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 semidesert areas of Mexico and used by Mexicans as a natural fortifying beverage. Multiple agave species including *A. atrovirens, Agave salmiana, Agave mapisaga*, and *Agave americana* are grown in the semidesert areas of Mexico [11]. Aguamiel is a colorless, sweet sap-like juice from the core of the agave plant containing (wt/wt on dry matter [DM] basis) glucose, 26.5%; sucrose, 8.8%; fructose, 32.4%; water; gum; protein; minerals; vitamins; and beneficial organisms such as *Kluyveromyces marcianus* var. *Bulgaricus* [12–14]. 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 antioxidant capacity and prebiotic effect of aguamiel during *in vitro* fermentation have been reported [11]. According to Tovar-Robles et al [15], aguamiel has been considered as a nutraceutical 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 (*A. 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°C. The agave plants, which were under commercial exploitation, were selected at random by the agave growers. The macronutrients and micronutrients of the aguamiel are shown in Table 2.

2.2. In Vitro Incubations

Before starting incubation, cecal contents (the inoculum source, 1 kg from each horse) were collected from the local slaughterhouse of Toluca, Mexico State, Mexico, from four adult female Criolla horses (3–4 years of age and weighing 300 ± 15 kg). Horses had about 8 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 vol/vol. 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

Table 1
Chemical composition (g/kg DM) of plant leaves species as the substrates used.

Substrate	*Avena sativa* (Oat Hay)	*Moringa oleifera*	*Salix babylonica*	*Eichhornia crassipes*	*Caesalpinia coriacea*
Chemical composition					
Organic matter	940.0	866.1	945.1	850.7	933.1
Crude protein	83.0	276.3	166.7	195.1	136.3
Ether extract	18.3	42.2	11.7	21.6	52.5
Neutral detergent fiber	530.0	223.0	364.1	507.7	247.7
Acid detergent fiber	361.0	194.6	205.9	481.2	201.2
Acid detergent lignin	309.0	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	73.4
Total saponins	Not determined	43.4	4.8	24.8	55.2

Abbreviation: DM, dry matter.

Table 2
Composition of the aguamiel (*Agave atrovirens*) used as a natural feed additive.

Item	g/kg DM
Crude protein	6.5
Ether extract	7.1
Ash	40
Mineral composition	mg/L
Mg	385
Ca	6,274
Na	66
P	4,329
K	1,867
Fe	1,314
Mesophilic bacterial count	8×10^6
Yeast count	4×10^6
Secondary metabolites	g/kg
Total phenolics	178.0
Total saponins	314.4

Abbreviation: DM, dry matter.

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 carbon dioxide (CO_2) and immediately closed with rubber stoppers, shaken and placed in an incubator set at 39°C. Gas production was recorded at 2, 4, 6, 8, 10, 12, 14, 24, 36, 48, 54, 60, and 72 hours using the Pressure Transducer Technique (Extech instruments, Waltham) of Theodorou et al [17]. The productions of CH_4 and CO_2 were recorded at 2, 6, 10, 14, 24, 36, 48, 54, 60, and 72 hours using Gas-Pro detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK).

As described in Rodriguez et al [18], at the end of incubation after 72 hours, 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 hours to estimate DM degradability (DMD) [19].

2.3. Chemical Analyses and Calculations

Samples of the substrates were analyzed for DM, ash, N, and ether extract according to Association of Official Analytical Chemists (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) 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 mineral 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°C to 30°C for 48 hours in closed jars of 20 L. After incubation, jars were heated at 39°C for 1 hour 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 gas production (GP), CH_4, and CO_2 results of GP, CH_4, and CO_2 (mL/g DM) were fitted using the NLIN option of SAS [22] according to the equation of France et al [23] as:

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

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

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, CH_4, and CO_2 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 ith substrate (R_i) with jth 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 $P < .05$.

