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RESEARCH ARTICLE

Effects of cellulase and xylanase enzymes mixed with increasing doses of *Salix babylonica* extract on *in vitro* rumen gas production kinetics of a mixture of corn silage with concentrate

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Abstract

An *in vitro* gas production (GP) technique was used to investigate the effects of combining different doses of *Salix babylonica* extract (SB) with exogenous fibrolytic enzymes (EZ) based on xylanase (X) and cellulase (C), or their mixture (XC; 1:1 v/v) on *in vitro* fermentation characteristics of a total mixed ration of corn silage and concentrate mixture (50:50, w/w) as substrate. Four levels of SB (0, 0.6, 1.2 and 1.8 mL g^{-1} dry matter (DM)) and four supplemental styles of EZ (1 μL g^{-1} DM; control (no enzymes), X, C and XC (1:1, v/v) were used in a 4×4 factorial arrangement. *In vitro* GP (mL g^{-1} DM) were recorded at 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h of incubation. After 72 h, the incubation process was stopped and supernatant pH was determined, and then filtered to determine dry matter degradability (DMD). Fermentation parameters, such as the 24 h gas yield (GY_{24}), *in vitro* organic matter digestibility (OMD), metabolizable energy (ME), short chain fatty acid concentrations (SCFA), and microbial crude protein production (MCP) were also estimated. Results indicated that there was a SB×EZ interaction (P<0.0001) for the asymptotic gas production (b), the rate of gas production (c), GP from 6 to 72 h, GP_2 (P=0.0095), and GP_4 (P=0.02). The SB and different combination of enzymes supplementation influenced (P<0.001) *in vitro* GP parameters after 12 h of incubation; the highest doses of SB (i.e., 1.8 mL g^{-1} DM), in the absence of any EZ, quadratically increased (P<0.05) the initial delay before GP begins (L) and GP at different incubation times, with lowering b (quadratic effect, P<0.0001) and c (quadratic effect, P<0.0001; linear effect, P=0.0018). The GP was the lowest (P<0.05) when the highest SB level was combined with cellulose. There were SB×EZ interactions (P<0.001) for OMD, ME, the partitioning factor at 72 h of incubation (PF_{72}), GY_{24}, SCFA, MCP (P=0.0143), and pH (P=0.0008). The OMD, ME, GY_{24} and SCFA with supplementation of SB extract at 1.8 mL g^{-1} DM were higher (P<0.001) than the other treatments, however,

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PF_{72} was lower (quadratic effect, P=0.0194) than the other levels. Both C and X had no effect (P>0.05) on OMD, pH, ME, GY_{24}, SCFA and MP. The combination of SB with EZ increased (P<0.001) OMD, ME, SCFA, PF_{72} and GP_{24}, whereas there was no impact on pH. It could be concluded that addition of SB extract, C, and X effectively improved the *in vitro* rumen fermentation, and the combination of enzyme with SB extract at the level of 1.2 mL g^{-1} was more effective than the other treatments.

Keywords: cellulase, degradability, gas production, *Salix babylonica*, xylanase

1. Introduction

The use of exogenous enzymes as a feed additive strategy have attracted growing attention and proved to be useful in improving production efficiency of ruminants (Beauchemin *et al.* 2003). Morgavi *et al.* (2000) reported that enzymes improved fiber degradation in the rumen by acting synergistically with the rumen microflora, thereby increasing their hydrolytic capacity in the rumen (Beauchemin *et al.* 2004). Moreover, the use of fibrolytic enzymes in ruminant diets is generally characterized by an increases dry matter (DM) intake, cellulose degradation and/or nutrient digestibility, and consequently increase animal performance (Yang *et al.* 2000). The major fibrolytic enzymes are cellulases and xylanases, which degrade cellulose and hemicellulose, respectively, with synergy occurring between their activities (Bhat and Hazlewood 2001). It is also well known that ruminal microorganisms perform numerous enzymatic activities which hydrolyze the complex structure of plant cell walls to its constituent monomeric components (Khattab *et al.* 2011, 2013). However, ruminal microbial fermentation may result in considerable energy and protein losses as methane and ammonia N (NRC 2001). Indeed, 2–12% of the digestible energy ingested by ruminants is lost in the rumen as methane, whereas from 75 to 85% of the nitrogen consumed by dairy cows is excreted in feces and urine (Busquet *et al.* 2006; Mitsumori and Sun 2008).

