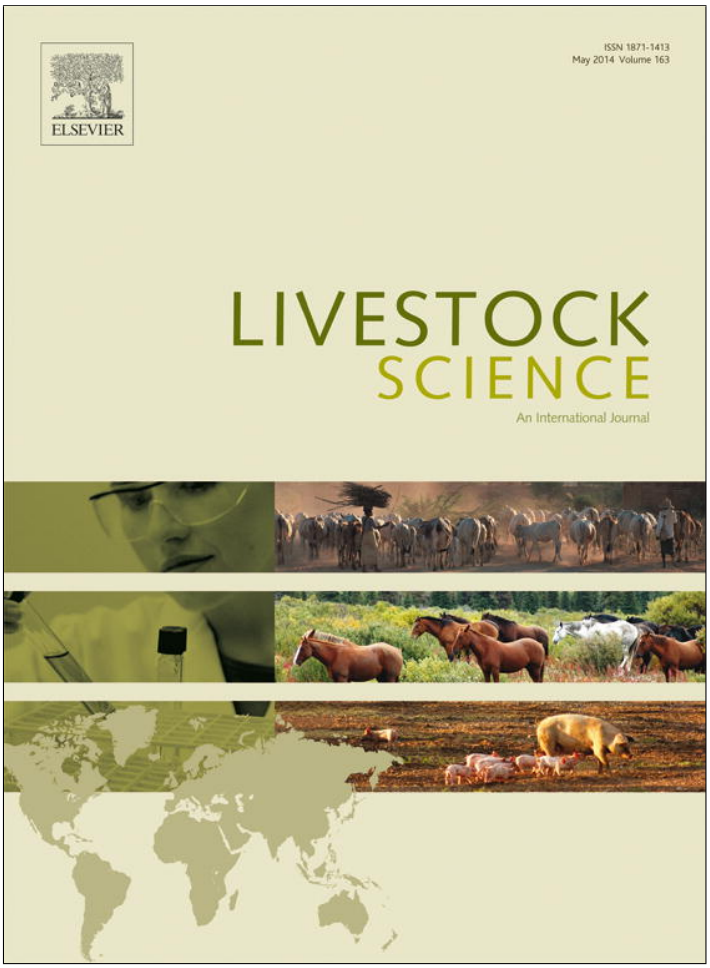

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Short communication

Influence of *Salix babylonica* and *Leucaena leucocephala* leaf extracts on ruminal fermentation characteristics, urinary purine derivative excretion and microbial protein synthesis of lambs

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ABSTRACT

Sixteen growing Katahdin × Pelibuey lambs (24 ± 0.3 kg body weight) were used to study the effects of oral administration of extracts of *Salix babylonica* (SB) and *Leucaena leucocephala* (LL) leaves on ruminal pH, total and individual volatile fatty acids (VFA) and NH_3-N concentrations, as well as ruminal protozoal counts, urinary purine derivatives (PD) excretion and estimated microbial protein synthesis. Lambs were fed a total mixed ration (TMR) containing 219 and 141 g kg^{-1} of CP and NDF, respectively. Four treatments (4 lambs/treatment) were Control (no extract added), SB (SB extract at 30 ml d^{-1}), LL (LL extract at 30 ml d^{-1}) and SBLL (mixture of SB at 15 ml d^{-1} + LL at 15 ml d^{-1}). Extracts were administered orally to each lamb before the morning meal daily. Measurements were on days 22 (P1), 43 (P2) and 63 (P3) of the experiment. Ruminal pH increased ($P=0.014$) with SBLL extract and decreased with SB. The ratio of acetate to propionate was higher ($P=0.042$) with SBLL compared to the control. Ruminal NH_3-N concentration was not affected by extract administration ($P=0.309$) or experimental period ($P=0.087$). Protozoal counts were not affected ($P=0.489$) by extract addition but decreased ($P<0.001$) in P3 compared with P1 and P2. Uric acid concentration increased ($P<0.001$) with individual extracts of SB and LL compared with the control or SBLL. Excretion of other purine derivatives (allantoin, xanthine, and hypoxanthine), creatinine and microbial protein synthesis were not changed ($P>0.05$) by extracts administration. Overall, oral administration of *S. babylonica* and *L. leucocephala* extracts, or their 1:1 mixture, to lambs for 63 d had only minor effects on ruminal fermentation, urinary purine derivative excretion and microbial protein synthesis.