3. Results

3.1. Chemical Composition and Secondary Metabolites

The CP content of *Moringa oleifera* forage was higher than that of the other forage species, while *Avena sativa* had the lowest CP content. NDF and ADF were lowest in *Moringa oleifera*, whereas both NDF and ADL, and ADF were highest in *Avena sativa* and *Eichhornia crassipes*, respectively. Concentrations of total phenolics and saponins were lowest in *Salix babylonica* and highest in *Caesalpinia coriacea*.

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)[a]			CH_4 Production (mL/g DM)[b]			CO_2 Production (mL/g DM)[c]			Fermentation Kinetics	
		b	*c*	*Lag*	*b*	*c*	*Lag*	*b*	*c*	*Lag*	pH	DMD
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	18.8	0.001	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	12.7	0.001	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		28.80	0.0125	0.344	0.940	0.0002	0.978	9.50	0.0005	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

Abbreviations: DM, dry matter; DMD, DM degradability; SEM, standard error of the mean.
[a] *b*, asymptotic gas production (mL/g DM); *c*, rate of gas production (/hour); *Lag*, initial delay before gas production begins (hour).
[b] *b*, asymptotic methane production (mL/g DM); *c*, rate of methane production (/hour); *Lag*, initial delay before methane production begins (hour).
[c] *b*, asymptotic carbon dioxide production (mL/g DM); *c*, rate of carbon dioxide production (/hour); *Lag*, initial delay before carbon dioxide production begins (hour).

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 Fig. 1). *Moringa oleifera* had the highest and lowest values for asymptotic GP and rate of GP, respectively, while lag time was highest for *Avena sativa*. 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 × aguamiel dose interactions had no effect ($P > .05$) on the asymptotic GP, fractional rate of GP, and lag time. Asymptotic methane (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 oleifera* forage. *Salix babylonica* reduced both the asymptotic and rate of CO_2 productions, while *Moringa oleifera* increased the lag time of CO_2 production. Although 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 × aguamiel dose interactions were pronounced ($P < .05$) for asymptotic CO_2, rate of CO_2, and lag time of CO_2 productions. *Moringa oleifera* increased (linear effect, $P < .001$) both the pH and DMD. Effects of aguamiel dose and forage species × aguamiel dose interactions were marginal ($P > .05$) for pH and DMD.

3.3. Proportional In Vitro Methane and Carbon Dioxide Productions

Methane production was linearly increased ($P = .05$) at 6, 24, and 48 hours incubations by *Avena sativa* (Table 4 and Fig. 2). Aguamiel dose and forage type × 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 degraded 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 sativa* forage increased (linear effect, $P < .05$) CO_2 production (mL/g degraded DM) at all hours. Forage type × aguamiel dose interaction effects were not ($P > .05$) significant for CO_2 production (mL/g degraded DM) at all hours. *Eichhornia crassipes* forage increased (linear, effect $P < .05$) proportional CO_2 production at all hours. Proportional CO_2 production at all hours was linearly and quadratically affected ($P < .05$) by aguamiel dose, with 34 μ/g DM dose having the lowest production at all hours (Fig. 3). Forage type × aguamiel dose interaction affected ($P < .05$) proportional CO_2 production.

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.

