PAPER

Effects of different doses of *Salix babylonica* extract on growth performance and diet *in vitro* gas production in Pelibuey growing lambs

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Abstract

Twenty Pelibuey 3-4 month old and 23.7±3.3 kg body weight male lambs were used in a randomised design to study the effects of daily oral administration of *Salix babylonica* (SB) extract on dry matter (DM), water intake, average daily gain (ADG), and feed efficiency for 72 days. Animals were divided into four groups fed the same total mixed ration with different doses of SB: 0 (Control), 20 (SB20), 40 (SB40) and 60 (SB60) mL/lamb/d. *In vitro* gas production (GP) of the same diet fed to lambs as a substrate was measured with different doses of SB (0, 0.3, 0.7, 1.0 mL/g DM). Daily administration of SB to lambs had no effects (P=0.05) on growth performance and DMI (linear effect, P=0.2805; quadratic effect, P=0.3747). Both low and moderate doses of SB (SB40>SB20) tended to increase (linear effect, P=0.4010; quadratic effect, P=0.9166) ADG. The asymptotic GP quadratically increased (P<0.001) with decreased GP rate and with increasing SB extract doses. *In vitro* GP increased (P<0.05) with advancing of incubation time in all SB doses. During the first 24 h of incubation, 0.3 mL SB/g DM had the highest GP, whereas 1.0 mL SB/g DM quadratically increased (P<0.001) GP. The low dose of SB extract increased ME (linear effect, P=0.024) and short chain fatty acids (SCFA) (linear effect, P=0.023). However, the highest dose quadratically decreased (P=0.02) DM degradability. In conclusion, administration of SB extract at 40 mL/lamb/d tended to increase DM intake, improve daily weight gain in growing lambs with increasing asymptotic *in vitro* ruminal GP and SB dose.

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Introduction

Public concern over use of antibiotics in livestock production has increased in recent years because of their possible contribution to emergence of antibiotic resistant bacteria (Busquet *et al.*, 2006) and their transmission from livestock to humans; hence, their use has been banned in the European Union. For this reason, ruminant microbiologists and nutritionists have to explore alternative methods of favourably altering ruminal metabolism to improve feed efficiency and animal productivity, including the use of yeasts, organic acids, plant extracts, probiotics, and antibodies (Calsamiglia *et al.*, 2007; Elghandour *et al.*, 2014). Accordingly, plant extracts contain specific secondary metabolites that give them potential alternatives as feed additives to manipulate rumen microbial population's activity (Jiménez-Peralta *et al.*, 2011; Salem, 2012; Salem *et al.*, 2014b). Plant secondary metabolites in *Salix babylonica* (SB) extract (Jiménez-Peralta *et al.*, 2011; Salem *et al.*, 2011, 2014b) or in herbs such as organic acids and essential oils (Hernández *et al.*, 2004) seem to be alternatives to replace chemical feed additives (Patra *et al.*, 2006; Jiménez-Peralta *et al.*, 2011).

A combination of positive impacts of secondary metabolites on ruminal microorganism activity (Jiménez-Peralta *et al.*, 2011; Salem *et al.*, 2014b), nutrient digestion (Mapiye *et al.*, 2010; Salem *et al.*, 2011, 2014b) increased supply of amino acid to the duodenum (Mueller-Harvey, 2006), and microbial protein production. This could improve and increase muscle deposition as well as carcasses weights and meat quality (Gleghorn *et al.*, 2004; Mapiye *et al.*, 2010). In addition, secondary metabolites have been shown to enhance protein metabolism, decrease CH_4 emission, and suppress or stimulate microbial growth (Makkar *et al.*, 1998). Some metabolites also reduce nutritional stress such as bloat and/or improve animal health and productivity (Xhomfulana *et al.*, 2009; Salem *et al.*, 2010) resulting in higher daily gain, voluntary feed intake and milk production (Salem *et al.*, 2011, 2014a). Also, it may have a protective effect on feed protein within the rumen with promoting duodenal absorption, minimising the excretion of N, modifying the acetate to propionate ratio, and it also has been shown to have anthelmintic effects (Athanasiadou and Kyriazakis, 2004; Mejía-Hernández *et al.*, 2014). Careful aspects should be considered during plant extract feeding. Consumption of large amounts of tannins or saponins may have a direct haemolytic effect and may even cause death (Athanasiadou and Kyriazakis, 2004). Moreover, long-term feeding of plants, rich in secondary compounds, may have detrimental effects on animal health (Mahgoub *et al.*, 2008).

This experiment was conducted to assess the effect of feeding diet with different doses of plant extract of *Salix babylonica* on feed intake, average daily gain and *in vitro* gas production (GP) of growing lambs.

