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Oral administration of leaf extracts to rumen liquid donor lambs modifies *in vitro* gas production of other tree leaves☆

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ABSTRACT

This study was conducted to determine if adaptation of lambs to ingestion of tree leaf extracts of *Salix babylonica* and *Leucaena leucocephala* can modify *in vitro* gas production of *Celtis ehrenbergiana*, *Ficus trigonata*, *Fraxinus excelsior* and *Prunus domestica*. Samples of leaves were collected in triplicate (*i.e.*, three individual samples of each tree leaf). Rumen inoculum was collected from 8 growing lambs fed a total mixed ration *ad libitum* (control; *RC*). Incubations were repeated with the rumen fluid collected from another 8 growing lambs of the same breed fed the same ration, but fed a daily dose of 30 ml/d of *S. babylonica* and *L. leucocephala* extracts in a 1:1 (v:v) mixture (treatment; RX). Leaf samples were incubated with each rumen fluid (*i.e.*, RC and RX inoculums) in 3 runs on different weeks. Data of each of the three runs within sample replicate were averaged and used as the mean value of each individual sample within tree species for statistical analysis in a 4 (tree species) × 2 (rumen inoculum) factorial design. *In vitro* gas production was recorded at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of incubation. After 72 h, the incubation was stopped and supernatant pH was determined, and then filtered to determine apparent degraded substrate (ADS). Fermentation parameters, such as the 72 h partitioning factor (PF_{72}), 24 h gas yield (GY_{24}), *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), short chain fatty acids concentration (SCFA), and microbial protein production (MP) were estimated. The crude protein content of the leaves ranged from 147 (*F. trigonata*) to 241 (*C. ehrenbergiana*) g/kg dry matter. The lowest fiber fraction values was in *P. domestica*, while *F. excelsior* had the highest, and *C. ehrenbergiana* and *F. trigonata* were intermediate. Secondary metabolites (*i.e.*, total phenolics, saponins, aqueous fraction) were lowest in *P. domestica* and highest in *F. trigonata*. Accumulated gas production was highest ($P<0.05$) in *F. excelsior* during the first 24 h of incubation. All fermentation parameters (*i.e.*, ADS, SCFA, GY_{24}, PF_{72}, IVOMD, ME, MP) varied ($P<0.01$) among tree leaves. The ADS, SCFA and MP were highest ($P<0.01$) in *F. excelsior*, lowest ($P<0.01$) in *F. trigonata*, and intermediate in *P. domestica* and *C. ehrenbergiana*. Incubation of tree leaves with RX inoculum did not affect gas production in the first 6 h, but it was lower ($P<0.05$) at 24–72 h, except for *F. trigonata*. *C. ehrenbergiana* had the highest ($P<0.05$) potential gas production, but rate of gas production and the discrete lag time did

Abbreviations: ADS, apparently degraded substrate; ADFom, acid detergent fiber; CP, crude protein; DM, dry matter; EE, ether extract; GY_{24}, gas yield at 24 h of incubation; IVOMD, *in vitro* organic matter digestibility; MP, microbial CP production; ME, metabolizable energy; NDFom, neutral detergent fiber; PF_{72}, partitioning factor at 72 h of incubation; SCFA, short chain fatty acids.

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not differ among leaves. As incubation of tree leaves with RX inoculum lowered (P<0.05) all fermentation parameters, oral administrated extracts of *S. babylonica* and *L. leucocephala* did not seem to adapt the rumen microbial population to better utilize these tree leaves.

1. Introduction

Browse trees and shrubs can be used as feed supplements in areas with long dry periods or harsh environmental conditions, such as northern and central Mexico, because they provide forage for grazing ruminants throughout the year, and/or at specific critical periods of the year, particularly after herbage senescence when the quantity and quality of herbaceous species is limited. Browse trees and shrubs can be an important component of goat and sheep diets (Papachristou and Nastis, 1996; Salem et al., 2006), and play an important role in nutrition of grazing ruminants in areas where few, or no, alternative feeds are available (Meuret et al., 1990). However use of tree and shrub leaves by ruminants may be restricted by negative effects on digestion of their generally high levels of secondary metabolites (Salem, 2005; Salem et al., 2006, 2007) and/or by their impacts on rumen microorganisms (McSweeney et al., 2001).