Substrate	Dose (μg/g DM)	CH_4 Production						CO_2 Production					
		mL/g Degraded DM			Proportional CH_4 Production			mL/g Degraded DM			Proportional CO_2 Production		
		6 hours	24 hours	48 hours	6 hours	24 hours	48 hours	6 hours	24 hours	48 hours	6 hours	24 hours	48 hours
Avena sativa	0	0.46	2.79	7.41	0.90	1.52	2.46	19.07	105.44	179.10	4.61	7.82	12.86
	34	0.52	3.63	6.34	0.81	1.35	2.16	13.09	90.81	144.90	0.71	1.32	2.31
	68	0.70	4.99	9.06	0.96	1.74	2.76	18.36	112.36	194.42	15.24	27.00	41.77
Moringa oleifera	0	0.24	1.98	4.09	1.33	1.69	2.21	1.49	41.24	112.80	10.13	12.81	16.57
	34	0.22	1.66	3.61	0.95	1.25	1.71	3.33	36.40	120.44	23.32	27.67	33.41
	68	0.22	1.67	4.86	0.61	0.79	1.07	1.96	23.35	104.41	8.71	10.71	13.52
Caesalpinia coriacea	0	0.43	2.13	3.24	0.54	0.96	1.52	6.53	22.95	50.21	5.35	9.74	15.30
	34	0.55	2.62	3.92	0.49	0.93	1.50	10.58	40.57	72.82	6.71	12.01	18.25
	68	0.27	1.68	2.91	0.72	1.04	1.51	3.50	20.92	38.35	6.05	8.22	11.42
Salix babylonica	0	0.40	2.45	4.83	1.10	1.49	2.07	3.65	26.17	96.90	20.24	27.08	36.20
	34	0.33	2.16	4.16	0.78	1.04	1.46	4.54	37.03	101.46	4.22	6.06	8.87
	68	0.30	2.12	6.25	2.44	2.61	2.84	7.77	56.99	173.72	8.89	12.86	18.32
Eichhornia crassipes	0	0.21	1.44	2.64	0.75	1.07	1.58	0.78	5.36	14.93	68.28	72.83	77.74
	34	0.32	1.72	2.72	0.57	0.93	1.44	3.97	24.50	50.20	4.64	8.30	14.15
	68	0.40	1.88	2.85	0.50	0.94	1.52	2.43	21.15	61.57	9.05	17.16	26.97
Pooled SEM		0.055	0.429	1.078	0.307	0.309	0.356	3.519	21.512	38.838	4.814	5.948	8.235
Substrate effect		<0.001	<0.001	<0.001	0.012	0.020	0.008	<0.001	0.003	0.002	<0.001	0.005	0.010
Dose effect													
Linear		0.403	0.261	0.283	0.530	0.697	0.897	0.824	0.625	0.342	0.004	0.007	0.083
Quadratic		0.417	0.850	0.270	0.127	0.103	0.136	0.778	0.848	0.827	0.006	0.006	0.015
Substrate × Dose		0.003	0.086	0.855	0.064	0.083	0.210	0.756	0.949	0.880	<0.001	<0.001	0.002

Abbreviation: DM, dry matter; SEM, standard error of the mean.