Materials and methods

The experiments of growth performance and *in vitro* evaluations were performed at the

experimental farm and laboratory of animal nutrition of the Centro Universitario UAEM-Temascaltepec, Universidad Autónoma del Estado de México at north latitude 19°02'04" and west longitude 100°02'14", 1720 m asl. The climate is moderately humid with an average temperature of 15 to 18°C and annual rainfall of 950 to 1000 mm (Borboa, 1987).

In vivo study: lamb's growth performance

Twenty Pelibuey male lambs 3 to 4 months of age and 23.7±3.3 kg body weight were used in a completely randomised design. Lambs were housed individually in pens of 1.24 m×0.82 m. After 2 weeks of adaptation consuming a total mixed ration (TMR) composed of [g/kg dry matter (DM) basis]: alfalfa hay, 150; sorghum grain, 530; soybean meal, 220; molasses, 20; fish meal, 35; salt, 20; mineral and vitamin premix, 25 (containing per kg of mineral premix: 19.60 g/kg calcium, 22.10 g/kg sulfur, 4 mg/kg cobalt, 15.93 mg/kg iodine, 15.49 mg/kg selenium; 860.73 mg/kg copper, 224.07 mg/kg zinc). For preventing the selection by animals, all the ingredients were granulated before mixing (2 to 5 mm) and then mixed very well. The TMR had the following nutritional composition [g/kg DM]: organic matter, 912.4; crude protein (CP), 173.6; neutral detergent fibre (NDF), 131.03, acid detergent fibre (ADF), 80.3; and hemicelluloses, 51.0.

This TMR was the same as the one fed to lambs of the experiment previously done at the same farm by Salem *et al.* (2011). Lambs were assigned to the following treatments: Control-TMR; SB20, SB40 and SB60 (as Control plus SB extract at 20, 40 and 60 mL/lamb/d, respectively) and contained in g/kg (Salem *et al*., 2011): 9.6, 12.8 and 76.8 g/kg of total phenolics, saponins and aqueous fraction of lectins, polypeptides, starch, respectively. Extract was orally administered daily before the 07.00 h feeding to each lamb during 72 days of the experimental period. A weekly stock volume (5 L) of the SB extract was prepared before oral administration to the animals. Lambs were fed at 07.00, 13.00 and 17.00 h with *ad libitum* TMR that was formulated to meet all of their nutrient requirements (National Research Council, 1985). Feed and water intake were recorded daily during the experimental period. Animals body weight was recorded weekly from start to end of the experiment. Individual lamb average daily gain (ADG) and feed efficiency were calculated.

Preparation and analysis of *S. babylonica* extract

Salix babylonica extract was prepared weekly as described previously in Salem (2012). Briefly, leaves collected randomly during summer season from several young and mature trees of SB were fresh chopped into 2 to 3 cm lengths and immediately extracted at 1 g leaf/8 mL of solvent which contained 10 mL methanol (99.8/100, analytical grade; Fermont, Monterrey, Mexico), 10 mL ethanol (99/100, analytical grade; Fermont) and 80 mL of distilled water. Plant materials were individually soaked and incubated in water in the laboratory temperature of 25 to 30°C for 72 h in closed jars of 5 L. After incubation, jars were heated at 39°C for 1 h, and then immediately filtered and the filtrates were collected and stored at 4°C for further use.

Secondary metabolites of SB extract were determined in triplicate according to the method described in Salem (2012). Briefly, 10 mL of extract were fractionated by funnel separation with a double volume of ethyl acetate to determine total phenolic compounds by drying and quantifying the phenolics layer in the funnel. After phenolic compouns separation, 20 mL of n-butanol were added to fractionate the saponins. The remaining solution in the funnel was considered to be the aqueous fraction that has the other secondary compounds such as lectins, polypeptides and starch (Cowan, 1999).

In vitro evaluation of the total mixed ration

Salix babylonica extract were examined at four doses (0, 0.3, 0.7, 1.0 mL/g DM of high grain diet) in four replicates for each treatment on the resultant *in vitro* fermentation kinetic profile of lambs diet (*i.e.,* TMR). The substrate was the same TMR fed to lambs during the growth performance experiment.

