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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*

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Abstract: This study was carried out to assess the effect of the chemical substances of *Salix babylonica* (SB) extract on in vitro rumen fermentation of five mixed rations with different maize silage to concentrate ratios. Fifty-nine compounds were identified in SB extract using the retention time and mass spectral technique. Interactive effects were noted ($P < 0.001$) for the asymptotic gas production (GP) (*b*), the rate of production (*c*), the initial delay before GP begins (*L*), pH, dry matter digestibility, metabolizable energy (ME), organic matter digestibility (OMD), short chain fatty acids (SCFAs), gas yield at 24 h (GY_{24}), microbial crude protein, and in vitro GP. Both 1.2 and 1.8 mL SB/g DM had the highest ($P < 0.05$) *b* and *c* values. Addition of 1.2 and 1.8 mL SB/g DM linearly improved ($P < 0.001$) ME, OMD, SCFAs, and GY_{24}. It could be concluded that, based on the highly detected interaction effects between ration types and extract doses for fermentation parameters and GP, the most effective dose of SB varied between incubated total mixed rations. However, the ration of 25% silage and 75% concentrate had the highest nutritive value, especially at doses of 1.2 to 1.8 mL SB/g DM total mixed ration.

Key words: Concentrate, silage, in vitro fermentation, *Salix babylonica*

1. Introduction

Salix babylonica (SB) is a tree of the willow family with slender leaves, native to dry areas of northern China. Trees of SB are commonly found along moist places and are often planted or cultivated as an ornamental tree. SB often cultivated to make high-quality wood chips, a renewable and carbon-neutral energy source. It was introduced to Mexico and many other countries other than China (1). Most of the members of the genus *Salix* were analyzed for their flavonoid, terpenoid, and phenolic constituents with diverse and important biological activities of improving ruminal fermentation (2). SB naturally contains benzyl ester of gentisic acid 2′-*O*-acetyl *β*-D-glucoside, along with trichocarpin, salicin, kaempferol-7-*O*-glucoside, apigenin-7-*O*-galactoside, and luteolin-4′-*O*-glucoside compounds and an ester of terephthalic acid (2). However, willows have phenolic glycosidic compounds based on the structure of salicin (3). Moreover, three flavonoids compounds were extracted from SB and identified as luteolin-7-*O*-*β*-D-glucopyranoside, luteolin, and chrysoeriol (2).

Plant secondary metabolites of some plants like SB seem to be alternatives to replace chemical feed additives (4–6). Extracts of SB were shown to have antimicrobial effects and can modulate rumen fermentation and improve nutrient utilization in ruminants (5,6). The antimicrobial activity of SB extracts is attributed to a number of chemical substances such as alkaloids, saponins, and phenolics (4). These chemical substances (i.e. secondary metabolites) have the ability to suppress or stimulate microbial growth, increase binding of ammonia during urea ammoniation, reduce odors from cattle manure in dairy barns, reduce nutritional stress such as bloat, and/or improve animal health and productivity (5,6). These resulting positive effects may improve daily gain, voluntary feed intake, and milk production (5), besides having protective effects on protein in the rumen, minimizing the excretion of nitrogen, modifying the acetate to propionate ratio in rumen fluid, and decreasing parasitic load (4). Plant extracts should be fed carefully because consumption of large amounts of tannins or saponins may have a direct hemolytic effect and may even cause death or detrimental effects on animal health (7).

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The silage to concentrate ratio, in ruminant rations, is one of the most important factors for efficient utilization of dietary nutrients for ruminant production (8). Microbial population, density, and activity depend on the roughage to concentrate ratio of diet fed to the host animal. With high levels of concentrates in diets, efficiency of microbial crude protein synthesis in the rumen is lower than in well-balanced roughage-based diets. However, diets higher in nonstructural carbohydrates such as starch normally cause a decrease in microbial growth efficiency due to a decreased rumen pH and a slowed rumen passage rate (9).

The aim of this study was to assess the effects of the chemical substances of SB extract at different doses on in vitro rumen fermentation of total mixed rations with different maize silage to concentrate ratios.

