



PAPER

Influence of *Salix babylonica* extract in combination or not with increasing levels of minerals mixture on *in vitro* rumen gas production kinetics of a total mixed ration

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Abstract

The aim of this study was to determine the effects of increasing levels of two feed additives composed of *Salix babylonica* (SB) extract and minerals mixture (MM) or their combination on *in vitro* gas production (GP) and dry matter (DM) degradability of total mixed ration (TMR; 50 concentrate: 50 corn silage, on DM basis). Combinations of four levels of SB extract (0, 0.6, 1.2 and 1.8 mL/g DM) with four levels of MM (0, 0.5, 1.5 and 2.5 g/100 g DM) were evaluated in a completely random design. Samples of TMR (1 g) were weighed in 120 mL serum bottles with addition of SB extract and/or MM. Then, 10 mL of particle free ruminal fluid were added followed by 40 mL of the buffer solution. The GP was recorded at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of incubation. Addition of SB extract, without MM, increased ($P < 0.05$) asymptotic GP (mL/g DM), the rate of GP (/h), GP and DM degradability (DMD).

Addition of MM, without SB, increased ($P < 0.05$) *b* and decreased *c* and ruminal pH. Increasing the levels of SB increased ($P < 0.05$) *b*, *c*, *L*, and GP in addition to linearly increase ($P < 0.001$) DMD. Increasing levels of MM increased ($P < 0.05$) *b* and *c* while decreased pH. An interaction occurred between different SB extract and MM levels: the most effective levels of SB extract and MM on ruminal fermentation and kinetics were 1.8 mL SB extract/g DM and 2.5 g MM/100 g DM.

Introduction

Plant extracts, with high concentrations of secondary metabolites, are good candidates for enhancing nutrient utilization (Patra *et al.*, 2006; Cedillo *et al.*, 2014). Administration of *Salix babylonica* (SB) extract to ruminants as feed additives modified *in vitro* ruminal fermentation in lambs (Jiménez-Peralta *et al.*, 2011; Salem *et al.*, 2014b), improved *in vivo* digestibility as well as average daily gain of lambs (Salem *et al.*, 2011a, 2014b) and milk production (Salem *et al.*, 2014a). Some plant extracts also improved animal growth and nutrient digestion (Mapiye *et al.*, 2010; Salem *et al.*, 2011a) due to positive impacts of their secondary metabolites on activity of ruminal microorganisms (Jiménez-Peralta *et al.*, 2011) and/or increased amino acid flow to the duodenum (Mueller-Harvey, 2006). This can result in more muscle deposition and, consequently, heavier carcasses (Gleghorn *et al.*, 2004) and improved meat quality (Mapiye *et al.*, 2010). Some secondary metabolites like tannins and saponins have also a protective effect on the protein in the rumen which can promote duodenal absorption; they also minimize the excretion of N via increasing feed intake and N retention (Miller *et al.*, 1995), modify the acetate to propionate ratio in rumen fluid, and decrease parasitic load (Asiegbu *et al.*, 1995; Hernandez *et al.*, 2014; Olmedo-Juárez *et al.*, 2014).

Digestibility and performance can be maximized with the addition of precursor nutrients such as minerals (Astawa *et al.*, 2011). Bal and Ozturk (2006) stated that the macro minerals (P, Ca and Mg) and micro minerals (Cu, Co, Zn, Mn and S) play an important role in protein synthesis of rumen microbes, feed degradation and various microbial enzyme activators. Feeding animals on minerals mixture (MM) containing elements like Zn may activate the carbonic anhydrase enzyme which has, among others, the function of maintaining acid-base

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balance of rumen fluid in a normal pH (5.5 to 7.2) and making ruminal environments more stable and neutral, as stated by Owen and Goetsch (1998). However, there are limited reports on the effects of tree extracts combined with MM on ruminal gas production (GP) and nutrient digestibility (Suharti *et al.*, 2010). The aim of this study was to determine the effects of using increasing levels of SB extract and a MM and their combination on *in vitro* GP and some ruminal fermentation parameters of a total mixed ration (TMR) made of concentrate with corn silage (50:50 DM basis).