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Abbreviations: ADF, acid detergent fiber; NDF, neutral detergent fiber; LL, *Leucaena leucocephala*; PD, purine derivatives; TMR, total mixed ration; SB, *Salix babylonica*; VFA, volatile fatty acid

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1. Introduction

Some extracts of tree leaves contain a high concentration of natural metabolites such as phenolic compounds, essential oils, saponins and alkaloids. These natural metabolites may affect rumen microbial activity *in vitro* (Jiménez-Peralta et al., 2011; Salem et al., 2007) and *in vivo* (Kamel, 2001; Salem et al., 2011a) and improve animal performance (Hart et al., 2008; Salem et al., 2011a, 2014). This group of compounds can modify key ruminal microbial communities (Domínguez-Bello and Escobar, 1997). For example, saponins, a class of the plant natural metabolites, can decrease ruminal ciliate protozoal populations and increase bacterial and fungal biomass (Makkar and Becker, 1997). Reducing ruminal ciliate protozoal numbers could increase ruminal bacterial counts and the total amount of microbial protein leaving the rumen (Hart et al., 2008; Navas-Camacho et al., 1993; Salem et al., 2011a).

Salix babylonica is a fast-growing tree of Salicaceae which is widely cultivated in Asia, Europe and America and grows rapidly (Surhone et al., 2010). *Leucaena leucocephala* is a native legume tree in southern Mexico and northern Central America, but also widely grown in South-East Asia. Leaves of these trees are an important source of feeds for ruminants, particularly in areas which experience harsh environmental conditions. Plant extracts from *S. babylonica* and *L. leucocephala* contain saponins and other secondary natural metabolites which can improve feed utilization, nutrient digestibility, lamb live weight gain and cow's milk production (Salem et al., 2011a, 2014). Salem et al. (2011b) detected 60 chemical compounds in the mixture of *S. babylonica* L. and *L. leucocephala* extracts. The mixed extracts are greatly different from the individual extracts of the two species. These constituents may have anthelmintic effects beneficial to lamb growth (Mejia-Hernandez et al., 2014) or as natural growth (Salem et al., 2011a) or milk production (Salem et al., 2014) promoters. However, little is known about their impacts on rumen fermentation, which is often characterized by ruminal pH, protozoal counts, volatile fatty acids (VFA) and NH_3-N concentrations, and rumen microbial synthesis estimated by urinary excretion of purine derivatives (PD; Chen et al., 1992).

Therefore the objective of our study was to evaluate effects of plant extracts of *S. babylonica* and *L. leucocephala*, and their 1:1 mixture, on parameters of ruminal fermentation, urinary excretion of PD and microbial protein synthesis in growing lambs.

2. Materials and methods

2.1. Animals and treatments

The handling of animals during the entire experiment was performed according to the international bioethical standard of NOM-062-ZOO-1999. Sixteen Katahdin × Pelibuey crossbreed male lambs at 3–4 months of age with 24 ± 0.3 kg of body weight were used. After 2 weeks of adaptation to a total mixed ration (TMR; Table 1), lambs were selected by weight and randomly allocated to one of the 4 treatments ($n=4$ lambs/treatment) being: Control (TMR); SB (Control plus *S. babylonica* leaf extract at 30 ml/d); LL (Control plus *L. leucocephala* leaf extract at 30 ml/d); SBLL (Control plus 30 ml/d of *S. babylonica* and *L. leucocephala* extracts in a 1:1 (vol:vol) mixture). Lambs in the four treatments were fed *ad libitum* a TMR formulated to meet all nutrient requirements (Australian Agricultural Council, 1990). Extracts were supplied to each lamb as oral administration before the morning meal each day. Measurements were recorded on d 22 (P1), 43 (P2) and 63 (P3) of the experiment. Fresh water was always available.