2. Materials and methods

2.1. SB extract and chemical constituent analysis

Collection of SB samples and analysis of chemical constituents were previously described by Salem et al. (5). Briefly, samples of young and mature leaves of SB were collected from several locations in the south of the State of Mexico. Samples were randomly collected from at least 7 trees at different sampling times. Leaf material was dried in a forced air oven at 23–25 °C until reaching a constant weight. Leaves were ground through a 1-mm sieve using a small laboratory mill (Wiley, UK), placed in plastic bags, and stored in the dark until laboratory analysis. One kilogram of leaves in 8 L of water was kept at room temperature for 48 h before being kept for 60 min at 30 °C in a water bath. The obtained solution was filtered with gauze, discarding the solid fraction, and the liquid fraction was retained at 4 °C.

For determination of active chemical constituents, subsamples of 100 g of dried SB leaves were coarsely powdered and soaked in 150 mL of methanol:acetone: hexane (1:1:1) at room temperature for 24 h. The crude extract was filtered through Whatman No. 1 filter paper and cleansed by filtration over activated charcoal to remove chlorophyll. The extract was concentrated by vacuum to 20 mL and then lyophilized to obtain dried extract and stored in a refrigerator until use (at 4 °C). Ten milliliters of the filtered liquid extract was kept at 4 °C until GC–MS analysis.

The chemical constituents of the leaf extract (20 mg) were dissolved in dichloromethane and analyzed by GC–MS (Varian Saturn 2100T 3900 GC/MS mass selective detector connected to an RTX 6890 gas chromatograph). Separation was achieved by a capillary column, RTX 5MS (5% phenyl methyl polysiloxane; 30 m long, 0.25 mm internal diameter, 0.25 μm film thickness). The column temperature was kept at 50 °C for 6 min and programmed to increase to 300 °C at a rate of 5 °C/min. The flow rate of helium as a carrier gas was 1 mL/min in the split 20 mL/min of 0 to 0.01. Aliquots (1 μL) of the solvent containing the SB extract were injected into the GC column with the injector heater at 300 °C. The MS was operated in full scan mode in the electron impact ionization (EI mode) at 70 eV. The ion source temperature was 230 °C. The MS was scanned from 40 to 650 *m/z* at a rate of two scans per second. The relative percentage of constituents was expressed as mg/g of peak area normalization. Identification of extract components was based on direct comparison of the retention times and mass spectral data, and computer search matching with the NIST MS Search 2.0 library, as well as by comparison of the fragmentation patterns of the mass spectra data with those reported in the literature (2).

2.2. Substrate and treatments

Three samples of five total mixed rations (TMRs) with different maize silage (F):concentrate (C) ratios (i.e. 0F:100C, 25F:75C, 50F:50C, 75F:25C, 100F:0C) were used in the current study (Table 1). A mineral/vitamin premix was included at 25 kg/t of TMR. Samples of the

Table 1. Chemical composition[1] of the rations with different silage and concentrate[2] ratios (g/kg DM).

Ration	OM	CP	NDF	ADF	ADL
0F:100C	927.4^{b}	172.0^{a}	145.1^{e}	70.3^{e}	8.1^{e}
25F:75C	932.6^{ab}	133.2^{b}	217.7^{d}	88.2^{d}	10.3^{d}
50F:50C	939.6^{a}	138.7^{b}	302.2^{c}	127.0^{c}	12.6^{c}
75F:25C	943.7^{a}	92.0^{c}	371.7^{b}	149.0^{b}	15.0^{b}
100F:0C	944.2^{a}	85.0^{c}	499.4^{a}	229.3^{a}	20.4^{a}
SEM	14.82	9.34	12.56	10.98	1.42

[1]OM: Organic matter, CP: crude protein, NDF: neutral detergent fiber, ADF: acid detergent fiber.

[2]Contained (g/kg): 200 maize grain flacked, 260 maize grain cracked, 154 sorghum grain, 100 molasses sugarcane, 100 distilled dry grain, 96 soy bean meal, 70 wheat bran, 10 $NaCOOH_3$, 10 mineral mixture[3].

[3]Mineral/vitamin premix (element/kg mineral/vitamin premix): vitamin A (12,000,000 IU), vitamin D3 (2,500,000 IU), vitamin E (15,000 IU), vitamin K (2.0 g), vitamin B1 (2.25 g), vitamin B2 (7.5 g), vitamin B6 (3.5 g), vitamin B12 (20 mg), pantothenic acid (12.5 g), folic acid (1.5 g), biotin (125 mg), niacin (45 g), Fe (50 g), Zn (50 g), Mn (110 g), Cu (12 g), I (0.30 g), Se (200 mg), Co (0.20 g).