Materials and methods

Substrate

The substrate used in the study was a TMR made of concentrate with corn silage (50:50 on DM basis). Samples ($n=3$) of the TMR, corn silage and concentrate mixture (Table 1) were dried at 60°C for 72 h in an oven to constant weight, ground in a hammer mill (Arthur H. Thomas Co., Swedesboro, NJ, USA) to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components and *in vitro* GP.

Treatments

The SB extract (containing g/kg DM: 16.4

total phenolics, 5.4 saponins and 76 aqueous fraction) was tested at four levels (0, 0.6, 1.2, 1.8 mL SB extract/g DM of TMR) in three replicates for each treatment on *in vitro* fermentation and kinetic profile of TMR. Addition of SB levels to the TMR was done without or in combination with 4 levels of a commercial MM (0, 0.5, 1.5 and 2.5 g/100g DM of TMR). The MM was prepared for feedlot lambs and contained per kg: Fe (853 mg), Zn (2000 mg), Mn (1448 mg), Cu (60 mg), I (19.8 mg), Se (3.6 mg), Co (3.7 mg), Mg (6479.8 mg), CaCO₃ (499 g), NaCl (180 g), NaHCO₃ (250 g), Na (6894 mg), K (5540 mg).

In vitro incubations

The method of Theodorou *et al.* (1994) was used for GP technique as described before in some publications of *in vitro* evaluation. Briefly, rumen fluid was collected from two Brown Swiss cows fitted with permanent rumen cannula and fed *ad libitum* a TMR with a proportion of 1:1, w/w of commercial concentrate (PURINA®, St. Louis, MO, USA) and corn silage. The handling of animals was made according to international bioethical standards and NOM-062-ZOO-1999 (SAGARPA, 1999). Ruminal contents, from each cow, were obtained before the morning feeding, mixed and strained through four layers of cheesecloth into a flask with O₂ free headspace. Samples of TMR (1 g) were weighed in 120 mL serum bottles with appropriate addition of SB and MM or their combination. Ten mL of particle free ruminal fluid were added to each bottle followed by 40 mL of the buffer solution according to Goering and Van Soest (1970), with no trypsinase added. Stock solution of each MM and SB levels was prepared previously. The MM was added to bottles containing weighed samples before buffered rumen liquor addition, and SB solution was added immediately before closing the bottles and infusing CO₂.

A total of 576 bottles (3 bottles of each triplicate sample in three runs in different weeks with 4 doses of SB (0, 0.6, 1.2, 1.8 mL SB/g DM), 4 doses of MM (0, 0.5, 1.5 and 2.5 g/100 g DM of substrate), 4 doses of SB+MM combinations, plus three bottles as blanks (*i.e.*, rumen fluid and buffer only), were incubated for 72 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39°C. The volume of gas produced was recorded at 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h of incubation using the pressure reading technique (Extech instruments, Nashua, NH, USA). At the end of incubation, bottles were uncapped, pH was measured using a pH meter (Conductronic pH15; Conductronic, Puebla, Mexico) and the con-

tents of each bottle were filtered to obtain the non-fermented residue for determination of degraded substrate.

Dry matter degradability

The DM degradability (DMD) was determined by filtration of bottles contents at the end of incubation (*i.e.*, after 72 h). The content of each serum bottle was filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100 to 160 µm; Pyrex, Stone, UK). Fermentation residues were dried at 65°C for 72 h overnight to estimate DM disappearance with loss in weight after drying being the measure of degradable DM.

Preparation of extract

The preparation of extract was provisory described in Salem (2012). Briefly, plant leaves of *S. babylonica* were collected randomly from several young and mature trees during summer. Leaves were freshly chopped into 1 to 2 cm length, then 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 soaked and incubated in solvent in the laboratory at 25 to 30°C for 72 h in closed flasks. After incubation, flasks were incubated in a water bath at 39°C for 1 h and then immediately filtered and the filtrates were stored at 4°C for further use.