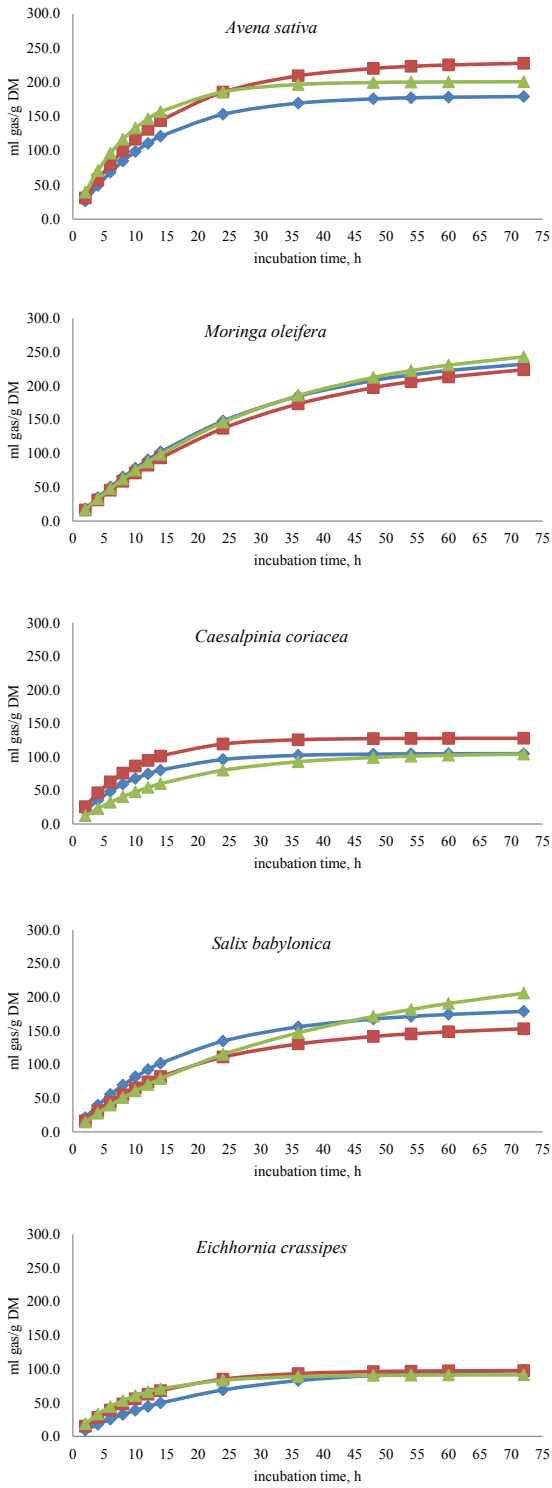

Fig. 1. *In vitro* cecal gas production (mL/g incubated DM) of plant species incubated in the inocula of horses in the presence of aguamiel at 0 (—◆—), 34 (—■—), and 68 (—▲—) μg/g DM of the substrate.

DM, dry matter.

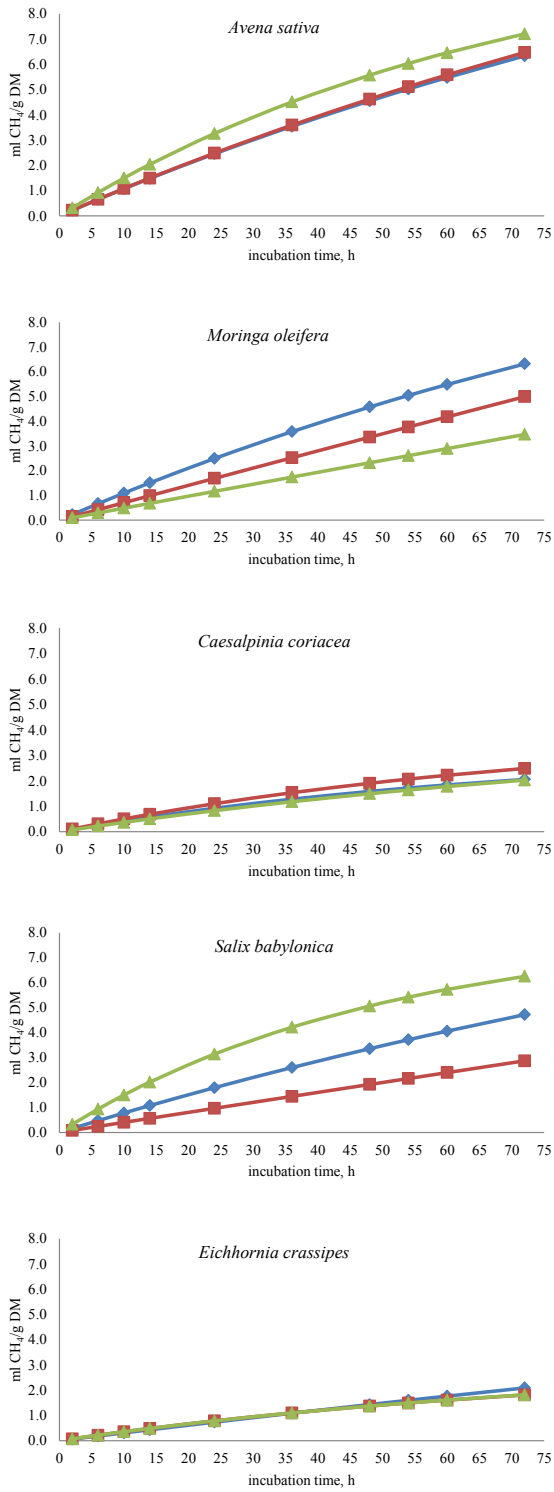

Fig. 2. *In vitro* cecal methane production (mL/g incubated DM) of plant species incubated in the inocula of horses in the presence of aguamiel at 0 (—◆—), 34 (—■—), and 68 (—▲—) μg/g DM of the substrate.