TMR were dried at 60 °C for 48 h in a forced air oven to constant weight, ground in a Wiley mill to pass through a 1-mm sieve, and stored in plastic bags for subsequent determination of chemical composition and in vitro gas production (GP). The extract of SB contained (g/kg dry matter (DM)): 12.80 total phenolics, 4.80 saponins, and 72.53 aqueous fraction. Four doses of SB extract (0, 0.6, 1.2, and 1.8 mL/g DM of substrate) were used in the current study. The actual doses of SB extract used were 0, 6, 12, and 18 µL SB/g DM of substrate. Because of the difficulty of mixing small doses of extract with the substrates, and to verify and get a good mixing of SB extract doses with the 1 g of TMR used, SB extract doses were diluted with distilled water before incubation to be 0, 0.6, 1.2, and 1.8 mL/g DM. Different amounts of distilled water were added to the control and the rest of the treatments so that the volumes of the liquid additive were equal before the incubation process (i.e. equal volumes of incubation medium).

2.3. In vitro gas production determination

Rumen inoculum was collected from two Brown Swiss cows (450 ± 20 kg body weight) fitted with permanent rumen cannula and fed ad libitum a total mixed ration made up of 1:1 commercial concentrate (PURINA, Toluca, Mexico) and alfalfa hay formulated to meet all of their nutrient requirements (10). Cows were housed in individual pens of 3 × 3 m and fed twice daily in equal amounts at 0630 and 1830 hours after milking at 0600 and 1800 hours. The average DM intake was about 17.8 ± 1.35 kg/day. Fresh water was available to cows at all times during the rumen inoculum collection phase. Cow handling during the collection phase was conducted according to official Mexican standards of animals care (NOM-051-ZOO-1995).

Rumen contents from each cow were obtained before the morning feeding, mixed, and strained through four layers of cheesecloth into a flask with O_2-free headspace. Samples (1 g) of each TMR were weighed into 120-mL serum bottles with appropriate addition of SB doses/g DM. Consequently, 10 mL of particle-free rumen fluid was added to each bottle followed by 40 mL of the buffer solution (i.e. rumen fluid and buffer at a ratio of 1:4) according to Goering and Van Soest (11), with no trypticase added.

A total of 180 bottles (three bottles for each SB dose for each TMR, in three different runs, in addition to three bottles as blanks (rumen fluid only)) were incubated for 72 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken, and placed in an incubator at 39 °C. The pressure of GP was recorded at 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h of incubation using the GP technique (Extech Instruments, Waltham, MA, USA) of Theodorou et al. (12). At the end of incubation after 72 h, bottles were uncapped, pH was measured using a pH meter (Conductronic pH15, Puebla, Mexico), and the contents of each bottle were filtered to obtain the nonfermented residue for determination of degraded substrate.

2.4. Dry matter degradability

At the end of incubation, the contents of each serum bottle were 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 overnight to estimate potential DM disappearance, with loss in weight after drying being the measure of undegradable DM.

2.5. Chemical analyses, secondary compound determination, and calculations

Samples of the TMRs were analyzed for DM (#934.01), ash (#942.05), N (#954.01), and EE (#920.39) according to the AOAC (13). The neutral detergent fiber (NDF) (14), acid detergent fiber (ADF), and lignin (13) (#973.18) analyses were carried out using an ANKOM200 Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA). NDF was assayed with the use of an alpha amylase and sodium sulfite. Both NDF and ADF are expressed without residual ash.

Secondary compounds were determined by taking 10 mL of SB extract 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. The remaining solution was considered to be the aqueous fraction, which contains the other secondary compounds such as lectins, polypeptides, and starch (15).

Results of kinetic parameters of GP (mL/g DM) were fitted using the NLIN option of SAS (16) according to France et al. (17) 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 (mL/h), and L (h) is the discrete lag time prior to initiation of GP.

Metabolizable energy (ME, MJ/kg DM) and in vitro organic matter digestibility (OMD, g/kg OM) were estimated according to Menke et al. (18) as:

ME = 2.20 + 0.136 GP (mL/0.5 g DM) + 0.057 CP (g/kg DM),

OMD = 148.8 + 8.89 GP + 4.5 CP (g/kg DM) + 0.651 ash (g/kg DM),

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

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

Gas yield (GY_{24}) = mL gas/g DM/g DMD.