Chemical and secondary compound analyses

Samples of the substrate were analyzed for DM (#934.01), ash (#942.05), crude protein (CP) (#954.01) and ether extract (EE) (#920.39) according to AOAC (1997). The neutral detergent fibre (NDF) (Van Soest *et al.*, 1991), acid detergent fibre (ADF) and lignin

(AOAC, 1997; #973.18) were analysed using an ANKOM²⁰⁰ Fibre Analyzer Unit (Ankom Technology, Macedon, NY, USA). Neutral detergent fibre was assayed without use of an alpha amylase but with sodium sulfite in the NDF. Both NDF and ADF are expressed without residual ash.

Secondary metabolites were determined by taking 10 mL of extract that were fractionated by funnel separation with a double volume of ethyl acetate to determine total phenolics by drying and quantifying the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol was added to fractionate the saponins (Makkar *et al.*, 1998). The remaining solution was considered to be the aqueous fraction having the other secondary compounds such lectins, polypeptides and starch (Cowan, 1999).

Calculations

All the following calculations were mentioned previously in Salem (2012). To estimate kinetic parameters of GP, results (mL/g DM) were fitted using the NLIN programme of SAS (2002), according to France *et al.* (2000) as:

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

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 *L* (h) is the discrete lag time prior to GP.

Metabolizable energy (ME; MJ/kg DM) and *in vitro* organic matter digestibility (OMD; %) were estimated according to Menke *et al.* (1979) as:

$$\begin{aligned} \text{ME} &= 2.20 + 0.136 \text{ GP} + 0.057 \text{ CP} \\ \text{OMD} &= 14.88 + 0.889 \text{ GP} + 0.45 \text{ CP} + 0.0651 \text{ XA} \end{aligned}$$

where: OMD is organic matter digestibility; CP

Table 1. Ingredient and chemical composition of the total mixed ration.

	Concentrate	Corn silage
Ingredients, g/kg DM		
Soybean meal	220	
Alfalfa hay	195	
Sorghum grain	550	
Fish meal	35	
Chemical composition, g/kg DM		
Crude protein	147	106
Ether extract	120	85
Neutral detergent fibre	160	445
Acid detergent fibre	28	111
Acid detergent lignin	8	18

n=3; standard deviation=0.43.

is crude protein (%); XA, ash (%); and GP is net GP in mL from 200 mg of dry sample after 24 h of incubation.

The partitioning factor at 24 h of incubation (PF₂₄; a measure of fermentation efficiency) was calculated as the ratio of *in vitro* DMD (mg) to the volume (mL) of GP at 24 h [*i.e.*, DMD/total gas production (GP₂₄)], according to Blümmel *et al.* (1997).

Gas yield (GY₂₄) was calculated as the volume of gas (mL gas/g DM) produced after 24 h of incubation divided by the amount of DMD (g) as:

$$\text{Gas yield (GY}_{24}\text{)} = \text{mL gas/g DMD}$$

Short chain fatty acid concentrations (SCFA) were calculated according to Getachew *et al.* (2002) as:

$$\text{SCFA (mmol/200 mg DM)} = 0.0222 \text{ GP} - 0.00425$$

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

Microbial CP (MCP) biomass production was calculated according to Blümmel *et al.* (1997) as:

$$\text{MCP (mg/g DM)} = \text{mg DMD} - (\text{mL gas} \times 2.2 \text{ mg/mL})$$

where: 2.2 mg/mL is a stoichiometric factor which expresses mg of C, H and O required for production of SCFA gas associated with production of one mL of gas.

Statistical analyses

The experimental design for the *in vitro* ruminal GP and fermentation parameters analysis was a completely random design, with SB and MM as fixed effects in the linear model (Steel *et al.*, 1997). Data of each of the three runs within the same sample was averaged prior to statistical analysis. Mean values of the triplicates within each extract level were used as the experimental unit. The statistical model was:

$$Y_{ijkl} = \mu + SB_j + MM_k + (SB \times MM)_{jk} + e_{ijkl}$$

where: Y_{ijk} is every observation of the i_{th} extract levels (SB_{*i*}) when incubated in the j_{th} minerals doses (MM); μ is the general mean; SB_{*i*} (*i*=1-4) is the extract doses; MM is the minerals doses effect (*j*=1-4); (SB*MM)_{*ij*} is the interaction between extract and minerals doses; and e_{ijk} is the experimental error. Linear and quadratic polynomial contrasts were used to examine responses of TMR of concentrate with corn silage (1:1, DM basis) to increasing

addition levels of SB extract and/or MM.