Many factors modify activities of rumen microorganisms which relate to fermentation of browse tree species and shrubs. These include animal species (Salem, 2005), diet composition (Getachew et al., 2005), shrub composition (Salem et al., 2006, 2007) and feed additives (Gado et al., 2009, 2011; Jiménez-Peralta et al., 2011; Salem et al., 2011a, 2012). Administration of leaf extracts to ruminants as feed additives have modified *in vitro* ruminal fermentation of high concentrate diets in lambs fed a daily dose of *Salix babylonica* and *Leucaena leucocephala* extract (Jiménez-Peralta et al., 2011), and improved *in vivo* digestibility and average daily gain of lambs (Salem et al., 2011a). Some plant extracts have also improved animal growth and nutrient digestion (Mapiye et al., 2010; Salem et al., 2011a) due to positive impacts of their secondary metabolites on activity of ruminal microorganisms (Jiménez-Peralta et al., 2011; Xu et al., 2010) and/or increased amino acid flow to the duodenum (Mueller-Harvey, 2006), which can result in more muscle deposition and, consequently, heavier carcasses (Gleghorn et al., 2004) and improved meat quality (Mapiye et al., 2010).

The aim was to determine if adaptation of lambs to ingestion of a tree leaf extract rich in plant secondary metabolites can modify or change *in vitro* digestion of some tree species leaves rich in secondary metabolites.

2. Materials and methods

2.1. Tree foliage species collection

Samples of leaves of the four species (*i.e.*, *Celtis ehrenbergiana*, *Ficus trigonata*, *Fraxinus excelsior* and *Prunus domestica*) were randomly and manually harvested from different parts of trees to obtain three individual samples of young and mature leaves from each tree species. Leaf samples were dried at 40 °C for 72 h in a forced air oven to constant weight, ground in a hammer mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components, secondary metabolites and *in vitro* gas production.

2.2. Animals as rumen inoculum donors

Rumen inoculum was collected by stomach tube from 8 growing Katahdin × Pelibuey lambs with a live weight of 24 ± 0.3 kg fed a total mixed ration (TMR) *ad libitum* based on soyabean meal, 220; alfalfa hay, 150; sorghum grain, 550; fishmeal, 35; mineral/vitamin premix, 25 and salt, 20 (g/kg dry matter (DM)) and used as the control inoculum (RC). Incubations were repeated with rumen inoculum collected from another 8 growing lambs of the same breed and fed the same TMR, but fed a daily 30 ml dose of extracts of *S. babylonica* and *L. leucocephala* in a 1:1 (v:v) mixture (RX). The RC and RX lambs were fed *ad libitum* a TMR formulated to meet all of their nutrient requirements (NRC, 1985). Extracts were orally administered daily to RX lambs before the 8:00 h feeding. Fresh water was always available.

2.3. Preparation of extracts for RX lambs

Tree leaves of *L. leucocephala* and *S. babylonica* were collected randomly from several young and mature trees during summer. Leaves were fresh chopped into 1–2 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®, Monterrey, Mexico) and 80 ml of distilled water. Plant materials were individually soaked and incubated in solvent in a laboratory at room temperature (*i.e.*, 25–30 °C) for 48–72 h in closed 20 L jars. After incubation, jars were heated at 30 °C for 1 h, and then immediately filtered and individual filtrates (extract) were collected. Extract was prepared weekly (stock volume of 2 L each) by mixing the *S. babylonica* and *L. leucocephala* extracts (500:500, v:v). This mixture was stored at 4 °C prior to daily oral administration to the lambs (Salem et al., 2011a).

2.4. In vitro incubations

Rumen fluid of RC and RX inoculums of lambs was collected by stomach tube. Ruminal contents of each lamb were obtained immediately before the morning feeding, mixed and strained through 4 layers of cheesecloth into a flask with O_2 free headspace. Samples (1 g) of each tree leaf species were weighed into 120 ml serum bottles. After that, 10 ml of particle-free ruminal fluid was added to each bottle and 40 ml of the buffer solution of Goering and Van Soest (1970), with no trypticase added, was immediately added in a proportion 1:4 (v/v).