Short chain fatty acid (SCFA) concentrations were calculated according to Getachew et al. (19) as:

SCFA (mmol/200 mg DM) = 0.0222 GP – 0.00425,

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

Microbial biomass production (MCP) was calculated (18) as:

MCP (mg/g DM) = mg DMD – (mL gas × 2.2 mg/mL),

where 2.2 mg/ mL is a stoichiometric factor that expresses mg of C, H, and O required for the SCFA gas associated with production of 1 mL of gas (20).

2.6. Statistical analyses

Data of each of the three runs within the same sample of each of the three individual samples of TMRs were averaged prior to 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 (16) as:

$$Y_{ije} = \mu + D_i + SB_j + \varepsilon_{ije},$$

where Y_{ije} is every observation of the *i*th ration (D_i) with *j*th extract (SBj), μ is the general mean, ε_{ije} is the experimental error, D_i (i = 1–5) is the total mixed rations of different silage concentrate ratios, and SB_j (j = 1–4) is the extract dose's effect. Linear and quadratic polynomial contrasts were used to examine responses of different silage to concentrate ratios to increasing addition levels of the SB extract.

3. Results

3.1. Chemical substance characterization of SB extract

Retention time and mass spectral study identified 59 compounds in SB extract. The main chemicals were tritetracontane, an aliphatic hydrocarbon, at 15.2%; 9-octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E), a trioleoylglycerol (11.1%); hexadecanoic acid-methyl ester, a saturated fatty acid (10.5%); 1,3-dioxane-4-(hexadecyloxy)-2-pentadecyl, a heterocyclic organic compound (10.3%); and phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) (9.7%). There were also some aliphatic hydrocarbons such as nonadecane (1.2%) and hexatriacontane (0.8%) (Table 2).

3.2. In vitro rumen gas kinetics

Addition of SB crude extract did not affect (P > 0.05) values of *b* and *L*. However, increasing doses of SB to both 0F:100C and 25F:75C increased (P < 0.05) *c* values compared to the other ratios, where addition of 1.2 and 1.8 mL/g DM had the highest (P < 0.05) values. Crude extract of SB at a dose of 1.8 mL/g DM in 0F:100C and 75F:25C rations had the highest values of GP. However, in the case of 25F:75C and 0F:100C rations, the doses of 1.2 and 1.8 mL/g DM improved GP compared to other doses of SB extract (Table 3).

Table 2. Principal chemical constituents identified[1] in *Salix babylonica* leaves extract by GC–MS analysis, adapted from Salem et al. (2).

Compound	Retention time (min)		Chemical formula	MW[2]	Concentration (mg/g)[3]
	Measured	Authentic			
2-Hydroxy-6-methyl- benzaldehyde	7.7	7.8	$C_8H_8O_2$	136.2	9.9
2-Methoxy-4-vinylphenol	9.8	9.8	$C_9H_{10}O_2$	150.0	3.6
Hexatriacontane	13.2	13.2	$C_{36}H_{74}$	507.0	7.7
Nonadecane	14.3	14.3	$C_{19}H_{40}$	268.5	11.7
Tridecanoic acid, 12-methyl, methyl ester	14.5	14.5	$C_{15}H_{30}O_2$	242.4	6.7
3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol)	15.7	15.6	$C_{20}H_{40}O$	296.5	97.2
Hexadecanoic acid, methyl ester (palmitic acid, methyl ester)	16.5	16.5	$C_{17}H_{34}O_2$	270.5	149.7
9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)	18.0	18.0	$C_{57}H_{104}O6$	885.4	110.5
Octadecanoic acid, methyl ester	18.2	18.2	$C_{19}H_{36}O2$	296.5	16.8
1,3-Dioxane, 4-(hexadecyloxy)-2-pentadecyl	18.7	18.7	$C_{35}H_{49}O_3$	517.0	103.3
Tritetracontane	19.5	19.5	$C_{43H}88$	605.2	152.1
1-Pentacontanol	21.6	21.6	$C_{50}H_{102}O$	719.3	9.7

[1]Components mentioned were possibly identical in both analyses.
[2]Molecular weight of the compound (g/mol).
[3]Concentration based on the total areas of the identified peaks.

Table 3. In vitro rumen gas kinetics of five different mixture ratios of maize silage with concentrate as affected by different levels of *Salix babylonica* crude extract.