Results

Results showed that individual addition of SB extract linearly and quadratically increased ($P < 0.05$) GP during all the incubation times, while the addition of MM linearly increased ($P < 0.05$) GP at 48 and 72 h of incubations (Table 2). In addition, the addition of MM quadratically increased asymptotic GP ($P = 0.038$). The SB×MM combination increased ($P = 0.01$) lag time and GP ($P < 0.05$) during the first 36 h of incubation. There were not quadratic effect ($P > 0.05$) on GP.

Addition of SB linearly and quadratically increased ($P < 0.05$) all fermentation parameters except DMD and the rumen pH without quadratic effect ($P > 0.05$) on all the fermentation parameters. The addition of MM only quadratically increased ($P < 0.05$) pH, PF₂₄, GY₂₄ and MCP. Combination of SB with MM increased ($P < 0.05$) DMD, OMD, ME, PF₂₄ and SCFA. The MM tended to affect the measured pH ($P = 0.074$), but it also influenced ($P < 0.05$) GP at 72 h, GY₂₄ and MCP with increasing doses of SB and levels of MM. However, opti-

Table 2. *In vitro* rumen gas kinetics and cumulative gas production after 72 h of incubation as affected by the combination of *Salix babylonica* extract doses and minerals levels.

SB dosage, mL/g DM	MM, DM g/100 g	GP parameters			<i>In vitro</i> GP, mL/g DM										
		<i>b</i> , mL/g DM	<i>c</i> , /h	<i>L</i> , h	GP ₂	GP ₄	GP ₆	GP ₈	GP ₁₀	GP ₁₂	GP ₂₄	GP ₃₆	GP ₄₈	GP ₇₂	
0	0	204	0.052	1.83	20.2	38.4	54.8	69.6	83.0	95.0	145.8	173.0	187.6	199.6	
	0.5	212	0.046	2.14	18.8	35.9	51.5	65.7	78.6	90.4	142.2	171.8	188.9	204.4	
	1.5	217	0.053	1.42	21.9	41.6	59.3	75.2	89.5	102.4	156.5	185.1	200.3	212.5	
	2.5	222	0.054	1.23	22.6	42.9	61.1	77.5	92.2	105.4	160.8	189.9	205.2	217.5	
0.6	0	210	0.053	1.88	21.1	40.0	57.0	72.4	86.1	98.5	150.8	178.6	193.3	205.4	
	0.5	211	0.056	2.01	22.5	42.6	60.5	76.5	90.8	103.6	156.2	183.0	196.7	207.4	
	1.5	215	0.057	1.68	23.0	43.6	62.0	78.4	93.0	106.1	159.9	187.2	201.1	211.6	
	2.5	215	0.057	1.83	23.2	44.0	62.5	79.0	93.7	106.8	160.5	187.5	201.1	211.4	
1.2	0	214	0.057	1.89	22.8	43.2	61.4	77.6	92.1	105.1	158.4	185.5	199.2	209.8	
	0.5	234	0.068	2.05	30.0	56.2	79.0	98.9	116.2	131.3	188.9	214.2	225.5	232.7	
	1.5	233	0.069	2.36	29.9	56.0	78.6	98.4	115.6	130.6	187.7	212.8	224.0	231.1	
	2.5	233	0.059	2.30	25.8	48.7	69.1	87.1	103.2	117.4	175.3	204.0	218.4	229.3	
1.8	0	242	0.081	2.97	36.0	66.7	92.8	115.0	133.9	150.0	207.0	228.7	236.9	241.3	
	0.5	251	0.068	2.55	31.7	59.3	83.4	104.4	122.8	138.8	200.4	227.9	240.3	248.5	
	1.5	239	0.073	3.13	32.3	60.2	84.3	105.2	123.2	138.8	197.0	221.4	231.8	238.0	
	2.5	242	0.067	2.83	30.5	57.2	80.5	100.9	118.7	134.2	193.9	220.6	232.5	240.2	
Pooled SEM		10.0	2.377	0.002	2.118	3.76	2.36	3.82	6.181	8.45	9.65	7.998	2.8319	2.6458	
P															
SB main effect		0.0250	<0.0001	<0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
Linear		0.0060	<0.0001	<0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
Quadratic		0.3740	0.0019	0.1210	0.0336	0.0153	0.0151	0.0147	0.0143	0.0138	0.0133	0.0098	0.0067	0.0048	
MM main effect		0.0740	0.0130	0.3860	0.8573	0.4353	0.4104	0.3832	0.3560	0.3270	0.2982	0.1465	0.0643	0.0325	
Linear		0.1160	0.0147	0.8710	0.4965	0.5330	0.4762	0.4218	0.3718	0.3249	0.2822	0.1096	0.0435	0.0225	
Quadratic		0.0380	0.1299	0.2610	0.6047	0.1403	0.1343	0.1286	0.1236	0.1187	0.1142	0.0961	0.0912	0.0961	
Interaction SB×MM		0.2840	0.2319	0.0101	0.1410	0.0142	0.0151	0.0161	0.0171	0.0183	0.0197	0.0321	0.0557	0.0919	