On the other hand, plant extracts, rich in secondary metabolites, have been used as feed additive in order to manipulate ruminal fermentation and improve the efficiency of nutrients use, by decreasing the amount of methane or ammonia N produced (Rezaei and Pour 2012). This strategy has achieved increasing interest since these compounds are generally recognized as safe for human consumption (Busquet *et al.* 2006). Previous studies reported that some secondary metabolites, such as essential oils, can modify rumen N metabolism by reducing degradation of proteins, and ammonia N production in the rumen (Jiménez-Peralta *et al.* 2011). This beneficial effect, however, could be offset by a decrease of volatile fatty acid production and feed digestibility (Martinez *et al.* 2006). It has been shown that the concentration of plant extracts and their secondary compounds is capable of imparting positive effects on rumen fermentation (Calsamiglia *et al.* 2007). However, all studies reported in the literature highlight effects of either exogenous enzymes or plant extracts feed additive on rumen fermentation, but there is little information on the effect of these feed additives used in combination.

Therefore, the objective of this study was to assess the effects of different doses of plant extracts when combined with exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of a mixture of corn silage with concentrate.

2. Results

There was a *Salix babylonica* extract×enzyme interaction (P<0.0001) for the asymptotic gas production (b; mL g^{-1} DM), the rate of gas production (c; h^{-1}), gas production (GP) from 6 to 72 h (mL g^{-1} DM), GP_2 (GP at 2 h) (P=0.0095), and GP_4 (P=0.02) with no effects (P>0.05) on lag time (L; Table 1). However, there were no effect (P>0.05) of extract at doses of 0, 0.6 and 1.2 mL g^{-1} DM of substrate on b, c, L and GP at different incubation times with exception of GP_2 (P<0.0001) at 1.2 mL g^{-1} DM of substrate. Addition of *S. babylonica* extract (i.e., SB) at the highest doses (i.e., 1.8 mL g^{-1} DM of substrate) and in the absence of any enzymes quadratically increased lag time (P=0.0003) and volumes of GP at different incubation times (linear and quadratic effects, P<0.05), which resulted in the lowest values for the asymptotic GP (linear effect, P=0.0149; quadratic effect, P<0.0001) and rate of gas production (linear effect, P=0.0018; quadratic effect, P<0.0001). The effect of treatments on the volumes of GP was more pronounced after 48 h of incubation and reached 80% for SB 1.8 mL g^{-1} with control enzyme and followed by a gradual decrease with fermentation times to reach only 5% at 72 h at the same precedent conditions. In general and except for values recorded at 12 h of incubation, GP was the lowest (P<0.05) when the highest SB level was combined with cellulase addition. For the other treatments, with the exception of b, GP_{48} and GP_{72}, there were no differences in terms of GP (Table 1).

There were SB extract×enzyme interactions (P<0.0001)

Table 1 *In vitro* rumen gas kinetics and cumulative gas production after 72 h of incubation as affected by the combination of different levels of *Salix babylonica* extracts (SB, mL g^{-1} DM) with exogenous enzymes (EZ, at 1 μL g^{-1} dry matter (DM)) of xylanase (X) and cellulase (C) or their mixture (XC, 1:1, v/v)