Rations	Extract (SB, mL/g DM of substrate)	Gas production parameters[1]			In vitro gas production (mL/g DM)									
		b	*c*	*L*	Gas2	Gas4	Gas6	Gas8	Gas10	Gas12	Gas24	Gas36	Gas48	Gas72
0F:100C	0	141.0	0.081	1.722	21.0	38.9	54.1	67.1	78.1	87.4	120.6	133.2	138.0	140.6
	0.6	146.7	0.083	1.753	22.6	41.7	57.8	71.4	83.0	92.7	126.7	139.2	143.9	146.3
	1.2	157.0	0.079	1.707	22.9	42.4	59.1	73.4	85.5	95.9	133.2	147.7	153.4	156.5
	1.8	208.2	0.154	3.017	55.6	96.3	126.0	147.7	163.6	175.3	202.7	207.2	208.0	208.2
	Linear	0.0007	<0.0001	0.0014	0.0004	0.0003	0.0003	0.0003	0.0002	0.0002	0.0003	0.0004	0.0006	0.0007
	Quadratic	0.0754	0.0016	0.0179	0.0108	0.01	0.0095	0.0093	0.0095	0.0099	0.0189	0.0376	0.0556	0.0717
25F:75C	0	145.6	0.080	1.982	21.5	39.7	55.3	68.6	80.0	89.6	124.0	137.3	142.4	145.1
	0.6	166.6	0.069	1.715	21.5	40.2	56.6	70.7	83.1	93.9	134.8	152.7	160.5	165.4
	1.2	275.7	0.051	2.052	26.9	51.2	73.1	92.8	110.6	126.7	194.9	231.8	251.7	268.5
	1.8	284.2	0.050	2.055	26.6	50.8	72.6	92.4	110.4	126.6	196.7	235.5	257.0	275.7
	Linear	<0.0001	<0.0001	0.8537	0.0045	0.0016	0.0006	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Quadratic	<0.0001	0.0036	0.9228	0.0362	0.0196	0.0104	0.0055	0.0029	0.0015	<0.0001	<0.0001	<0.0001	<0.0001
50F:50C	0	271.3	0.031	2.317	16.5	32.0	46.6	60.3	73.1	85.1	143.5	183.5	210.9	242.7
	0.6	292.4	0.026	2.499	15.1	29.3	42.9	55.7	67.9	79.5	137.4	179.5	210.2	248.8
	1.2	183.4	0.043	0.689	15.2	29.2	42.0	53.7	64.4	74.3	118.5	144.8	160.4	175.2
	1.8	179.8	0.040	2.245	14.0	26.8	38.7	49.7	59.8	69.1	111.6	137.8	153.9	170.0
	Linear	<0.0001	0.0002	0.7767	0.027	0.0203	0.0152	0.0114	0.0086	0.0065	0.0013	0.0003	<0.0001	<0.0001
	Quadratic	0.0001	0.0002	<0.0001	0.9749	0.8679	0.7631	0.6633	0.5698	0.484	0.1524	0.0423	0.0126	0.0019
75F:25C	0	193.5	0.038	1.691	14.1	27.3	39.4	50.7	61.1	70.8	115.6	144.1	162.1	180.8
	0.6	210.8	0.033	1.117	13.6	26.4	38.3	49.4	59.9	69.6	116.2	147.4	168.4	191.8
	1.2	190.2	0.032	2.578	11.7	22.6	32.9	42.5	51.6	60.1	101.2	129.3	148.5	170.7
	1.8	198.0	0.040	2.293	15.0	28.9	41.7	53.5	64.4	74.5	120.7	149.4	167.4	185.7
	Linear	0.5549	0.5154	0.3551	0.3312	0.3289	0.3266	0.3243	0.322	0.32	0.3136	0.3238	0.3536	0.4374
	Quadratic	0.4105	0.0097	0.302	0.0039	0.0037	0.0036	0.0034	0.0033	0.0032	0.0031	0.0042	0.0082	0.042
100F:0C	0	316.5	0.026	6.688	16.0	31.2	45.6	59.3	72.3	84.6	146.6	192.0	225.3	267.5
	0.6	393.5	0.021	4.459	14.7	28.7	42.1	55.0	67.3	79.1	140.3	188.1	225.6	279.0
	1.2	264.4	0.037	5.228	18.7	36.0	52.2	67.1	81.1	94.0	154.6	193.6	218.8	245.5
	1.8	284.3	0.031	4.768	17.0	33.0	48.0	62.0	75.3	87.7	148.1	189.7	218.5	252.2
	Linear	0.6102	0.2683	0.0005	0.2916	0.3054	0.3208	0.3388	0.3593	0.3834	0.6898	0.538	0.297	0.3225
	Quadratic	0.5136	0.0478	0.1338	0.0219	0.0228	0.0237	0.0248	0.0258	0.0271	0.0461	0.3973	0.5761	0.2841
SEM pooled		40.238	0.0075	0.5619	3.854	6.3031	7.8472	8.8127	9.411	9.7775	10.25	10.335	10.939	14.025
Interactions (P-value)														
Ration														
Linear		<0.0001	<0.0001	0.5721	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.219	<0.0001	<0.0001
Quadratic		0.343	0.0381	0.8022	0.2822	0.0818	0.0156	0.0021	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SB														
Linear		0.206	<0.0001	0.9517	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Quadratic		0.4318	<0.0001	0.0074	0.0101	0.0125	0.0166	0.0232	0.0334	0.0486	0.2666	0.4533	0.4309	0.2824
Ration× SB		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1]*b* is the asymptotic gas production (mL/g DM); *c* is the rate of gas production (mL/h); *L* is the initial delay before gas production begins (h).