Gas production assay was carried out as described by Theodorou *et al.* (1994) with arrangements of Mauricio *et al.* (1999). Samples (1±0.002 g) of substrate (*i.e.,* TMR) were weighed in quadruplicate into 160-mL serum bottles. Extract doses of SB were applied directly onto the substrate inside the bottles immediately before adding buffer medium and rumen fluid. Rumen fluid was collected by stomach tube from 4 of the growing lambs fed the same TMR during the growth performance experiment for 72 days (Control group). Ruminal contents of each animal were obtained immediately before the morning feeding, mixed and strained through four layers of muslin and then kept at 39°C under a continuous CO_2 stream. Ten mL of particle-free ruminal fluid and 90 mL buffer medium (containing micro- and macro-elements, a reducing agent and a reduction indicator of resazurin) were added to each bottle. Negative controls containing buffered rumen fluid with or without SB extract but no substrate, were also included in triplicate for correction of gas produced from small particles present in the ruminal fluid. Cumulative GP (mL/g DM) was recorded at 2, 4, 6, 8, 10, 12, 15, 19, 24, 30, 36, 48, 72 and 96 h post incubation at 39°C. Volume of gas (mL/g DM) produced after 24 h of incubation (GP_{24}) was used as an index of energy feed value. At the end of incubation (*i.e.,* 96 h), the contents of each serum bottle were filtered using sintered glass crucibles (porosity 1, 100- to 160-µm pore size; Pyrex, Washington, UK) under vacuum. Fermentation residues were dried at 105°C overnight to estimate the DM disappearance.

Pressure generated by the gas accumulated in the upper part of the incubation serum bottles was measured through a pressure transducer connected to a digital reader. The equation was previously obtained using PROC REG of the SAS (2002) programme:

$$Y=-0.807+6.86X+0.083X^2$$

where Y is volume (mL), X is pressure (psi). R^2=0.99.

Then, GP data (mL/g DM) were fitted using the NLIN option of SAS (2002) to the model of France *et al.* (2000) as:

$$A=bx[1-^{e-c(t-L)}]$$

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 lag time.

Metabolisable energy (ME; MJ/kg DM) was estimated according to Menke and Steingass (1988) as:

$$ME=2.20+0.136IVGP_{24}(mL/0, 2g\ DM)+0.057CP$$

where $IVGP_{24}$ was 24 h gas volume and CP (% DM) was that of tree leaves.

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

$$mmol\ SCFA=-0.00425+0.0222\ (mL\ gas\ at\ 24\ h)$$

Proximate analysis of total mixed ration

Samples of TMR were analysed for DM, ash, CP according to the AOAC (1990). Neutral detergent fibre and ADF content were analysed using the ANKOM F-57 filter bags in an Ankom^{200}Fibre Analyzer unit (Ankom Technolgy, Macedon, NY, USA) according to Van Soest *et al.* (1991). For NDF analysis, samples were treated with a-amylase (Sigma A-

3403; Sigma-Aldrich Co., St. Louis, MO, USA), and the neutral detergent solution contained sodium sulfite and the residues were not corrected for residual ash. Hemicellulose content was calculated from the difference between NDF and ADF.

Statistical analysis

The experimental design of the growth performance experiment was completely randomised, where lambs were the experimental units. The statistical model used for the analysis was:

$$y_{ijk}=\mu+d_i+a(d)_{j(i)}+ _{ijk}$$

where y_{ijk} is the value measured at period k on the j^{th} lamb assigned to the i^{th} diet (extract dose), μ is the overall mean effect, d_i is the i^{th} fixed diet (extract) effect, a $(d)_{j(i)}$ is the random effect of the j^{th} lamb within the i^{th} diet, ε_{ijk} is the random error associated with the j^{th} lamb assigned to the i^{th} diet.

Tukey's test was used for the multiple comparisons among mean values and linear and quadratic effects were calculated at $P<0.05$. Data of *in vitro* ruminal GP, fermentation parameters were analysed as a completely randomised design (four extract doses) using the GLM option of SAS (2002) with methods of Steel and Torrie (1980), to determine differences due to extract levels. Tukey's test was used for the multiple comparisons among mean values for each run and linear and quadratic effects were calculated at $P<0.05$.

Results

No significant differences ($P>0.05$) were observed for the daily oral administration of SB to lambs. However, DM intake (DMI; kg/d) tended to be increased (linear effect, P=0.2805; quadratic effect, P=0.3747) with low (*i.e.,* SB20) and moderate (*i.e.,* SB40) doses of SB extract (SB20>SB40) compared to Control and the high SB extract dose (*i.e.,* SB60). All SB lambs (*i.e.,* SB20, SB40 and SB60) with different doses tended to increase (linear effect, P=0.1961; quadratic effect, P=0.8287) water intake (L/d) by about 8.1, 13.5, and 5.1% *vs* no SB lambs (*i.e.,* SB0). During the 72 days of the experiment, both low and moderate doses of SB extract (*i.e.,* SB20, SB40) tended to increase (linear effect, P=0.4010; quadratic effect, P=0.9166) the ADG (g/d) (SB40>SB20) by about 5 and 8.2%, respectively. Feed efficiency (g DMI/kg ADG) was the same among all groups with the exception of SB20 group which tended to increase (linear effect, P=0.9736; quadratic effect, P=0.6510) the efficiency by about 4.3% compared to other diets (Table 1).