A total of 219 bottles (3 bottles of each triplicate sample within each of the 4 tree species in 3 runs on different weeks for each rumen inoculum (*i.e.*, RC and RX) with 3 bottles as blanks (*i.e.*, rumen fluid only), were incubated for 72 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39 °C. The volume of gas produced was recorded at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h of inoculation using the reading pressure technique (RPT; DELTA OHM, Italy) of Theodorou et al. (1994). At the end of incubation (*i.e.*, 72 h), bottles were uncapped, pH was measured immediately with a pH meter (GLP 22, Crison Instruments, Barcelona, Spain), and fermentation was stopped by swirling the bottles in ice. Contents of each bottle were transferred as filtered fermentation residue for determination of apparently degraded substrate.

2.5. Apparently degraded substrate

At the end of the incubation (*i.e.*, 72 h), contents of each serum bottle were filtered through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100–160 μm, Pyrex, Stone, UK) under vacuum. Fermentation residues were dried at 105 °C overnight to estimate potential DM disappearance. Loss in weight after drying was the measure of undegradable DM. The DM degradability at 72 h of incubation (*i.e.*, apparently degraded substrate, ADS; mg/g DM) was calculated as the difference between DM content of substrate and its undegradable DM.

2.6. Chemical analyses and secondary metabolites

Tree leaf samples were analyzed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997). The neutral detergent fiber (NDFom, Van Soest et al., 1991), acid detergent fiber (ADFom) and lignin(sa) (AOAC, 1997); #973.18) analyses used an ANKOM200 Fibre Analyzer unit (ANKOM Technology Corporation, Macedon, NY, USA). NDFom was assayed without use of an alpha amylase but with sodium sulfite in the NDFom. Both NDFom and ADFom are expressed without residual ash.

Plant secondary metabolites were determined in the leaves from each tree species. Ten ml of extract was 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®, Monterrey, Mexico), was added to fractionate saponins (Makkar et al., 1998; Ahmed et al., 1990). The remaining solution was considered to be the aqueous fraction which has other secondary metabolites such as lectins and polypeptides (Cowan, 1999).

2.7. Calculations

To estimate kinetic parameters of gas production, gas production results (ml/g DM) were fitted using the NLIN option of SAS (2002) to the France et al. (2000) model as:

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

where A is the volume of gas production at time t; b the asymptotic gas production (ml/g DM); c is the rate of gas production (/h), and L (h) is the discrete lag time prior to gas production.

Metabolizable energy (ME, MJ/kg DM) and *in vitro* OM digestibility (IVOMD, g/kg OM) were estimated according to Menke et al. (1979) as:

$$\text{ME} = 2.20 + 0.136\,\text{GP} + 0.0057\,\text{CP}\,(\text{g/kg DM})$$
$$\text{IVOMD} = 148.8 + 8.89\,\text{GP} + 0.45\,\text{CP}\,(\text{g/kg DM}) + 0.65\,\text{ash}\,(\text{g/kg DM})$$

where GP is net gas production in ml from 200 mg 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 apparent degraded substrate *in vitro* (ADS, mg) to the volume of gas (ml) produced at 72 h (*i.e.*, ADS/total gas production (GP_{72})) according to Blümmel et al. (1997). Gas yields (GY_{24}) were calculated as the volume of gas (ml gas/g DM) produced after 24 h of incubation divided by ADS (g) as:

$$\text{Gas yields}\,(GY_{24}) = \text{ml gas/g DM/g ADS}$$

Table 1
Chemical composition and secondary metabolites levels (g/kg DM) of the tree leaf species.

	Species				SEM
	C. ehrenbergiana	*F. trigonata*	*F. excelsior*	*P. domestica*	
Organic matter	891.3[d]	933.7[c]	996.4[a]	969.0[b]	2.51
Crude protein	241.4[a]	146.5[d]	174.1[c]	207.3[b]	4.55
Ether extract	11.6	12.4	12.6	12.3	0.30
Neutral detergent fiber(om)	344.5[b]	318.0[b]	408.3[a]	199.3[c]	8.36
Acid detergent fiber(om)	159.8[c]	196.7[b]	221.5[a]	138.4[d]	3.71
Lignin(sa)	91.5[b]	131.2[ab]	142.1[a]	82.4[b]	3.99
Total phenolics	37.3[c]	65.6[a]	58.9[b]	7.6[d]	0.42
Saponins	33.2[a]	20.4[b]	12.4[c]	13.3[c]	0.87
Aqueous fraction[a]	156.5[a]	96.5[c]	116.8[cb]	135.2[b]	5.56

[a,b,c,d] Different superscripts following means in the same row indicate differences at P<0.05.
[a] Cowan (1999).