The responses to different maize silage:concentrate ratios for in vitro GP were varied (linear effect, P < 0.001). Relative to the 0F:100C ration, increasing the ratio of silage linearly increased (P < 0.001) *b* values (mL/g DM) with decreasing *c* values (/h) (linear, P < 0.0001; quadratic P = 0.0381). However, it did not affect (P > 0.05) *L* values (h). With the exception of the 25F:75C ration, all other rations had decreased (P < 0.001) GP compared to the 0F:100C ration. However, ration 25F:75C had the highest (P < 0.001) GP (Table 3).

3.3. In vitro rumen fermentation profile

The mixture of silage and concentrate affected the values of pH (linear, P < 0.001; quadratic P = 0.0309), DMD, ME, OMD, SCFA (linear and quadratic, P < 0.001), and GY_{24} without affecting (P > 0.05) MCP. Compared to the ration of 0F:100C, both 25F:75C (linear, P < 0.001; quadratic P = 0.0038) and 50F:50C (linear, P = 0.004) lowered pH values. However, the other rations had almost the same values. The rations of 75F:25C and 100F:0C had lower (P < 0.01) DMD and OMD. However, the rations of 50F:50C and 100F:0C had improved (P < 0.01) SCFA and GY_{24} compared to other rations. Increasing portions of silage lowered (P > 0.05) the yield of MCP (Table 4). The dose of SB extract of 1.8 mL/g DM had the highest (linear, P < 0.0001) values of DMD, ME, OM, SCFA, and GY_{24} when added to rations 0F:100C and 75F:25C. However, the doses of 1.2 and 1.8 mL SB/g DM had improved (linear, P < 0.0001) DMD, ME, OM, SCFA, and GY_{24} (linear, P < 0.0001) when added to ration 25F:75C (Table 4).

3.4. Interaction effects between ration and SB extracts

Several interactive effects were noted (P < 0.0001) between different ration types and different extract doses for values of *b*, *c*, *L*, pH, DMD, ME, OMD, SCFA, GY_{24}, MCP, and in vitro GP (Tables 3 and 4).

4. Discussion

Limited data are available about the effect of the chemical substances of SB extract on ruminal microorganisms and ruminal fermentation kinetics, in addition to their modes of action and optimal concentrations. In the current study, analysis of SB leaf extract showed that C_{10} to C_{60} compounds were the dominant compounds. Examples included fatty acids and their methyl esters such as hexadecanoic acid, octadecatrienoic acid, octadecenoic acid, and pentadecanoic acid, which are relatively common essential oils in higher plants. Hexadecanoic acid is one of the major odd- and branched-chain fatty acids in rumen microorganisms (21).

Plant secondary metabolites include a vast array of compounds that to date sum up to more than 200,000 defined structures. There is limited information about effects of secondary plant metabolites on rumen microbial fermentation, their mechanism of action, and optimal doses to improve the efficiency of nutrient utilization. However, plant secondary metabolites have important roles as feed additives. These compounds have antimicrobial activity in the rumen of ruminants. The antimicrobial activity of plant extracts is attributed to a number of secondary plant metabolites, which include saponins (extracts of *Trigonella foenum-graecum*, fenugreek, or *Yucca schidigera*), terpenoids (such as carvacrol, carvone, thymol, or terpinen-4-ol), and phenylpropanoids (such as cinnamaldehyde, eugenol, or anethol) compounds, present in the essential oil fractions of many plants (22). Rumen microorganisms have the ability to degrade low concentrations of these compounds without negative effects on rumen fermentation.