CH_4, methane; DM, dry matter.

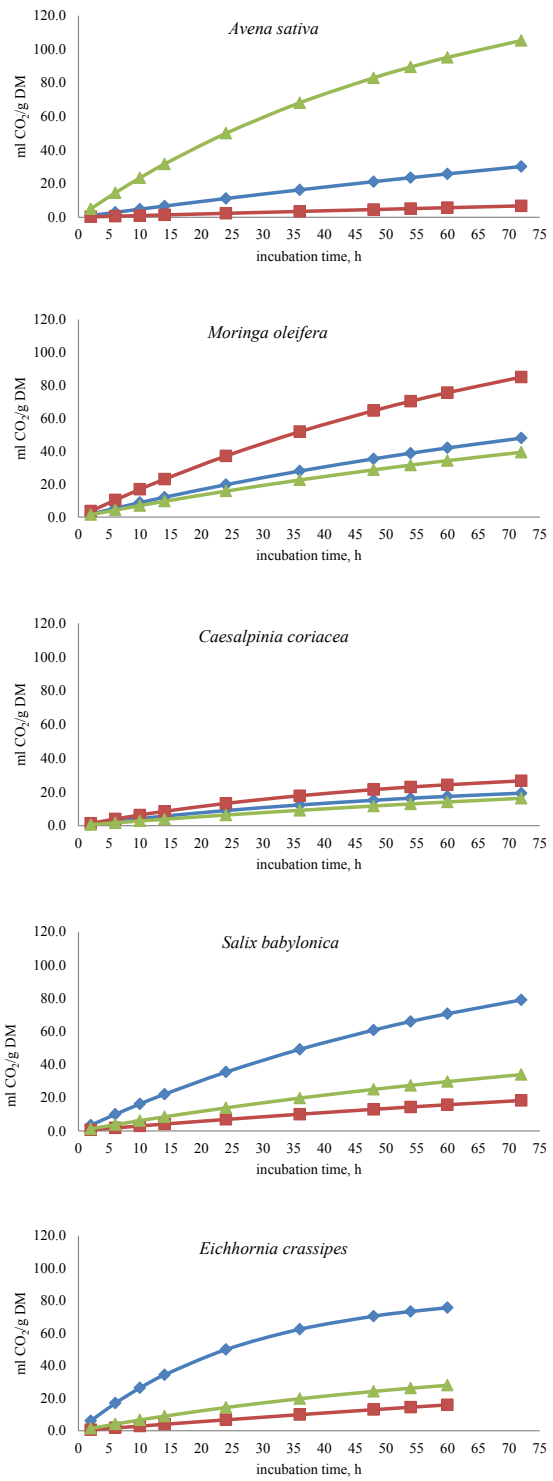

Fig. 3. *In vitro* cecal carbon dioxide production (mL/g incubated DM) of plant species incubated in the inocula of horses in the presence of aguamiel at 0 (▬◆▬), 34 (▬■▬), and 68 (▬▲▬) μg/g DM of the substrate. CO_2, carbon dioxide; DM, dry matter.