Short chain fatty acids concentration (SCFA) was calculated according to Getachew et al. (2002) as:

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

where GP is the 24 h net gas production (ml/200 mg DM).

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

$$\text{MP (mg/g DM)} = \text{mg ADS} - (\text{ml gas} \times 2.2\,\text{mg/ml})$$

where 2.2 mg/ml is a stoichiometric factor which expresses mg of C, H and O required for the SCFA gas associated with production of one ml of gas (Blümmel et al., 1997).

2.8. Statistical analyses

Data of *in vitro* ruminal gas production and fermentation parameters were analyzed as a 4 × 2 factorial experiment (*i.e.*, 4 tree leave species (random effect) and 2 rumen inocula (fixed effect)) according to a randomized block design using the PROC MIXED procedure of SAS (2002). Data of each one of the 3 runs within the same sample were averaged. Mean values of each individual sample within each species (3 samples of each) were the experimental unit (Udén et al., 2011), and the statistical model was:

$$Y_{ijk} = \mu + S_i + R_j + S_i * R_j + \varepsilon_{ijk}$$

where Y_{ijk} represents every observation of the *i*th browse species when incubated in the *j*th rumen inoculum (extract), S_i (i = 1–4) the browse tree species effect, R_j (j = control (RC) or extract (RX) lambs) is the rumen inoculum effect, $S_i{}^*R_j$ is the interaction between tree species and rumen inoculum, and ε_{ijk} is the experimental error. Tukey's test was used for the multiple comparisons among mean values for the four plant species.

3. Results

The CP content of the leaves ranged from 147 (*F. trigonata*) to 241 (*C. ehrenbergiana*) g/kg DM (Table 1). Fiber levels (*i.e.*, NDFom, ADFom, lignin(sa)) were lowest in *P. domestica*, *F. excelsior* had the highest, and *C. ehrenbergiana* and *F. trigonata* were intermediate. Total phenolics were lower in *P. domestica* and higher in *F. trigonata*, while saponins were lower in *P. domestica* and *F. excelsior*, whereas they were highest in *C. ehrenbergiana*. Aqueous fraction was lowest in *F. trigonata* had the lowest and *C. ehrenbergiana* had the highest.

Tree species × rumen inoculum interactions (P<0.05) occurred for gas production (ml/g DM) at all incubation times of measures (Table 2). Accumulated gas production was highest (P<0.05) in *F. excelsior* during the first 24 h of incubation (*i.e.*, 6 and 24 h). At 48 h, *P. domestica* had the highest (P<0.05) values, whereas at 72 h *C. ehrenbergiana* had the highest (P<0.05) values. Gas production of leaf species was not affected during the first 6 h of incubation in all tree species with RX inoculum, while it was decreased (P=0.016) after 12 h only in *F. excelsior* and *P. domestica*. From 24 to 72 h of incubation, gas production was lower (P<0.05) for all leaf species with RX inoculum, except *F. trigonata*. Highest potential gas production (*i.e.*, fraction *b*) was in *C. ehrenbergiana* had the highest (P<0.05), but the rate of gas production (*i.e.*, *c*) and discrete time lag (*i.e.*, *L*) did not differ among leaves. Incubation of tree leave species with RX inoculum decreased (P<0.05) fraction b in all leaves, except *F. trigonata*, without impacts on *c*, and *L*, overall (Table 2).

All fermentation parameters (*i.e.*, ADS, SCFA, GY_{24}, PF_{72}, IVOMD, ME and MP) varied (P<0.01) among tree leaves. The ADS, SCFA and MP were highest (P<0.001) in *F. excelsior*, lowest (P<0.001) in *F. trigonata*, and intermediate in *P. domestica* and *C. ehrenbergiana*. Incubation of tree species with RX inoculum decreased (P<0.05) all the fermentation parameters, except pH (Table 3).