Extract doses of SB (i.e. plant metabolites) are expected to be beneficial to rumen function on the basis of their stimulating effects on fermentation (5,6), and this may be also due to increasing degradabilities of CP and cell-wall constituents, as well as increasing microbial protein. These metabolites may act as catalysts for fiber degradation through increasing access of fibrolytic bacteria to the cell-wall components (4). These metabolites also have a protective effect on the protein in the rumen to promote duodenal absorption, minimize the excretion of nitrogen, modify acetate:propionate ratio in the rumen fluid, and decrease parasitic load (7).

Gas production is generally a good indicator of digestibility, fermentability, and rumen microbial protein production (23). SB extract addition was expected to be beneficial to rumen function based on its stimulating effect on fermentation, by increasing degradabilities of crude protein and plant cell-wall constituents, and by increasing microbial protein production (5,6). Salem et al. (6) stated that addition of SB extract to samples of TMRs improved ruminal GP and fermentation activities at moderate and high doses of SB extract (i.e. 1.2 and 1.8 mL/g DM). Moreover, increased GP was in parallel with increasing extract doses administrated. They also noted that DMD, OMD, ME, SCFAs, and MCP were increased (P <0.05) with the increasing SB extract doses. This could be attributed to the positive impacts of different chemical molecules of the extract on rumen fermentation (6). The same trends were observed in another study that used SB extract as a means to improve ruminal fermentation (6).

Rumen microorganisms have the ability to degrade secondary metabolites such as alkaloids, saponins, and phenolics and utilize them as an energy sources without negative effects on rumen fermentation (4). Cedillo et al. (24) illustrated that alkaloids, saponins, and phenolics increased digestibility and gas production of TMRs in vitro while improving rumen fermentation kinetics such as ME and SCFAs.

Table 4. In vitro rumen fermentation profile[1] of five different mixture ratios of maize silage with concentrate as affected by different levels of *Salix babylonica* crude extract.

Ration	Extract (SB, mL/g DM of substrate)	pH	DMD	ME	OMD	SCFAs	GY_{24}	MCP
0F:100C	0	6.7	837.2	6.5	45.0	2.66	144.2	571.9
	0.6	6.7	793.3	6.7	46.0	2.79	160.5	514.5
	1.2	6.7	880.1	6.9	47.2	2.94	152.9	587.0
	1.8	6.67	839.4	8.7	59.6	4.48	241.6	393.4
	Linear	0.8468	0.3157	0.0003	0.0003	0.0003	0.0026	0.0712
	Quadratic	0.6573	0.1263	0.019	0.0189	0.019	0.0182	0.0298
25F:75C	0	6.7	853.3	6.5	44.8	2.73	145.4	580.4
	0.6	6.6	847.5	6.8	46.7	2.97	159.1	550.9
	1.2	6.4	862.8	8.4	57.4	4.31	225.9	434.0
	1.8	6.4	860.9	8.5	57.7	4.34	228.4	428.3
	Linear	<0.0001	0.4613	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Quadratic	0.0038	0.5223	<0.0001	<0.0001	<0.0001	<0.0001	0.0003
50F:50C	0	6.3	836.7	6.9	46.9	3.16	171.6	521.0
	0.6	6.4	819.3	6.7	45.8	3.03	167.6	517.2
	1.2	6.5	689.0	6.2	42.4	2.61	173.5	428.3
	1.8	6.7	765.0	6.0	41.2	2.46	145.8	519.5
	Linear	0.004	0.0437	0.0013	0.0013	0.0013	0.0627	0.9642
	Quadratic	0.3417	0.0026	0.1519	0.1524	0.1519	0.19	0.0145
75F:25C	0	6.7	800.3	6.1	41.6	2.55	144.4	545.9
	0.6	6.7	737.1	6.1	41.7	2.56	158.0	481.4
	1.2	6.7	762.6	5.7	39.0	2.23	132.7	540.0
	1.8	6.7	793.6	6.2	42.5	2.66	152.2	528.2
	Linear	0.2029	0.698	0.313	0.3136	0.3144	0.4364	0.3413
	Quadratic	0.0062	0.0438	0.0031	0.0031	0.0031	0.8778	0.0463
100F:0C	0	6.7	732.5	6.7	45.3	3.23	200.2	409.9
	0.6	6.7	762.1	6.5	44.2	3.09	184.1	453.4
	1.2	6.7	752.8	6.9	46.8	3.41	205.4	412.7
	1.8	6.7	756.9	6.7	45.6	3.27	195.7	431.1
	Linear	0.1991	0.0062	0.6899	0.6896	0.6893	0.3412	0.0339
	Quadratic	0.4423	0.1926	0.0465	0.0461	0.046	0.0892	0.3089
SEM pooled		0.0676	35.966	0.2788	1.8225	0.2276	15.718	46.37
Ration								
Linear		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0715	0.2207
Quadratic		0.0309	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.5635
SB								
Linear		0.3664	0.9965	<0.0001	<0.0001	<0.0001	<0.0001	0.0006
Quadratic		0.0309	0.1678	0.2662	0.2665	0.2664	0.9062	0.591
Ration × SB		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1]DMD is the DM degraded substrate (mg/g DM); ME is metabolizable energy (MJ/kg DM); OMD is in vitro organic matter digestibility (g/kg DM); SCFAs is short chain fatty acids (mmol/g DM); GY_{24} is gas yield at 24 h (mL gas/g DMD); MCP is microbial CP production (mg/g DM).