SB, *Salix babylonica*; MM, minerals mixture; DM, dry matter; GP, gas production; *b*, asymptotic gas production; *c*, rate of gas production; *L*, initial delay before gas production begins.

Table 3. *In vitro* rumen fermentation profile as affected by the combination of *Salix babylonica* extract doses and minerals levels.

SB dosage, mL/g DM	MM, g/100 g DM	pH	DMD, mg/g DM	OMD, %	ME, MJ/kg DM	PF ₂₄ , mg DMD:mL gas	GY ₂₄ , mL gas/g DMD	SCFA, mmol/g DM	MCP, mg/g DM
0	0	6.51	732.46	45.20	6.68	5.03	199.06	3.22	411.69
	0.5	6.50	743.87	44.55	6.58	5.24	191.08	3.13	431.14
	1.5	6.47	736.51	47.10	6.97	4.54	258.32	3.45	377.88
	2.5	6.50	758.62	47.86	7.09	4.72	211.94	3.55	404.92
0.6	0	6.53	762.14	46.08	6.82	5.06	197.88	3.33	430.37
	0.5	6.49	755.78	47.04	6.96	4.87	206.89	3.45	412.23
	1.5	6.48	740.21	47.71	7.07	4.63	216.02	3.53	388.38
	2.5	6.49	749.76	47.81	7.08	4.67	214.05	3.54	396.68
1.2	0	6.48	752.83	47.43	7.02	4.75	210.40	3.49	404.41
	0.5	6.47	742.38	52.86	7.85	3.95	254.64	4.17	326.84
	1.5	6.49	748.56	52.65	7.82	4.01	250.99	4.15	335.62
	2.5	6.47	767.31	50.44	7.48	4.41	228.31	3.87	381.70
1.8	0	6.48	756.86	56.07	8.35	3.66	273.50	4.57	301.54
	0.5	6.46	753.96	54.90	8.17	3.78	265.80	4.43	313.17
	1.5	6.47	743.01	54.30	8.08	3.77	265.21	4.35	309.61
	2.5	6.48	733.63	53.75	7.99	3.79	264.45	4.28	307.00
Pooled SEM		0.006	3.637	0.533	0.082	0.081	6.697	0.067	8.119
P value									
SB main effect		0.0250	0.2017	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Linear		0.0060	0.4369	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Quadratic		0.3741	0.0471	0.0098	0.0098	0.0076	0.0085	0.102	0.0019
MM main effect		0.0736	0.2128	0.1471	0.1541	0.0177	0.0484	0.1465	0.0465
Linear		0.1159	0.9602	0.1099	0.1116	0.0302	0.1843	0.1080	0.1580
Quadratic		0.0381	0.0541	0.0966	0.1021	0.0172	0.0170	0.0970	0.0144
Interaction SB×MM		0.2841	0.0419	0.0322	0.0335	0.0413	0.1409	0.0324	0.1024