4. Discussion

Except for *Avena sativa* which is a grass fodder, the other forage species are nongrass fodders. The studied forage species had a good nutrient profile except for *Avena sativa*, which had the lowest CP content of < 90 g/kg DM and the highest NDF and ADL contents. With the exception of *Avena sativa*, 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 sativa* [24,25]. Low CP and high fiber contents generally have some implications on the nutritive value of a diet. All the nongrass fodders, especially *Caesalpinia coriacea* 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 coriacea* 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 hours 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 hours [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 coriacea* 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. Kholif 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 coriacea* was unexpected because secondary metabolites have been reported to depress degradability and hence GP [24,28]. *Moringa oleifera* 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]. Nutrient 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 × 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 coriacea* 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 sativa* 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 babylonica* indicates that the forage facilitates the access of microorganisms and promotes faster microbial adaptation, in consonance with previous reports [29,32]. The low dose of aguamiel (34 μg/g DM) increased the lag time relative to the control dose, whereas 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 coriacea* 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 oleifera*. Production of CH_4 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 sativa* 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 coriacea* with highest concentrations of total phenolics and saponins produced the least CH_4. These two secondary metabolites are antimethanogens and have been used to suppress methanogenesis in ruminants [25,28]. However, *Avena sativa* increased CH_4 production (mL/g incubated DM and mL/g degraded DM) at all hours of incubation, while *Caesalpinia coriacea* decreased the proportional CH_4 production at all hours. The high fiber of *Avena sativa* and high secondary metabolite concentrations of *Caesalpinia coriacea* are likely responsible for the results, in agreement with earlier reports [1,28]. The reduced CH_4 production by *Caesalpinia coriacea* has some implications for the availability of dietary energy to the horse. Methane production in horses is between that of swine and ruminant animals and accounts for 3% to 4% and 2% to 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 × 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 coriacea* 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 sativa* could be due to its relatively fibrous nature, while lower rate and lag time of CO_2 productions of *Caesalpinia coriacea* 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 × 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 crassipes* suggests low level of nonfibrous carbohydrate in this forage. Increased DMD of *Moringa oleifera* 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, CH_4 and CO_2 productions, pH and DM degradability with the results not following a particular trend. *A. sativa* had lowest CP and highest fiber levels resulting in the highest CH_4 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, CH_4 and CO_2 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 for plane of nutrition and energy availability assuming the same situation occurs in *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.

References

[1] Kholif AE, Baza-García LA, Elghandour MM, Salem AZM, Barbabosa A, Dominguez-Vara IA, Sanchez-Torres JE. *In vitro* assessment of fecal inocula from horses fed on high-fiber diets with fibrolytic enzymes addition on gas, methane and carbon dioxide productions as indicators of hindgut activity. J Equine Vet Sci 2016; 39:44–50.

[2] Elghandour MM, Kholif AE, Lopez S, Mendoza GD, Odongo NE, Salem AZM. *In vitro* gas, methane and carbon dioxide productions of high fibrous diet incubated with fecal inocula from horses fed live yeasts in response to the supplementation with different yeast additives. J Equine Vet Sci 2016;38:64–71.

[3] Rowe JB, Lees MJ, Pethick DW. Prevention of acidosis and laminitis associated with grain feeding in horses. J Nutr 1994;124: 2742S–4S.

[4] Longman AC. Nutritional assessment of forage quality. In: Saastamoinen M, Fradinho MJ, Santos S, Miraglia N, editors. Forages and grazing in horse nutrition. EAAP publication no. 132. Wageningen, The Netherlands: Wageningen Academic Publishers; 2012. p. 101–5.

[5] Khattab HM, Gado HM, Salem AZM, Camacho LM, El-Sayed MM, Kholif AM, El-Shewy AA, Kholif AE. Chemical composition and *in vitro* digestibility of *Pleurotus ostreatus* spent rice straw. Anim Nutr Feed Technol 2013;13:507–16.

[6] Kholif AE, Khattab HM, El-Shewy AA, Salem AZM, Kholif AM, El-Sayed MM, Gado HM, Mariezcurrena MD. Nutrient digestibility, ruminal fermentation activities, serum parameters and milk production and composition of lactating goats fed diets containing rice straw treated with *Pleurotus ostreatus*. Asian-Australas J Anim Sci 2014;27:357–64.

[7] Salem AZM, Alsersy H, Camacho LM, El-Adawy MM, Elghandour MMY, Kholif AE, Rivero N, Alonso MU, Zaragoza A. Feed intake, nutrient digestibility, nitrogen utilization, and ruminal fermentation activities in sheep fed *Atriplex halimus* ensiled with three developed enzyme cocktails. Czech J Anim Sci 2015;60:185–94.