Table 1
Concentrations of secondary metabolites of *Salix babylonica* and *Leucaena leucocephala* leaf extracts and ingredients and chemical composition of the control diet for the growing lambs (previously published in Salem et al., 2011a).

	Leaf extracts	
	S. babylonica	*L. leucocephala*
Secondary metabolites (g/kg dry matter)		
Total phenolics	12.8	24.8
Saponins	4.8	13.2
Aqueous fraction[a]	72.5	116.5
Diet ingredients (g/kg dry matter)		
Soya bean meal	220	
Alfalfa hay	150	
Sorghum grain	550	
Fish meal	35	
Mineral/vitamin premix[b]	25	
Salt	20	
Chemical composition (g/kg dry matter)		
Organic matter	911	
Crude protein	219	
Ether extract	119	
Neutral detergent fiber	141	
Acid detergent fiber	59	
Lignin	21	

[a] Aqueous fraction contained lectins, polypeptides and starch (Cowan, 1999).

[b] Mineral/vitamin premix in 1 kg contained 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), iron (50 g), zinc (50 g), manganese (110 g), copper (12 g), iodine (0.30 g), selenium (200 mg) and cobalt (0.20 g).

2.2. Preparation of extracts

Extract of *L. leucocephala* or *S. babylonica* leaves was prepared as described before in Salem et al. (2011a). Briefly, leaves of each tree species were collected randomly from several young and mature trees during summer. Leaves (1.25 kg) were fresh chopped into 1 to 2 cm lengths and immediately extracted in 10 l of a solvent mixture containing 1 l of methanol, 1 l of ethanol and 8 l of distilled water. Extraction was at 25–30° C for 48–72 h in 20 l closed jars, followed by incubation at 39° C for 1 h, and then filtered. Filtrates were collected and stored at 4° C for further use. Plant extracts were weekly prepared. The 1:1 mixture extract was also prepared weekly by mixing the SB and LL extracts (0.5:0.5, vol:vol).

2.3. Rumen and urine sampling and sample pre-treatment

Rumen contents were sampled using a stomach tube at 0, 3 and 6 h after feeding on d 22, 43 and 63. Approximately 20 ml of ruminal content were strained through two layers of cheesecloth and pH value immediately measured using a pH meter (GLP 22, Crison Instruments, Barcelona, Spain). A subsample of 3 ml was preserved in 3 ml of 0.2 M HCl for NH_3-N analysis and 0.8 ml of rumen liquor was combined with 0.2 ml of 250 g l^{-1} of metaphosphoric acid for VFA analyses. Subsamples were stored at −20° C until laboratory analyses. Ruminal samples (0.8 ml) for protozoa count were fixed in 1 ml of formaldehyde saline solution containing 370 g l^{-1} of formaldehyde and 9 g l^{-1} of NaCl (Dehority, 1984).

A 15 ml spot urine sample was collected on d 22 (P1) and 63 (P3) and mixed with 40 ml of 100 ml l^{-1} of HCl to keep the final pH below 3. Urine samples were stored at −20° C for later PD (*i.e.*, allantoin, uric acid, xanthine and hypoxanthine) and creatinine analyses.

2.4. Laboratory analyses

Samples of TMR offered were collected weekly, pooled, mill ground (1 mm screen) and analyzed for dry matter (DM; #934.01, AOAC, 1997), ash (#942.05), N (#954.01) and ether extract (EE; #920.39). The neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin (#973.18) analyses were performed by using $ANKOM_{200}$ Fiber Analyzer unit (ANKOM Technology Corporation, Macedon, NY, USA). Neutral detergent fiber was assayed without use of α-amylase but with sodium sulfite. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites were determined as described before in Salem et al. (2011a) by fractionating 10 ml of the extract of each plant (LL and SB) in a funnel with a double volume of ethyl acetate. The total phenolics were measured by drying and quantifying the ethyl acetate layer in the funnel. After total phenolics separation, a double volume of *n*-butanol was added to fractionate saponins. The remaining solution was considered to be the aqueous fraction.