The asymptotic GP (*b,* mL/g DM) was quadratically increased ($P<0.001$) with increasing doses of SB extract, whereas the rate of GP (*c,* /h) was quadratically decreased ($P<0.001$) with increasing SB doses. The dose of 0.7 mL/g DM caused a linear increased (P=0.003) in the lag time (h) while the dose 0.3 mL/g DM was linearly decreased (P=0.003) compared to control (0 mL/g DM). *In vitro* GP was increased ($P<0.05$) with the advancing of incubation time with all SB doses. During the first 24 h of incubation, the 0.3 mL SB extract/g DM had the highest GP. However, after 24 h of incubation the highest dose of SB extract (*i.e.,* 1 mL SB extract/g DM) had quadratically increased ($P<0.001$) GP *vs* the other doses. Supplementation of 0.3 mL SB extract/g DM increased DMD (quadratic effect, P=0.020), ME (linear effect, P=0.024), SCFA (linear effect, P=0.023) *vs* the other doses (Table 2).

Discussion

Feed and water intakes

Daily oral administration of SB extract to lambs at doses of 20 and 40 mL/lamb/d tended to increase DMI by 10.8 and 6.4%, respectively, compared to Control and SB60 (*i.e.,* high dose of SB extract). This tendency could be associated with the improved rumen fermentation kinetics of the same diet used as a substrate *in vitro*. This improvement may be due to positive impacts of plant secondary metabolites on ruminal fermentation and nutrient digestibility (Salem *et al.*, 2011, 2014b). This action possibly leads to increase rates of dry matter disappearance in the rumen and rates of passage and consequently increased feed intake (Conrad, 1966). It is well known that some tree extracts reduced microbial protein degradability (Mueller-Harvey, 2006). The tendency to increase water consumption with administration of SB extract with SB20 and SB40 *vs* SB60 may be related to SB extract content of secondary metabolites. Dearing *et al.* (2000) and Salem *et al.* (2013) stated that ingestion of secondary metabolites increases water intake and could act as diuretics, resulting in more water consumption.

Growth performance of lambs

Oral administration of SB extract at appropriate doses of 20 and 40 mL/lamb/d tended to increase lambs ADG *vs* SB60 (*i.e.,* high dose) or Control (SB0). Appropriate doses of plant extract, rich in secondary metabolites, ensure improved ruminal fermentation kinetics may be through reduction of CH_4 emission of GP during fermentation. This action could insure more energy available for growth and increase the SCFA and ME density of the diet. Improved ADG may be due to improved GP and ruminal fermentation as well as increased microbial protein synthesis of the same diets used as substrate *in vitro* studies performed in our laboratory. Moreover, secondary metabolites of SB extract such as phenolic compounds or saponins could improve synchronisation between energy and N release and improve microbial protein synthesis (Salem *et al.*,

Table 1. Dry matter intake, average daily gain and feed efficiency in growing lambs fed concentrate diet with different doses of *S. babylonica* extract.

Intake	SB doses, mL/lamb/d				SEM	P	
	SB0	SB20	SB40	SB60		Linear	Quadratic
DMI, kg/d	1.48	1.64	1.62	1.47	0.355	0.2805	0.3747
Water, L/d	3.7	4.0	4.2	3.9	1.11	0.1961	0.8287
Initial BW, kg	22.7	25.6	22.4	24.2	5.89	0.9087	0.1079
Final BW, kg	41.5	45.4	42.8	43.0	7.16	0.6052	0.1569
ADG, g/d	216.4	227.2	234.2	216.0	59.02	0.4010	0.9166
g DMI/kg ADG	6.9	6.6	6.9	6.9	2.21	0.9736	0.6510

SB, *S. babylonica*; DMI, dry matter intake; SB0, 0 mL/lamb/d *S. babylonica* (Control); SB20, 20 mL/lamb/d *S. babylonica*; SB40, 40 mL/lamb/d *S. babylonica*; SB60, 60 mL/lamb/d *S. babylonica*; BW, body weight; ADG, average daily gain. P values of all parameters were more than 0.05.

Table 2. *In vitro* gas production parameters, gas volume accumulated after different hour of incubation and rumen fermentation profile of diet with different doses of *S. babylonica* extract.