Extract (SB)	Enzyme (EZ)	Gas production parameters[1]			*In vitro* gas production (mL g^{-1} DM)[2]									
		b	*c*	*L*	GP_2	GP_4	GP_6	GP_8	GP_{10}	GP_{12}	GP_{24}	GP_{36}	GP_{48}	GP_{72}
0	Control	146.7	0.087	1.753	36.2	64.3	80	99.9	113.2	126.4	154.2	170.6	180.6	190.8
	X	145.6	0.07	1.930	34.2	55.4	71.6	90.2	104.6	117.8	145.1	162.4	178.8	186.6
	C	140.7	0.078	1.921	36.9	56.7	73.7	92.1	106.2	119.5	145.7	160.9	175.3	183.6
	XC	152.1	0.072	1.402	40.8	65.5	81.6	99.5	113.2	126.3	153.5	169.8	185.5	199.1
	SEM	2.24	0.002	0.064	1.27	1.52	1.57	1.56	1.59	1.65	1.94	2.22	2.51	2.17
	P	0.41	0.28	0.064	0.355	0.103	0.146	0.156	0.186	0.215	0.266	0.349	0.5708	0.1383
0.6	Control	141	0.081	1.722	39.7	61.3	76.6	94.7	109.5	122.3	150	163.4	176.1	183.9
	X	147.7	0.076	1.530	37.5	62.2	76.9	96	109.1	121	150	165.4	180.1	189.4
	C	153.3	0.077	1.330	39	64.4	82.2	101.1	114.4	126.2	157.7	173.7	189.2	197.7
	XC	150.5	0.079	1.786	37.5	62.5	82.4	101.5	114.6	125.3	156.3	171.9	186.9	196.7
	SEM	1.89	0.0010	0.1000	0.95	1.43	1.35	1.41	1.34	1.62	1.56	1.49	1.55	1.62
	P	0.2029	0.6488	0.4919	0.8045	0.89	0.31	0.28	0.34	0.65	0.25	0.11	0.06	0.052
1.2	Control	157.0	0.079	1.707	36.2 a	59.4	79.5	99.9	114.4	127	157.3	173.7	189.2	199.3
	X	154.3	0.083	1.744	33.3 a	57.5	77.6	98.1	112.4	124.6	155.5	170.1	183.9	193.9
	C	162.6	0.072	1.666	26.3 b	51.4	69.5	88.8	104.3	117	148.8	167	184.4	195.9
	XC	186.8	0.080	1.293	29.1 b	54	80.2	100.7	119.5	131.9	164.4	185	204.9	217
	SEM	4.64	0.0040	0.1730	0.39	0.04	2.35	2.6	2.68	2.75	2.87	2.57	2.92	2.98
	P	0.1298	0.8044	0.7821	<0.0001	0.88	0.4	0.4	0.31	0.34	0.35	0.15	0.1	0.08
1.8	Control	208.2 a	0.1551 a	3.017a	31.0 a	56.6 a	103.3 a	149.9 a	172.3 a	184.8 a	215.6 a	229.5 a	242.7 a	254.3 a
	X	149.7 c	0.095 b	1.964 b	29.1 ab	54.2 ab	73.0 b	95.5 b	112.4 b	123.9 b	153.7 b	165.4 b	176.0 b	186.6 b
	C	149.9 c	0.087 b	1.841 b	25.7 b	47.4 b	66.2 b	90.2 b	105.1 b	116.2 b	144.0 b	157.6 b	170.0 b	183.6 b
	XC	160.0 b	0.073b	1.885 b	24.7 b	46.6 b	66.8 b	88.1 b	101.4 b	113.5 b	146.4 b	164.0 b	181.0 b	192.4 b
	SEM	3.95	0.004	0.083	0.49	0.84	1.01	3.38	3.82	3.89	3.82	3.97	4.32	4.38
	P	0.002	0.0006	0.0029	0.006	0.006	<0.0001	0.0005	0.0005	0.0006	0.0005	0.0011	0.0011	0.0013
Pooled SEM		16.92	0.0022	0.0576	4.2	6.07	8.2	11.8	12.7	13.2	13.4	13.6	14.9	14.9
P values														
SB														
Linear		0.0149	0.0018	0.132	0.0081	0.0094	0.0109	0.0126	0.0145	0.0165	0.0317	0.0458	0.0491	0.0314
Quadratic		<0.0001	<0.0001	0.0003	0.9639	0.7271	0.4515	0.2449	0.1152	0.047	<0.0001	<0.0001	<0.0001	<0.0001
EZ		0.0098	<0.0001	0.0487	0.0273	0.0289	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	0.0002
SB×EZ		<0.0001	<0.0001	0.0836	0.0095	0.022	<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^{-1} DM); *c* is the rate of gas production (h^{-1}); *L* is the initial delay before gas production begins (h).
[2] GP, gas production.
Different letters following means among enzymes in the column within each extract level indicate differences at $P<0.05$.