Ruminal fluid samples prepared for VFA determination were thawed and centrifuged at 9,000 × *g* for 10 min at 10° C, and then analyzed according to Erwin et al. (1961) using a gas chromatograph (Agilent Technologies 6890 N (G153ON) Network GC System (Series U510314041); Agilent Technologies, Inc., NY, USA), equipped with a flame ionization detector and a capillary column (Elite-FFAP, Perkin Elmer Instruments, Shelton, USA) 30 m long, 0.32 mm i.d., 0.25 μm film thickness). The NH_3-N concentration was determined using the method of McCullough (1967). Ciliate protozoal counts were determined using a 0.1 mm depth Burker counting chamber (Burker Blau Brand; Wertheim, Germany).

Urinary PD and creatinine were determined following the procedure described by Belenguer et al. (2002), using HPLC analysis, which consisted of a multisolvent delivery system (model 515B; Waters, Milford, MA, USA), an injector (model 717B; WISP), a multiwavelength detector (model 2487; Lambda-Max, Waters; set to 205 nm) and a double 4.0-mm × 250-mm S5 ODS 2 analytical column (Waters Sphericorb). Purine derivatives and creatinine were quantified by peak integration using the Waters HPLC systems software Millennium 32.

2.5. Calculations and statistical analyses

Microbial N supply (g N d^{-1}) was calculated according to Chen et al. (1992) using the equation:

$$\text{Microbial N(g N d}^{-1}\text{)} = (\text{PD(mmol d}^{-1}\text{)} \times 70)/(0.116 \times 0.83 \times 1000)$$

where, PD is the total absorbed purine derivatives in mmol d^{-1}; digestibility of microbial purines is assumed to be 0.83; the N content of purines is 70 mg N $mmol^{-1}$; the ratio of purines N: total N in mixed rumen microbes is 11.6:100.

Data were analyzed using the MIXED procedure of SAS (2002) according to a split-plot design involving two experimental factors (fixed effects) being plant extract (*i.e.*, the control, SB, LL, SBLL) and experimental period (*i.e.*, P1, P2 and P3). Data from rumen fluid samples collected at different times after feeding (*i.e.*, 0, 3, 6 h) were averaged for each lamb within each experimental period before statistical analysis. Plant extract was the whole-plot factor and experimental period the subplot factor within each whole plot. Random effects were animal within extract as the whole-plot error to test plant extract effects, and pooled residual error to test experimental period effects and extract × period interaction. Mean values of each parameter and standard error of the mean are reported in tables. As interactions treatment × period were never significant ($P > 0.05$), only mean values for the main effects are presented in the tables.

3. Results

Total phenolics, saponins and aqueous fraction concentrations were 50% lower ($P < 0.05$) in *S. babylonica* (SB) *versus L. leucocephala* (LL) extracts (Table 1).

Ruminal pH was lower in P1 than in P2 and P3 ($P < 0.001$). Total and individual VFA concentrations were not affected ($P > 0.05$) by extract administration. Acetate to propionate ratio was higher ($P < 0.05$) with SBLL compared with the control. Molar proportions of propionate decreased ($P = 0.008$) by extracts administration, while butyrate increased ($P = 0.013$) compared with the control. Acetate molar proportion in total VFA was lower ($P = 0.003$) in P3 than in P1 and P2, whereas propionate was not affected ($P = 0.762$) by experimental period. Butyrate was decreased ($P = 0.047$) in P2 and P3 than in P1. Concentration of NH_3-N was not affected by extract administration ($P = 0.309$) or experimental period ($P = 0.087$). Protozoal counts were not changed by extract addition ($P = 0.489$) but it was affected by experimental period ($P < 0.001$) and increased in P1 and P2 than P3 (Table 2).