Table 2
In vitro gas production parameters and gas volume accumulated after different hour of incubation of some tree leaf species in rumen liquor of control (RC) or extract administrated (RX) lambs.

Species (S)	*C. ehrenbergiana*		*F. trigonata*		*F. excelsior*		*P. domestica*		SEM	P		
Rumen liquor (R)	RC	RX	RC	RX	RC	RX	RC	RX		S	R	S × R
In vitro gas production (ml/g DM)												
Gas6	30.8[b]	33.8[a]	37.8[b]	40.4[a]	44.9	44.8	48.7[a]	27.5[b]	2.43	0.02	0.14	0.01
Gas24	97.1[a]	81.0[b]	80.6	85.4	107.4[a]	89.7[b]	127.7[a]	67.2[b]	4.77	0.11	0.02	0.01
Gas48	146.2[a]	97.2[b]	90.2	95.6	128.7[a]	97.7[b]	163.3[a]	84.1[b]	5.36	0.01	<0.01	0.01
Gas72	171.1[a]	100.6[b]	91.3	96.9	133.2[a]	98.4[b]	173.3[a]	89.8[b]	5. 56	0.01	<0.01	0.01
Gas production parameters[a]												
b (ml/g DM)	196.8[a]	101.7[b]	91.5	97.1	134.4[a]	98.5[b]	177.2[a]	93.8[b]	5.58	<0.01	<0.01	<0.01
c (/h)	0.029	0.070	0.089	0.093	0.068	0.102	0.053	0.059	0.0061	0.01	0.01	0.11
L (/h)	1.47	1.98	1.69	2.48	1.75	2.74	1.60	1.81	0.154	0.06	0.01	0.33

[a,b] Different superscripts following means within tree leaf species in the same row indicate differences at P<0.05.
[a] *b* is the asymptotic gas production (ml/g DM); *c* is the rate of gas production (/h); *L* is the initial delay before gas production begins (h).

4. Discussion

4.1. Effect of tree species

The variation in the chemical composition of our tree species has occurred in other studies with shrubs and tree species (Salem et al., 2007; Mbugua et al., 2008). The high CP content of *P. domestica* and *F. excelsior* was similar to those of other high quality shrubby forages such as *Calycotum villosa* (Gasmi-Boubaker et al., 2005). While the CP content *per se* should not be the sole criteria of judging the relative importance of a particular feedstuff. The difference in CP content among species may be due to inherent characteristics of each species related to their ability to extract and accumulate nutrients from the soil, as well as atmospheric N. Other factors causing variation in the chemical composition of browse forages include planting location, plant part, age of leaf and season. With regard to geographical location, some authors have reported that browse plants in arid zones have higher N than plants of the same species in humid zones (Rittner and Reed, 1992; Salem, 2005).

Differences among browse trees and shrubs in NDFom and ADFom contents are consistent with Khanal and Subba (2001) and Salem et al. (2007). In our study, the ADF/NDF ratio was highest for *P. domestica* (mean: 0.69), lowest for *C. ehrenbergiana* (0.46) and *F. trigonata* (0.53) and *F. excelsior* (0.54) had the intermediate. For all samples, the ADFom fraction is a large proportion of NDFom, suggesting a high content of cellulose and lignin, but lower levels of hemicellulose (Salem et al., 2007).

Gas production parameters suggested differences in nutritional value which were generally closely related to chemical composition (Kamalak et al., 2005; Salem, 2005; Salem et al., 2007). The high gas production during the first 24 h in *P. domestica* and *F. excelsior* suggests a higher extent of fermentation in the first 24 h of fermentation (Gas24) *versus* the other leaves, especially *F. trigonata* (Table 2). Differences in gas production among leaves could be due to the proportion, and nature, of their fiber (Rubanza et al., 2003). Indeed the higher fiber levels, as well as high levels of secondary metabolites in *F. trigonata* (Salem et al., 2006, 2007), are almost certainly responsible for its reduced gas production *versus* the other leaves. However, differences in degradability among leaves could also be due to the extent of lignification of NDFom (Van Soest, 1994; Fonesca et al., 1998), and/or negatively correlated with both NDFom and lignin(sa) (Salem et al., 2007).