	SB doses, mL/g DM				SEM	P	
	0	0.3	0.7	1.0		Linear	Quadratic
GP parameters							
b, mL/g DM	254.4^{c}	262.7^{bc}	278.6^{b}	313.4^{a}	18.64	0.2107	<0.0001
c, /h	0.068^{a}	0.07^{a}	0.05925^{b}	0.047^{c}	0.0046	0.2253	<0.0001
L, h	2.008^{ab}	1.213^{c}	2.255^{a}	1.575^{bc}	0.4786	0.0003	0.8056
In vitro GP, mL/g DM							
GP_6	60.4^{b}	75.0^{a}	55.2^{b}	58.9^{b}	8.70	0.0003	0.0046
GP_{12}	125.5^{b}	139.4^{a}	121.7^{b}	121.4^{b}	11.08	0.0028	0.0052
GP_{19}	174.3	187.2	174.7	175.3	13.00	0.0123	0.1764
GP_{24}	197.3	209.5	201.2	204.2	13.88	0.0231	0.8538
GP_{48}	243.2^{c}	252.8^{bc}	259.7^{b}	278.0^{a}	16.27	0.1070	<0.0001
GP_{72}	252.2^{c}	260.8^{bc}	273.9^{b}	301.9^{a}	17.59	0.1699	<0.0001
GP_{96}	253.9^{c}	262.3^{bc}	277.4^{b}	309.6^{a}	18.26	0.1983	<0.0001
Rumen fermentation profile							
DMD, mg/g DM	868.5	870.3	864.0	844.3	32.10	0.8741	0.0199
ME,° MJ/kg DM	8.6	8.9	8.7	8.7	0.38	0.0244	0.8428
SCFA,# mmol/g DM	0.872	0.926	0.889	0.902	0.0616	0.0232	0.8530

SB, *S. babylonica*; GP, gas production; DM, dry matter; *b*, asymptotic gas production; *c*, rate of gas production; *L*, initial delay before gas production begins; DMD, apparent degraded substrate; ME, metabolisable energy; SCFA, short chain fatty acids. °Calculated according to Menke and Steingass (1988) based on gas production; #calculated according to Getachew *et al.* (2002) based on gas production. $^{a-c}$Means in the same row with different superscripts indicate significant differences at P<0.05.

2011). Saponins have antimicrobial properties on ciliate protozoa growth, peptidase-producing bacteria, and cellylolytic bacteria (Francis *et al.*, 2002) which normally effect SCFA production (*i.e.,* acetate, not propionate; Wallace *et al.*, 1994) which could improve ADG. Secondary metabolites of SB extract may improve the lamb's health during the 72 days of experiment by their anti-helminthic effects (Xhomfulana *et al.*, 2009; Mejía-Hernández *et al.*, 2014) that could improve nutrient digestibilities, ruminal fermentation, and animal health. Mejía-Hernández *et al.* (2014) at the same experimental farm recently reported an elimination of about 40% of intestinal worm burdens when lambs administrated orally with 30 mL of SB extract daily for 60 days. Xhomfulana *et al.* (2009) showed that steers fed *A. karroo* leaves had low mean total faecal egg counts, *Haemonchus contortus* and *Oesophagostomum colombianum* worm burdens.

In vitro gas production

The asymptotic GP was increased with decreased rate of production with increasing SB extract. This phenomenon was observed with all doses of SB extract supplementation. These effects are related to secondary metabolites content of SB extract. Higher *in vitro* GP and ruminal fermentation patterns of TMR fed to lambs with SB could be, at least partly, due to its lower secondary metabolites concentrations (Salem *et al.*, 2006), and/or higher soluble sugars in the extracts (Patra *et al.*, 2006) which could positively affect ruminal microorganism activity. At low secondary metabolites concentrations, as it was in the current study, rumen microorganisms could degrade and use them as energy source. Rumen microorganisms may degrade alkaloids (Wachenheim *et al.*, 1992), saponins (Hart *et al.*, 2008) and phenolics (Varel *et al.*, 1991). The low and moderate doses of secondary metabolites can increase cell-wall constituent's degradability, and improve synchronisation between energy and N release in the rumen resulting in more energy available for increasing SCFA production and ME density (Salem *et al.*, 2014b).

Conclusions

Daily oral administration of SB extract to lambs tended to increase DMI with a concomitant tendency to improve lamb daily weight gain compared to control. *In vitro* GP and ruminal fermentation results demonstrated that SB extract with doses up to 1 mL/g DM of diet improved *in vitro* GP. Results of our study indicate that the use of SB extract with a rate of 40 mL/lamb/d can improve performance of lambs.

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