for organic matter digestibility (OMD), metabolizable energy (ME), the partitioning factor at 72 h of incubation (PF_{72}), the 24 h gas yield (GY_{24}), short chain fatty acid concentration (SCFA), microbial crude protein production (MCP) ($P=0.0143$), and pH ($P=0.0008$), with no effect on dry matter degradability (DMD) ($P>0.05$). However, there were no effect ($P>0.05$) of treatments on final pH (6.6–6.7) and DMD (79–88%). The highest SB doses, and in absence of any enzyme addition, control recorded the highest *in vitro* organic matter digestibility (OMD) (linear effect, $P=0.0315$; quadratic effect, $P<0.0001$), ME (linear effect, $P=0.0317$; quadratic effect, $P<0.0001$), GY_{24} (quadratic effect, $P=0.034$) and SCFA (linear effect, $P=0.0316$; quadratic effect, $P<0.0001$). With the exception of the highest SB doses and within the same doses, there was no effect ($P>0.05$) of enzyme addition on all the parameters measured. However, at SB 1.8 mL g^{-1} DM, with the exception of MCP, an effect ($P<0.05$) of enzyme addition on the pattern of fermentation was observed only with the control treatment (no enzyme addition) giving rise to a higher fermentation than that recorded when enzymes were added alone or in a combination with SB (Table 2).

3. Discussion

Natural plant extracts represent one of the options to replace the use of antibiotic as growth promoters in animal feeds (Salem *et al.* 2012). Indeed, there has been very limited research on the effect of these extracts when combined with exogenous enzymes on ruminal microbial fermentation. The intent of the present screening was to identify the optimal dose of *S. babylonica* extract and the amount of fibrolytic enzyme (xylanase/cellulose) needed to improve *in vitro* ruminal fermentation. To achieve this, four doses of SB (i.e., 0, 0.6, 1.2 and 1.8 mL g^{-1} DM) were tested *in vitro* combined

Table 2 *In vitro* rumen fermentation profile as affected by the combination of different levels of *S. babylonica* (SB, mL g^{-1} DM) extracts with exogenous enzymes (EZ, at 1 μL g^{-1} DM) of xylanase (X) and cellulase (C) or their mixture (XC, 1:1, v/v)