The low gas production of *F. trigonata* could also be due to its NDFom being bound by polyphenolics (Ndlovu and Nherera, 1997). Higher *in vitro* gas production from *P. domestica* could be due to its lower secondary metabolite levels (Salem et al., 2006, 2007), although some variation among leaves could be due to genotypic characteristics and relative to the type of

Table 3
In vitro rumen fermentation profile[a] of some tree leave species incubated in rumen liquor from control (RC) or extract administrated (RX) lambs.

Species (S)	*C. ehrenbergiana*		*F. trigonata*		*F. excelsior*		*P. domestica*		SEM	P		
Rumen liquor (R)	RC	RX	RC	RX	RC	RX	RC	RX		S	R	S × R
pH	7.04	7.04	7.11	7.11	6.95	6.95	7.05	7.05	0.009	0.01	1.00	1.00
ADS	671	606	604	624	713a	641[b]	796[a]	550[b]	19.4	0.11	0.02	0.01
SCFA	2.13[a]	1.78[b]	1.77	1.88	2.36[a]	1.97[b]	2.81[a]	1.47[d]	0.106	0.11	0.01	0.01
GY_{24}	144.6	133.4	133.4	135.7	150.6[a]	139.8[b]	160.5[a]	120.4[b]	3.75	0.29	0.01	0.01
PF_{72}	6.9[b]	7.5[a]	7.5	7.4	6.7	7.2d	6.2[b]	8.4[a]	0.23	0.38	0.03	0.02
IVOMD	43.7[a]	40.9[b]	36.2	37.1	41.8[a]	38.7[b]	47.1[a]	36.4[b]	0.85	0.01	0.02	0.01
ME	6.2[a]	5.8[b]	5.2[b]	5.4[a]	6.1[a]	5.6[b]	6.9[a]	5.2[b]	0.13	0.01	0.02	0.01
MP	457.6[a]	427.5[b]	426.7[b]	435.7[a]	476.9[a]	443.6[b]	514.7[a]	401.7[b]	8.92	0.11	0.02	0.01

[a,b] Different superscripts following means within tree leaf species in the same row indicate differences at P<0.05.
[a] ADS is the apparent degraded substrate (mg/g DM); SCFA is the short chain fatty acids (mmol/g DM); GY_{24} is the gas yield at 24 h (ml gas/g ADS); PF_{72} is the partitioning factor at 72 h of incubation (mg ADS: ml gas); IVOMD is the *in vitro* organic matter digestibility (g/kg MS); ME is the metabolizable energy (MJ/kg DM); MP is the and microbial protein production (mg/g DM).

secondary metabolite activity on digestibility (Muetzel and Becker, 2006; Salem et al., 2006, 2007). This suppressing effect probably resulted from a reduction in microbial attachment to feed particles (McAllister et al., 1994) and inhibition of microbial growth and enzyme activity (McSweeney et al., 2001). Salem et al. (2010) found that *L. plantarum* was more susceptible than *E. faecium* to secondary metabolites in the medium, and had a higher response to polyethyleneglycol (PEG) addition, which reduced toxicity in both bacterial strains. Lactic acid bacteria would be affected to a different extent by secondary metabolites reaching the small intestine, whereas PEG may neutralize inhibitory effects of secondary metabolites on these bacterial species.

Increased IVOMD, ME and ruminal fermentation parameters of *P. domestica* reflect its higher fermentation and lower secondary compound levels. In contrast, lower values in *F. trigonata* and *F. excelsior* represent less fermentation and higher secondary compound levels (Salem et al., 2006, 2007). Similar results were reported by Peng et al. (2005) who showed reduced IVOMD of Lucerne chaff incubated with secondary compounds extracted from *L. sativus*, and suggested that oxalyl-diaminobutyric acid, rather than phenolics, were responsible for inhibition of cellulolytic bacteria. However digestibility of tree leaves was adversely affected by secondary metabolites *in vitro* (Peng et al., 2005) and *in vivo* (Salem et al., 2006, 2007).

The lowest microbial protein production (MP) occurred in leaves with higher levels of secondary metabolites (*i.e.*, *F. trigonata*) where lower gas production suggests a role of phenolics as an anti-methanogen which stimulates growth of propionate producing bacteria. Lower methane production, with higher propionate, is consistent with higher MP (McCrabb et al., 1997).