SB, *Salix babylonica*; MM, minerals mixture; DM, dry matter; DMD, DM degradability; OMD, *in vitro* organic matter digestibility; ME, metabolizable energy; PF₂₄, partitioning factor at 24 h of incubation; GY₂₄, gas yield at 24 h of incubation; SCFA, short chain fatty acids; MCP, microbial crude protein production.

rumen fermentation profiles in terms of SCFA and MCP were, however, appeared at minimum MM levels combined with the SB extract (Table 3).

Discussion

Salix babylonica extract positively modified rumen GP and fermentation activities as improved nutrients utilization (Jiménez-Peralta *et al.*, 2011; Salem *et al.*, 2014a). It was reported that this could also be used as growth promoter (Patra *et al.*, 2006). *Salix babylonica* complexes can protect dietary proteins from degradation in the rumen and made them available for digestion as a protein within the stomach. The observed GP increase in this study with increasing SB extract levels may be explained by the presence of soluble sugars in the extracts (Patra *et al.*, 2006), which apparently positively influence on the activity of rumen microorganism. The *in vitro* DMD increased when 0.6 mL/g DM of SB extract was added, but declined with increasing doses of SB extract. The highest DMD (76.2%) was

obtained at concentration of 0.6 mL SB extract/g DM, which might indicate that this level is the optimum concentration of the extract for rumen microbes for stimulating rumen microbes to efficient breakdown the substrate (Table 3). The decline beyond the optimum levels of the SB extract was in agreement with Salem *et al.* (2006) who reported that high concentration of plant metabolites negatively affects the activities of rumen microorganisms. At low or moderate doses of secondary metabolites, the rumen microorganisms have the ability to degrade and utilize them as an energy source (Varel *et al.*, 1991; Hart *et al.*, 2008). However, high doses of plant extracts with antimicrobial activity will decrease microbial activity and diet fermentability (Jiménez-Peralta *et al.*, 2011). *Salix babylonica* extract was expected to be beneficial to rumen function due to its stimulating effect on fermentation (Jiménez-Peralta *et al.*, 2011) as revealed by protection dietary proteins from rumen degradation increased cell-wall constituents and MCP. However, the observed increased MCP, PF₂₄ and GY₂₄ may possibly be due to improved synchronization between energy and protein release in the

rumen with SB and/or MM combination (Montemayor *et al.*, 2009).

Some of phenolic compounds in plant extract may interact with biosynthesis of aromatic amino acids, as both biosynthesis pathways are linked through cinnamic acid, a natural compound in plant secondary metabolites (Mandal *et al.*, 2010). Salem *et al.* (2011b) identified 59 compounds in *S. babylonica* leaves. Phenylpropanoic acid and phenylacetic acid, as a phenolic compounds in plant extract, have been reported to enhance cellulose degradation and growth of several strains of *Ruminococcus albus* (Stack and Cotta, 1986). The biological activities of organic substances other than sugars, fatty acids or amino acids, have been identified in plant extracts such *Achillea millefolium*, *Arnica montana*, *Lavandula officinalis*, *Equisetum arvense*, *Fagopyrum esculatum*, *Sativa officinalis* and *Solidago virgaurea* (Duke, 1992).

Suharti *et al.* (2010) found that addition of lerak (*Sapindus rarak*) fruit extract up to 0.18% from total ration in the presence of mineral block mixture was not effective to depress protozoa population, but could modify fermentation characteristics *in vitro*. From all above

one can infer that plant secondary metabolites at the optimum doses can enhance GP, degradability, and general fermentation kinetics.