Administration of extracts to lambs did not change ($P > 0.05$) urinary excretion of PD of allantoin, xanthine and hypoxanthine or creatinine and microbial protein

Table 2
Effects of *Salix babylonica* (SB) and *Leucaena leucocephala* (LL) leaf extracts and their mixture (SBLL, 1:1, vol/vol) on ruminal pH, protozoa count, and volatile fatty acid (VFA) and NH_3-N concentrations in growing lambs after 22 (P1), 43 (P2) and 63 (P3) days of treatment ($n=4$ lambs/treatment)*.

	Treatment				SEM	*P* value	Period			SEM	*P* value
	Control	SB	LL	SBLL			P1	P2	P3		
pH	6.59^{ab}	6.43^{b}	6.68^{ab}	6.88^{a}	0.133	0.014	6.33^{b}	6.90^{a}	6.70^{a}	0.115	< 0.001
Total VFA (mmol/l)	66.1	64.9	61.2	57.7	6.23	0.546	66.5	65.4	55.4	4.90	0.060
Acetate (C2; mmol/l)	37.6	38.0	35.6	34.2	2.98	0.577	38.7^{a}	39.3^{a}	31.1^{b}	2.59	0.003
Propionate (C3; mmol/l)	20.2	17.0	17.0	14.1	2.58	0.195	17.9	16.9	16.5	1.97	0.762
Butyrate (C4; mmol/l)	8.3	9.8	8.4	9.4	1.03	0.427	9.97^{a}	9.18^{ab}	7.75^{b}	0.855	0.047
C2 in total VFA (mmol/100 mmol)	57.3	58.7	59.2	59.2	1.43	0.523	58.5^{ab}	60.5^{a}	56.8^{b}	1.25	0.018
C3 in total VFA (mmol/100 mmol)	29.9^{a}	25.8^{ab}	26.4^{ab}	24.5^{b}	1.54	0.008	26.0^{ab}	25.1^{a}	28.9^{b}	1.33	0.017
C4 in total VFA (mmol/100 mmol)	12.8^{b}	15.0^{ab}	14.3^{ab}	16.3^{a}	0.88	0.013	15.1	14.4	14.3	0.59	0.379
C2:C3	2.01^{b}	2.39^{ab}	2.35^{ab}	2.52^{a}	0.184	0.052	2.35^{ab}	2.56^{a}	2.04^{b}	0.160	0.009
NH_3-N (mg/100 ml)	10.5	11.7	11.5	13.1	1.35	0.309	13.1	11.6	10.4	1.17	0.087
Protozoal counts ($\times 10^5$/ml)	6.2	7.6	6.4	8.7	1.25	0.489	10.0^{a}	9.8^{a}	1.9^{b}	1.09	< 0.001

^{a,b}Mean values within extract species or experimental periods with different letters differ ($P < 0.05$).
* Interaction of treatment × period was $P > 0.30$ in all cases.

Table 3
Effects of *Salix babylonica* (SB) and *Leucaena leucocephala* (LL) extracts and their mixture (SBLL, 1:1, v-v) on purine derivatives and microbial protein synthesis in growing lambs after 22 (P1) and 63 (P3) days of treatment ($n=4$ lambs/treatment)*.

	Extract				SEM	*P* value	Period		SEM	*P* value
	Control	SB	LL	SBLL			P1	P3		
Total purine derivatives (PD), mmol/l	21.7	22.7	45.6	30.0	8.75	0.266	29.8	30.1	6.18	0.974
Allantoin (A)	17.1	17.6	37.9	25.5	7.66	0.263	24.2	24.8	5.42	0.943
Uric acid	1.53^{b}	4.47^{a}	4.32^{a}	1.02^{b}	0.339	< 0.001	1.72	2.45	0.240	0.064
Xanthine	2.28	2.72	1.11	2.11	0.646	0.401	2.35	1.76	0.457	0.386
Hypoxanthine	1.75	1.92	2.24	1.34	0.634	0.445	1.51	1.11	0.335	0.138
PD/C	2.12	1.71	1.74	1.62	0.233	0.484	1.85	1.74	0.165	0.650
A/C	1.66	1.38	1.37	1.37	0.188	0.669	1.48	1.42	0.125	0.722
Creatinine (C)	14.6	15.8	21.4	22.0	8.80	0.458	18.4	22.0	6.22	0.689
Microbial N supply (g N/d)	21.0	19.9	42.9	28.5	10.49	0.435	21.6	34.6	7.41	0.249