Extract (SB)	Enzyme (EZ)	pH	DMD (mg g^{-1} DM)[1)]	OMD (mg g^{-1} DM incubated)[2)]	ME (MJ kg^{-1} DM)[3)]	PF_{72} (mg DMD mL^{-1} gas)[4)]	GY_{24} (mL gas g^{-1} DMD)[5)]	SCFA (mmol g^{-1} DM)[6)]	MCP (mg g^{-1} DM)[7)]
0	Control	6.68	793	460	6.68	6.29	160.5	2.79	514.5
	X	6.70	836	449	6.51	6.95	143.0	2.65	571.3
	C	6.71	823	446	6.46	6.95	144.0	2.61	562.7
	XC	6.69	845	456	6.61	6.82	147.1	2.74	571.6
	SEM	0.005	80.6	30.8	0.058	0.148	3.59	0.047	10.72
	P	0.362	0.197	0.547	0.555	0.395	0.372	0.557	0.259
0.6	Control	6.69	837	450	6.51	6.96	144.2	2.65	571.9
	X	6.70	824	455	6.60	6.67	150.2	2.72	552.4
	C	6.70	839	464	6.74	6.51	153.7	2.84	555.5
	XC	6.68	853	462	6.71	6.67	149.9	2.81	571.9
	SEM	0.011	76.4	23.9	0.036	0.198	2.12	0.029	8.23
	P	0.952	0.636	0.187	0.197	0.097	0.5	0.183	0.755
1.2	Control	6.69	880	472	8.85	6.64	152.9	2.93	586.0
	X	6.80	867	472	6.86	6.5	154.3	2.94	573.8
	C	6.67	819	472	6.85	6.18	162.8	2.93	526.7
	XC	6.67	842	508	7.40	5.54	182.3	3.38	504.8
	SEM	0.007	169.9	56.2	0.086	0.217	5.43	0.07	20.51
	P	0.716	0.616	0.132	0.133	0.3504	0.275	0.1327	0.4837
1.8	Control	6.67	840	596 a	8.75 a	4.19 b	241.61 a	4.47 a	393.4 b
	X	6.64	913	474 b	6.88 b	6.80 a	147.26 b	2.95 b	617.8 a
	C	6.64	812	467 b	6.78 b	6.22 a	162.76 b	2.87 b	524.9 ab
	XC	6.63	850	468 b	6.80 b	6.47 a	154.58 b	2.89 b	560.9 ab
	SEM	0.008	185.7	70.9	0.108	0.152	5.89	0.088	19.99
	P	0.495	0.331	0.0005	0.0005	0.0013	0.0016	0.0005	0.022
Pooled SEM		0.03	168.8	25.0	1.38	0.79	12.26	1.031	17.91
P value									
SB									
Linear		0.9345	0.3624	0.0315	0.0317	0.0854	0.1352	0.0316	0.0854
Quadratic		0.3564	0.001	<0.0001	<0.0001	0.0194	0.034	<0.0001	0.0194
EZ		0.0006	0.299	0.0002	0.0002	0.0297	0.0026	0.0002	0.066
SB×EZ		0.0008	0.441	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	0.0143

[1)] DMD is dry matter degradability.
[2)] OMD is *in vitro* organic matter digestibility.
[3)] ME is metabolizable energy.
[4)] PF_{72} is the partitioning factor at 72 h of incubation.
[5)] GY_{24} is gas yield at 24 h.
[6)] SCFA is short chain fatty acids.
[7)] MCP is microbial CP production.

with individual or a mixture of exogenous fibrolytic enzymes. The SB extract used in this study contained moderate levels of phenolic compounds and saponins (Jiménez-Peralta *et al.* 2011). With the exception of the highest SB doses (1.8 mL g^{-1} DM), treatment with exogenous fibrolytic enzymes at the different SB doses had no effect ($P>0.05$) neither on parameters of GP nor on *in vitro* degradation and pattern of fermentation. No effect of dietary enzyme supplementation on ruminal fermentation parameters was also noted in other studies (Beauchemin *et al.* 1999).

Inconsistent results were observed with exogenous fibrolytic enzymes. Baah *et al.* (2005) indicated positive effects on activity of rumen bacteria, total bacterial growth rate, volatile fatty acids (VFA), GP, DM intake and milk production in cattle. However, Kim *et al.* (2005) reported no significant effects on VFA production, DM degradation rates and methane production. Fibrolytic enzymes may improve the nutritive value of feeds due to enhanced attachment by rumen microorganisms to feeds particles (Nsereko *et al.* 2002), creation of a stable enzyme feed complexes (Kung *et al.* 2000), and/or the possibility of alteration in the fiber structure, which could stimulate microbial colonization (Giraldo *et al.* 2004). The effects of enzymes, however, seem to be dependent on many factors such as source, type and dose of enzyme, type of diets fed to the animals, enzymes application methods, and method of administration (Beauchemin *et al.* 2003; Carro *et al.* 2007).