4.2. Effect of ruminal liquor inoculum

Reduced ruminal fermentation activities of tree species in RX rumen fluid probably due to the negative impacts of a chemical, possibly formed by mixing of the two extract species which were orally administered to the lambs (*i.e.*, RX lambs). Salem et al. (2011b) detected 60 chemical constitutes formed by a mixture of an *S. babylonica* extract with an *L. leucocephala* extract. The main compounds in the extract mixture were 9,12,15-octadecatrienoic acid-ethyl ester with concentration of 294 mg/g followed by 9,12-octadecadienoic acid-methyl ester (112 mg/g), and hexadecanoic acid-methyl ester a saturated fatty acid, with a concentration of 90 mg/g. Hexacontanoic acid (21 mg/g), 5,8,11,14,17-eicosapentaenoic acid-methyl ester (20 mg/g) and octadecanoic acid-16-methyl-methyl ester (10 mg/g) were the major fatty acids. However, there was 129 mg/g of 1,3-dioxane, 4-(hexadecyloxy)-2-pentadecyl and the aliphatic hydrocarbon in the tritetracontane form was 32 mg/g (Salem et al., 2011b). These chemicals were very different from those in the individual extracts and could negatively affect ruminal microbes. Jiménez-Peralta et al. (2011) used the same lambs as used in our experiment as rumen fluid donors to study impacts of type of ruminal liquor (*i.e.*, RC or RX) on *in vitro* fermentation of a high concentrate diet fed to the same lambs with or without different levels of tree extracts, and found that mixing extracts of *S. babylonica* and *L. leucocephala* reduced gas production and ruminal fermentation of the high concentrate diet, suggesting formation of a hydroxyl radical in a reaction with the extract mixture which reduced ruminal microbial activities and their ability to degrade deoxyribose (Jirovetz et al., 2007). Salem et al. (2011a) studied impacts of the same individual extracts of *S. babylonica* and *L. leucocephala* or their mixture (1:1, v/v) on average daily gain and diet digestibility of the same lambs as used in our study and found that mixing of the two extracts 24 h before oral administration reduced average daily gain and nutrient digestibility compared to the individual extracts, but without differences from the control diet (without extract).

Another explanation for the reduced ruminal fermentation activities of tree species in RX rumen fluid could be due to the lambs which received the combined extract for 63 d not producing salivary proteins to protect tree leaves from the negative impacts of secondary metabolites, and/or that a feedback control mechanism may not have evolved or may have been lost. This effect could be due to changes in parotid saliva composition as, for instance, K secretion was lower in RX lambs (Salem, 2002). Based on our results, lambs administrated the extract mixture would not secrete salivary proteins, and/or the activity of their saliva to deactivate tannins would be negligible. Our results are consistent with Ammar et al. (2011), who assessed the nutritional influence of pre-incubation in saliva collected from sheep fed alfalfa hay with 50 g quebracho/kg DM for 60 d on *in vitro* fermentation of some tanniniferous shrubs in batch cultures of mixed ruminal microorganisms. The authors did not find differences between incubation of shrub species in the saliva between the control and quebracho sheep in any of the variables studied based on gas production. Results reported in a previous study (Ammar et al., 2009) provided evidence that microbial populations in the rumen of sheep fed a diet supplemented with quebracho tannins may evolve mechanisms of adaptations to counteract condensed tannins in browse species, and a symbiotic relationship between the host animal and the tannin-resistant bacteria may be established (Sasaki et al., 2005). These changes in ruminal populations would enable ruminants to overcome the antinutritional effects of tanniferous species. It appears that the major mechanism to detoxify tannins depends upon changes in the ruminal microbial population to offset the activity of phenolic compounds (Ammar et al., 2009). This was an unexpected finding because it precludes the probability that any defensive mechanism induced by adaptation to regular ingestion of tannins has been developed (Makkar, 2003), thereby changing the salivary protein profile (Costa et al., 2008).

5. Conclusions

F. excelsior and *P. domestica* leaves had the highest *in vitro* fermentation among four tree species with lower secondary metabolite concentrations. Incubations of tree species with rumen inoculum of lambs fed plant extracts for 60 d reduced

ruminal fermentation of all tree species. It is clear that the orally administrated extract mixture could modify rumen gas production and fermentation of browse trees specie, which may affect their utilization by growing lambs.

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