^{a,b} Mean values within three extract species or experimental periods with different letters differ ($P < 0.05$).
* Interaction of treatment × period was $P > 0.05$ in all cases.

supply. Uric acid concentration increased ($P < 0.001$) with extracts of SB and LL compared with the control or SBLL. Overall, no changes in all urinary excretion of PD and microbial protein supply occurred or in creatinine among periods (Table 3).

4. Discussion

Antimicrobial properties of natural plant extracts may provide an alternative way to manipulate ruminal fermentation for the improvement of energy and protein utilization in ruminants (Kamel, 2001). Jiménez-Peralta et al. (2011) conducted an *in vitro* study using rumen liquor from the same lambs used in our study and found that individual extracts of LL and SB could positively modify *in vitro* gas production and ruminal fermentation. However, total VFA concentration was not affected by the extracts compared with the control in our study, suggesting that these extracts as additives at the dose used (*i.e.*, 30 ml/lamb/d) did not modify diet fermentability. The level of dose could be increased in future studies.

Although the molar proportion of acetate in total VFA was not affected by the extracts compared with the control, propionate and butyrate proportions were changed and, as a result, the ratio of acetate to propionate increased, especially with the mixture of the extracts. This is consistent with *in vitro* and an *in vivo* study by Singer et al. (2008) who used saponins from *Yucca schidigera* and found that a high ratio of acetate to propionate is associated with more CH_4 produced in the rumen. It seems that addition of these plant extracts would not mitigate enteric CH_4 emissions.

It is noteworthy that concentrations of acetate, propionate and butyrate decreased from P1 to P3 thereby suggesting that, although these additives had an effect on ruminal microbial fermentation, ruminal microbes adapted. Thus these additives have little long term benefit *in vivo* showing the limitations of short term *in vitro* studies to predict long term *in vivo* responses.

Urinary PD excretion is often used to estimate ruminal microbial protein synthesis. Duodenal purine bases, as a microbial marker, are efficiently absorbed at the small intestine and the majority of their metabolites are excreted

via the kidney with urinary recovery (Belenguer et al., 2002). Furthermore, the ratio of PD:creatinine concentrations in spot urine from sheep fed *ad libitum* can be an indicator of microbial protein supply status (Chen et al., 1992). In our study, the ratio was similar for the treatments and the control, indicating that microbial protein supply was not improved by the addition of the plant extracts.

In our study, allantoin was the major PD, accounting for 78–85% of total PD. The value is consistent to the finding by Chen et al. (1992) who fed sheep with a mixed hay and barley diet. Hypoxanthine and xanthine can be converted to uric acid and further to allantoin by enzymes in ruminants, which may be the reason for allantoin being predominantly present in urine.

Increased uric acid excretion was associated with a decreased ruminal protozoal count from P1 to P3 (Tables 2 and 3) in our study, while the others PD of allantoin, xanthine and hypoxanthine or microbial N supply were not changed by the extract administration or experimental period. However, protozoa counts were not affected by the extracts compared with the control. Thus it seems unlikely that the protozoa substantively contributed to the estimated increase in duodenal flow of microbial N as estimated from the urinary excretion of PD, especially uric acid, which were used to estimate microbial N (Fujihara et al., 2003). Our results are similar to those of Pérez-Maldonado and Norton (1996) who fed *Desmodium intortum* to sheep and goats and did not find a change in microbial protein using ^{35}S as a microbial marker.

5. Conclusions

Oral administration of *S. babylonica* and *L. leucocephala* leaf extracts or their 1:1 mixture for 63 d had only minor effects on ruminal fluid fermentation characteristics and microbial protein synthesis. The use of these extracts as a feed additive in the diet may be of little benefit to growing lambs.

Conflict of interest

None.

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