It is well reported that enzyme activity measurements

must be conducted under conditions closely defined with respect to temperature, pH, ionic strength, substrate concentration, and substrate type, since all of these factors will affect the activity of an enzyme. The optimal temperature and pH for assessing enzyme activity are generally not representative of the conditions in the rumen, which are closer to a pH of 6.0–6.7 and 39°C (van Soest 1994). Studies reported by Gashe (1992) indicated that at pH values ranging between 4.5 and 5.5, exogenous enzymes could make a contribution to ruminal fiber digestion. This condition could explain partly the lack of enzymatic effect on *in vitro* fermentation since pH varied between 6.63 and 6.70. Thus, the activities quoted for commercial enzyme products are considerably higher than for those that would be measured at a pH and temperature similar to that of the rumen. In discrepancy with our results, Colombatto *et al.* (2003) reported a significant relationship between xylanase activity and feed digestion. However, the relationship was positive with alfalfa hay, but negative with corn silage. It appears therefore that it may not be possible to predict the potential of increasing cell wall digestion in the rumen using exogenous feed enzymes based only on their biochemical characterization of substrate. This observation is not surprising because enzyme activities are measured on model substrates that do not represent the complexity of plant cell wall material. From this finding, we suggest that there might be an ideal ratio between the major enzymatic activities to achieve further improvement of degradation when enzymes are combined with other additives such as plant extracts.

Furthermore, because the conditions of the assays and method of expressing enzyme activity vary among manufacturers, it is difficult to compare enzyme products or predict the efficacy of the product in ruminant diets. In some studies, the response to enzymes has been substantial. For example, Lewis *et al.* (1999) treated forage with a cellulase/xylanase mixture (Loveland Industries, Greeley, CO, USA; supplying 1 mL kg^{-1} of total mixed ration, DM basis) and observed that cows in early lactation produced 16% more milk. However, higher and lower levels of the same enzyme product were less effective. It is clear that exogenous enzymes can be effective for ruminants, but it is important to determine the conditions that are most likely to result in positive responses. In fact, it was hypothesized that method of application has an impact on ruminal fermentation.

The ability of cellulases and xylanases to increase the extent of fiber digestion may be limited by the lack of enzymes that degrade the core structure of lignin-cellulose complexes in low quality forages (Carro *et al.* 2007). Krueger *et al.* (2003) showed that an enzymatic complex containing high esterase, cellulose and endogalacturonase activities enhanced the digestion of tropical hays, and suggested that the use of enzymes such as ferulic acid esterases could made the digestible xylans in the cell wall more susceptible to enzymatic degradation.

Irrespectively of enzymes addition and by the exception of the highest doses (1.8 mL g^{-1} DM) of SB, treatments with different SB doses, in general, had not significant effect on pattern of fermentation and *in vitro* degradation.

Previous studies reported by Jiménez-Peralta *et al.* (2011) pointed out that administration of high doses (i.e., 1.2 and 1.8 mL g^{-1} DM) of SB to high concentrate diet affected potentially ruminal fermentation and *in vitro* GP parameters. These authors suggested that ruminal bacteria of lambs are capable of metabolizing phenolic compounds (Chen *et al.* 1988), and may act as catalysts for fiber degradation by increasing access of fibrolytic bacteria to the cell wall polysaccharides in the high concentrate diet. In our study, rumen liquid used for the *in vitro* incubations was collected from cows fed on commercial concentrate and alfalfa hay, with no preliminary experience with SB extract.

In our study, pH varied between 6.63 and 6.70 and did not affected neither by SB doses nor by enzymes addition. Only high doses of (1.8 mL g^{-1} DM) of SB without any enzymes addition resulted generally in slightly beneficial effect on *in vitro* fermentation. Under these conditions, there was a particular increase of cumulative GP recorded at different incubation times and most parameters of pattern of fermentation. This was expected since SCFAs are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants (van Soest 1994). It is possible that plant extracts at these doses and levels of secondary compounds did not exhibit any antimicrobial effects (Cowan 1999) possibly due to the ability of rumen microorganisms to degrade them. Busquet *et al.* (2006) observed similar responses with some plant extracts when doses ranged from 3 to 30 mg L^{-1} in a dairy cow-type environment. This could be due to the stimulating effect of plant extracts on degradation of OM and synchronization between energy and protein release in the rumen in the presence of some chemical constituents of the plant extracts (Jiménez-Peralta *et al.* 2011). Nevertheless, in presence of an exogenous fibrolytic enzyme (xylanase/cellulose), the beneficial effect of plant extract was offset and a significant decrease (P<0.05) of *in vitro* GP parameters and pattern of fermentation was recorded. Perhaps high doses of SB would be needed to achieve an improvement of ruminal fermentation in cows.