[8] Togtokhbayar N, Cerrillo MA, Rodríguez GB, Elghandour MMY, Salem AZM, Urankhaich C, Jigjidpurev S, Odongo NE, Kholif AE. Effect of exogenous xylanase on rumen *in vitro* gas production and degradability of wheat straw. Anim Sci J 2015;86:765–71.

[9] Salem AZM, Elghandour MMY, Kholif AE, Odongo NE, Jiménez FJ, Montes-de-Oca R, Domínguez IA, Dibarrat JA. The effect of feeding horses a high fiber diet with or without exogenous fibrolytic enzymes supplementation on nutrient digestion, blood chemistry, fecal coliform count, and *in vitro* fecal fermentation. J Equine Vet Sci 2015;35:735–43.

[10] Elghandour MMY, Chagoyán JCV, Salem AZM, Kholif AE, Castañeda JSM, Camacho LM, Buendía G. *In vitro* fermentative capacity of equine fecal inocula of 9 fibrous forages in the presence of different doses of Saccharomyces cerevisiae. J Equine Vet 2014; 34:619–25.

[11] Romero-Lopez MR, Osorio-Díaz P, Flores-Morales A, Robledo N. Mora-Escobedo, R. Chemical composition, antioxidant capacity and prebiotic effect of *aguamiel* (*Agave atrovirens*) during *in vitro* fermentation. Revista Mexicana de Ingeniería Química 2015;14: 281–92.

[12] Estrada GAR, Cruz GAE, Lappe P, Ulloa M, García GM, Gómez RL. Isolation and identification of killer yeast from Agave sap (aguamiel) and pulque. World J Microbiol Biotechnol 2001;17:557–60.

[13] Cruz GAE, Olvera JL, García GM, Gómez RL. Inulinase-hyperproducing strains of Kluyveromyces sp. isolated from aguamiel (Agave sap) and pulque. World J Microbiol Biotechnol 2006;22:115–7.

[14] Ortiz BR, Pourcelly G, Doco T, Williams P, Dornier M, Belleville M. Analysis of the main components of the aguamiel produced by the maguey-pulquero (*Agave mapisaga*) throughout the harvest period. J Agric Food Chem 2008;56:3682–7.

[15] Tovar-Robles CL, Perales-Segovia C, Cedillo AV, Valera-Montero LL, Gómez-Leyva JF, Guevara-Lara F, Hernández-Duque JLM, Silos-Espino H. Effect of aguamiel (agave sap) on hematic biometry in rabbits and its antioxidant activity determination. Ital J Anim Sci 2011;10:106–10.

[16] Goering MK, Van Soest PJ. Forage fibre analysis (apparatus, reagents, procedures and some applications). Washington, DC, USA: Agricultural Research Service, USDA; 1970.

[17] 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 Technol 1994;48:185–97.

[18] Rodriguez MP, Mariezcurrena MD, Mariezcurrena MA, Lagunas BC, Elghandour MMY, Kholif AM, Kholif AE, Almaráz EM, Salem AZM. Influence of live cells or cells extract of *Saccharomyces cerevisiae* on *in vitro* gas production of a total mixed ration. Ital J Anim Sci 2015; 14:590–5.

[19] Ørskov ER, McDonald L. The estimation of protein degradability in the rumen from incubation measurements weighted according to the rate of passage. J Agric Sci Camb 1979;92:499–503.

[20] Association of Official Analytical Chemists (AOAC). Official methods of analysis. 16th ed. Arlington, VA, USA: AOAC; 1997.

[21] Salem AZM, Olivares M, López S, González-Ronquillo M, Rojo R, Camacho LM, Cerrillo SMA, Mejia HP. Effect of natural extracts of *Salix babylonica* and *Leucaena leucocephala* on nutrient digestibility and growth performance of lambs. Anim Feed Sci Technol 2011; 170:27–34.

[22] SAS. User's guide: statistics, version 9.0. Cary, NC: SAS Institute; 2002.

[23] France J, Dijkstra J, Dhanoa MS, 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.