The presence of some active chemical constituents of the plant extracts can improve synchronization between energy and protein release in the rumen, resulting in higher microbial protein synthesis. Some of these phenolic compounds may interact with biosynthesis of aromatic amino acids, as biosynthesis pathways are linked through cinnamic acid (4). In addition, phenylpropanoic acid and phenylacetic acid have been reported to enhance cellulose degradation and growth of several strains of *Ruminococcus albus* (4).

Feeding high-concentrate or restricted-roughage rations was found to increase ruminant productivity and decrease methanogenesis of feed ingested. High-roughage rations may decrease readily available energy and protein contents and increase structural carbohydrate content, which might impair microbial growth and fermentation (8). This is basically due to less nutrient digestibility causing less volatile fatty acid (VFA) production and less GP. Lowered digestibility may be due to high cell-wall and lignin material present in the silage, which might have suppressing effects resulting in decreased attachment of rumen microbes to feed particles (25). However, concentrate diets shift the rumen fermentation towards propionogenesis, whereas fibrous diets result in the preferential production of acetate, butyrate, and methane (CH_4) production (26). Per mole of propionate, less gas will be produced in the higher gastrointestinal tract than per mole of acetate, since in addition to the CO_2 developing from the VFA-bicarbonate buffering reaction, more waste gases (CH_4, CO_2) are produced from acetate (27). The higher GP of rations containing higher proportions of concentrates might have resulted from the increased production of propionate as CO_2 is produced, when propionate is made by rumen bacteria via the succinate:propionate pathway (8). Another explanation might be that the time necessary for degradation is longer than that for concentrate feed, and thus less gas is produced in the short term, which would confirm the obtained results, as after 72 h more gas was produced with the silage-based rations. Reddy (28) found a decrease in gas volume as the roughage level increased in the complete ration while replacing the concentrate proportion. However, Kumar et al. (26) reported that total GP was not affected by silage:concentrate ratio.

Increased cell-wall content was considered to suppress microbial activity through a reduction in the availability of rapidly fermented carbohydrates (29). Baah et al. (30) indicated positive effects of increased cell-wall content on activity of rumen bacteria, total bacterial growth rate, VFA, GP, DM intake, and milk production in cattle. However, Kim et al. (21) reported no significant effects on VFA production, DM degradation rates, and CH_4 production. In general, GP appeared to be related to the chemical composition of the feeds, and in particular to the fiber content.

It could be concluded that addition of SB extract, rich in active chemical molecules, showed a marked improvement in in vitro rumen gas kinetics and cumulative gas production. However, the highly detected interaction effects between diet types and extract doses for fermentation parameters and gas production make it difficult to define the most effective dose of SB extract for all rations. The most effective dose of SB varied between incubated TMRs. However, the most practical diet is that consisting of 25% silage and 75% concentrate at doses of 1.2 to 1.8 mL SB/g DM TMR, which could enhance the productive performance in some further in vivo experiments in ruminants.

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