4. Conclusion

In vitro gas production and ruminal fermentation results contribute to highlight that extract of *S. babylonica*, when added at high doses (1.8 mL g^{-1} DM of diet), are promising potential

modifiers of *in vitro* ruminal fermentation. However, when added simultaneously with fibrolytic enzymes (individual or mixture) they generally lose their beneficial effects on pattern of fermentation and *in vitro* gas production parameters. However, the dose of 1.2 mL g^{-1} DM will be more effective than the other doses when xylanase and cellulase or their combination will be added to diet.

5. Materials and methods

5.1. Substrate and treatments

A total mixed ration of corn silage and concentrate mixture (50:50, w/w) was prepared and contained g kg^{-1} DM: 939.6 organic matter (OM), 138.7 crude protein (CP), 302.2 neutral detergent fiber (NDF), 127.0 acid detergent fiber (ADF) and 12.6 acid detergent lignin (ADL). Diet sample was dried at 60°C for 48 h in a forced air oven until constant weight, ground in a Wiley mill to pass a 1-mm sieve and stored in plastic bags for subsequent determination of chemical composition and *in vitro* gas production (GP) parameters. Four doses of *Salix babylonica* extract (SB, i.e., 0, 0.6, 1.2 and 1.8 mL g^{-1} DM of substrate) were used in the absence (control) or presence of 1 μL g^{-1} DM of xylanase (X) and cellulase (C) or their combination (XC; 1:1, v/v) as an exogenous fibrolytic commercial enzymes (Dyadic® PLUS, Dyadic international, Inc., Jupiter, FL, USA) in a liquid form. Activities of the exogenous fibrolytic enzymes were provided by the manufacturers, and were cellulase 30 000 to 36 000 U g^{-1} and beta-glucanase from 7 500 to 10 000 U g^{-1} for cellulase plus, and xylanase from 34 000 to 41 000 U g^{-1}, beta-glucanase from 12 000 to 15 000 U g^{-1} and cellulose at 45 000 to 55 000 U g^{-1} for xylanase plus. Our analysis for extract of SB showed that it contained (g kg^{-1} DM): 164 total phenolics, 54 saponins and 763 aqueous fractions.

5.2. *In vitro* incubation

Effects of enzymes on ruminal fermentation of forages were widely determined using the *in vitro* GP technique (Eun *et al.* 2006). 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 50:50 commercial concentrate (PURINA®, Toluca, Mexico) and alfalfa hay formulated to meet all of their nutrient requirements (NRC 2001). Fresh water was available to cows at all times during the rumen inoculum collection phase.

Ruminal contents from each cow were obtained before the morning feeding, flushed with CO_2 mixed and strained through four layers of cheesecloth into a flask with O_2 free headspace. Samples (500 mg) of each feed were weighed into 120 mL serum bottles with appropriate addition of SB doses g^{-1} DM. Consequently, 10 mL of particle free ruminal fluid was added to each bottle followed by 40 mL of the buffer solution according to Goering and van Soest (1970), with no trypticase added, in a 1:4 (v/v) proportion. Exogenous fibrolytic enzymes of X and C, or XC were added on bottle contents (i.e., substrate and buffered rumen fluid) immediately before closing.

A total of 144 bottles (3 bottles in three runs with each four doses of SB (i.e., 0, 0.6, 1.2, 1.8 mL SB g^{-1} DM) and four exogenous fibrolytic enzymes (i.e., control, X, C or XC (1:1, v/v)) plus three bottles as blanks (i.e., rumen fluid only) were incubated for 72 h. Once all bottles were filled, flushed with CO_2, they were immediately closed with rubber stoppers, shaken and placed in an incubator at 39°C. The pressure of gas produced was recorded at 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h during incubation using the GP technique (Extech instruments, Waltham, USA) of Theodorou *et al.* (1994). At the end of incubation (i.e., at 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 non-fermented residue for determination of degraded substrates.