The increasing SB×MM combination levels showed an increasing effect on rumen fermentation and resulted in increased DMD, SCFA, ME and MCP. The MM might enhance the GP as a result of the improved fermentation ability of ruminal microorganisms to digest secondary metabolites contained in the extract (Hu *et al.*, 2005; Hart *et al.*, 2008). Supplementation with minerals can improve rumen microbial activity in the metabolism of carbohydrates and protein in the rumen, so that the rumen fermentation process would be more efficient. The SCFA produced in the rumen will increase and in turn lead more nutrient availability for microbes and animals (Astawa *et al.*, 2011). Moreover, Kamra (2005) stated that availability of minerals like Zn and S in the rumen will improve ruminal fermentation. The presence of Zn mineral in the rumen will increase microbial protein synthesis through the activation of microbial enzymes. It will also stimulate growth of rumen protozoa because Zn can easily penetrate protozoa and was utilized in the body for growth. However, the presence of mineral S in the rumen can be used as a component of sulfur amino acid by rumen microbes.

The addition of minerals might have provided the necessary nutrients required by the microbes to effectively degrade the TMR. The presence of some trace mineral elements such as Cu and Zn are involved in many enzymatic systems and may improve metabolic utilization of major dietary nutrients such as proteins and carbohydrates (Ramírez, 2007). Although severe Zn deficiency is uncommon in ruminants, symptoms related to marginal Zn deficiencies may occur more often than expected (Smart *et al.*, 1981). Moreover, in herds reared at pasture, grazing ruminants in many parts of the world often experience Cu deficiency or toxicity, which may affect growth and digestibility of nutrients (Sharma *et al.*, 2004). Phosphorus can be considered, worldwide, the major mineral deficiency causing economic impact (Underwood and Suttle, 1999).

Proper level of MM is important for ruminants. Although no effects of MM addition on pH values were registered, addition of minerals to a high concentrate diet may help to maintain an adequate pH for cellulolytic activity by microorganisms and enhance the efficiency and quantity of the microbes produced (Durand and Komisarczuk, 1988) during fermentation. Elements like Zn may activate the carbonic anhydrase enzyme which can maintain acid-base balance of rumen fluid in normal pH,

resulting in a more stable and neutral ruminal environment (Owen and Goetsch, 1998). However, it is well known that moderate mineral deficiencies or improper proportions of minerals in the ruminant's diet often induce depressed feed intake leading partly to the impaired activity of rumen microorganisms (Komisarczuk-Bony and Durand, 1991). Durand and Kawashima (1980) reported that not only was MM added to meet the microbial requirements for Na, K and Ca for ruminants, but it also has some indirect effects on microbial metabolism either by interacting with organic compounds or by altering the physicochemical characteristics of the rumen medium (Mackie and Therion, 1984). These indirect effects offer some possibilities for manipulating rumen fermentation and would therefore be one of the reasons for inducing the improved *in vitro* fermentation as observed in this study (Table 3). Mg is essential for all microorganisms to activate many bacterial enzymes phosphohydrolase and phosphotransferase pathways involving adenosine triphosphate and thiamin pyrophosphate reactions. Cellulase from *Ruminococcus flavefaciens* in the rumen was shown to be activated by Mg²⁺ (Pettipher and Latham, 1979) making this supplement beneficial for rumen microbe's activity.

Conclusions

In the current study, the interactions between SB extract and MM for *in vitro* GP parameters and *in vitro* DM and OM disappearances suggest that it is important to identify appropriate supplemental levels of both SB extract and MM. Interactive effects between SB extract and MM for *in vitro* DM and OM disappearances suggest that the appropriate supplemental levels of SB extract and MM were 1.8 mL extract/g DM and 2.5 g MM/100 DM of TMR composed of concentrate and corn silage (TMR - 50:50, on DM basis).

Moreover, addition of SB extract to the rumen medium containing MM at supplemental levels of 1.8 mL extract/g DM and 2.5 g MM/100 DM of TMR composed of concentrate and corn silage (TMR - 50:50, on DM basis) showed a marked improvement in *in vitro* rumen gas kinetics and cumulative GP.

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