[24] Okunade SA, Olafadehan OA, Isah OA. Fodder potential and acceptability of selected tree leaves by goats. Anim Nutr Feed Technol 2014;14:489–98.

[25] Olafadehan OA, Okunade SA. Fodder value of three browse forages for growing goats. J Saudi Soc Agric Sci (In press), http://dx.doi.org/10.1016/j.jssas.2016.01.001; 2016.

[26] Tisserand JL. Microbial digestion in the large intestine in relation to monogastric and polygastric herbivores. Acta Vet Scand Suppl 1989; 86:83–92.

[27] Agazzi A, Ferroni M, Fanelli A, Maroccolo A, Invernizzi G, Dell'Orto V, Savoini G. Evaluation of the effects of live yeast supplementation on apparent digestibility of high fiber diet in mature horses using the acid insoluble ash marker modified method. J Equine Vet Sci 2011; 31:13–8.

[28] Salem AZM. Oral administration of leaf extracts to rumen liquid donor lambs modifies *in vitro* gas production of other tree leaves. Anim Feed Sci Technol 2012;176:94–101.

[29] Elghandour MMY, Kholif AE, Bastida AZ, Martínez DLP, Salem AZM. 2015. *In vitro* gas production of five rations of different maize silage and concentrate ratios influenced by increasing levels of chemically characterized extract of *Salix babylonica*. Turk J Vet Anim Sci 2015; 39:186–94.

[30] Vallejo LH, Salem AZM, Kholif AE, Elghangour MMM, Fajardo RC, Rivero N, Bastida AZ, Mariezcurrena MD. Influence of cellulase or xylanase on the *in vitro* rumen gas production and fermentation of corn stover. Indian J Anim Sci 2016;86:70–4.

[31] Khazaal KA, Parissi Z, Tsiouvaras C, Nastis A, Ørskov ER. Assessment of phenolics-related antinutritive levels using the *in vitro* gas production technique: a comparison between different types of polyvinylpyrrolidone or polyethylene glycol. J Sci Food Agric 1996; 71:405–14.

[32] Ferraro SM, Mendoza GD, Miranda LA, Gutierrez CG. *In vitro* ruminal fermentation of glycerol, propylene glycol and molasses combined with forages and their effect on glucose and insulin blood plasma concentrations after an oral drench in sheep. Anim Feed Sci Technol 2016;213:74–80.

[33] Elghandour MMY, Kholif AE, Salem AZM, Montes de Oca R, Barbabosa A, Mariezcurrena M, Olafadehan OA. Addressing sustainable ruminal methane and carbon dioxide emissions of soybean hulls by organic acid salts. J Clean Prod 2016;135:194–200.

[34] Tang SX, Zou Y, Wang M, Salem AZM, Odongo NE, Zhou CS, Han XF, Tan ZL, Zhang M, Fu YF, Huang SQ, He ZX, Kang JH. Effects of exogenous cellulase source on *in vitro* fermentation characteristics and methane production of crop straws and grasses. Anim Nutr Feed Technol 2013;13:489–505.

[35] Kirchgessner M. Animal nutrition. 6th ed. Frankfurt: DLG publisher; 1985. p. 488.
[36] Wolin MJ, Miller TL, Stewart CS. Microbe–microbe interactions. In: Hobson PN, Stewart CS, editors. The rumen microbial ecosystem. 2nd ed. London, UK: Chapman & Hall; 1997. p. 467–91.
[37] Kholif AE, Gouda GA, Morsy TA, Salem AZM, Lopez S, Kholif AM. *Moringa oleifera* leaf meal as a protein source in lactating goat's diets: feed intake, digestibility, ruminal fermentation, milk yield and composition, and its fatty acids profile. Small Rumin Res 2015;129: 129–37.
[38] Kholif AE, Morsy TA, Gouda GA, Anele UY, Galyean ML. Effect of feeding diets with processed *Moringa oleifera* meal as protein source in lactating Anglo-Nubian goats. Anim Feed Sci Technol 2016;217: 45–55.