5.3. Dry matter degradability

At the end of incubation, the DM degradability was determined according to Ørskov and McDonald (1979). Contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter. Fermentation residues were dried at 105°C overnight to determine DM disappearance, with loss in weight after drying being the measure of undegradable DM (Ørskov and McDonald 1979).

5.4. Chemical analyses and secondary metabolites determination

Samples of the total mixed ration were analyzed for DM (#934.01), ash (#942.05), N (#954.01) and ether extract (EE) (#920.39) according to AOAC (1997). NDF (van Soest *et al.* 1991), ADF and lignin (AOAC 1997; #973.18) analyses were carried out using an ANKOM200 Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA). The NDF was assayed with use of an alpha amylase and with sodium sulfite in the NDF solution. Both NDF and ADF are expressed without residual ash.

Secondary compounds were determined by taking 10 mL of *S. babylonica* extract fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont®, Monterrey, Mexico) 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 (99.9/100, analytical grade, Fermont®), was added to fractionate the saponins (Makkar *et al.* 1998). The remaining solution was considered to be the aqueous fraction which contains the other secondary compounds such as lectins, polypeptides and starch (Cowan 1999).

5.5. Calculations

All the following calculations were previously mentioned in Salem *et al*. (2013). To estimate kinetic parameters of GP, results (mL g^{-1} DM) were fitted using the nonlinear model option 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 (mL g^{-1} DM) at time t; b is the asymptotic GP (mL g^{-1} DM); c is the rate of GP (h^{-1}), and L (h) is the discrete lag time prior to initiation of GP.

Metabolizable energy (ME, MJ kg^{-1} DM) and *in vitro* organic matter digestibility (OMD, g kg^{-1} OM) were estimated according to Menke *et al.* (1979) as:

ME=2.20+0.136GP (mL 0.5 g^{-1} DM)+0.057CP (g kg^{-1} DM)

OMD=148.8+8.89GP+4.5CP (g kg^{-1} DM)+0.651Ash (g kg^{-1} DM)

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

The partitioning factor at 72 h of incubation (PF_{72}; a measure of fermentation efficiency) was calculated as the ratio of DM degradability *in vitro* (DMD, mg) to the volume (mL) of GP at 72 h (i.e., DMD/Total gas production (GP_{72})) according to Blümmel *et al.* (1997). Gas yield (GP_{24}) was calculated as the volume of gas (mL gas g^{-1} DM) produced after 24 h of incubation divided by the amount of DMD (g) as:

Gas yield (GY_{24})=Gas (mL g^{-1} DM)/DMD (g^{-1})

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

SCFA (mmol 200 mg^{-1} DM)=0.0222GP-0.00425

Where, GP is the 24 h net gas production (mL 200 mg^{-1} DM).

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

MP (mg g^{-1} DM)=DMD (mg)–Gas (mL)×2.2 (mg mL^{-1})

Where, 2.2 (mg mL^{-1}) is a stoichiometric factor which expresses mg of C, H and O required for production of SCFA gas associated with production of 1 mL of gas.

5.6. Statistical analyses

Mean values of each run were used as the experimental unit. Results of *in vitro* GP and ruminal fermentation parameters were analyzed as a 4×4 factorial experiment (i.e., 4 levels of SB extract being 0, 6, 1.2 and 1.8 mL SB g^{-1} DM) with 4 exogenous fibrolytic enzymes (i.e., no enzyme, C, X or XC (1:1, v/v), using the PROC GLM option of SAS (2002) as:

$Y_{ij}=\mu+SB_i+EZ_j+(SB_i\times EZ_j)+ijk$

Where, Y_{ijk} is every observation of the ith extract (SB_i) when incubated with the jth EZ types (EZ_j; type enzyme preparation); μ is the general mean; SB_i (i=1–4) is the extract doses effect; EZ_j is the enzyme dose effect (j=1–4); $SB_i\times EZ_j$ is the interaction between extract and enzyme dose.

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