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Effect of pre-incubation in sheep and goat saliva on *in vitro* digestibility and gas production kinetics of the foliage of some Spanish shrubs

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SUMMARY – *In vitro* dry matter digestibility (IVDMD) and gas production kinetics of the foliage of some browse plants were studied after soaking the samples in saliva obtained from three sources: artificial saliva and saliva obtained either from sheep or from goats. The study was carried out on leaves of four shrub species: *Erica australis* (Spanish heather), *Cistus laurifolius* (laurel-leaved rock-rose), *Quercus pyrenaica* (hoary oak) and *Rosa canina* (wild dog rose) collected from uplands in the province of León (north-west Spain) in two seasons, in spring and in autumn. Samples were pre-incubated in each saliva for 4 h at 39°C, followed by an incubation in buffered rumen liquid to determine their IVDMD and gas production kinetics. Rumen fluid for all treatments was obtained from sheep. The extent of degradation in the rumen and rate of gas production were determined from the gas production parameters. Pre-incubation with sheep and goat saliva did not significantly affect IVDMD, with the exception of mature leaves of *Q. pyrenaica* and *R. canina*, for which the higher values ($P < 0.05$) were obtained with sheep saliva. In all the browse species, the rate of gas production was similar for both sheep and goat saliva treatments, except for mature leaves of *E. australis* and *Q. pyrenaica* where values were lower ($P < 0.05$) for goat saliva. Gas production after 24 h was significantly higher ($P < 0.05$) with sheep than with goat saliva for most of the species. The estimated extent of degradation in the rumen was similar with sheep and goat saliva for *C. laurifolius* and *Q. pyrenaica*, but in the case of *R. canina* it was higher ($P < 0.05$) in goats. *In vitro* ruminal fermentative activity seemed to be affected by the pre-incubation of the substrate in different saliva sources.

Key words: *S*aliva, sheep, goats, *in vitro* digestibility, gas production, shrub.

RESUME – *"Effet de la pré-incubation en salive de mouton ou de chèvre sur la digestibilité et la cinétique de production de gaz* in vitro *du feuillage de quelques arbustes espagnols". La digestibilité* in vitro *de la matière sèche (IVDMD) et les cinétiques de production de gaz du feuillage de quelques arbustes espagnols ont été étudiées après une pré-incubation des échantillons avec un tampon ou avec salive animale provenant de mouton ou de chèvre pendant 4 h à 39°C. Les feuilles de quatre espèces arbustives ont été utilisées :* Erica australis, Cistus laurifolius, Quercus pyrenaica *et* Rosa canina*. Elles ont été prélevées dans la montagne de la province de León (Nord-Ouest d'Espagne) au printemps et en automne. Après pré-incubation, les échantillons ont été incubés dans du jus de rumen mélangé avec un tampon pour déterminer leurs IVDMD et les cinétiques de production de gaz. Du jus de rumen de mouton a été utilisé avec tous les échantillons de cette étude. La dégradation théorique et la vitesse de production de gaz ont été calculées à partir des paramètres de production de gaz. La pré-incubation dans la salive de mouton et de chèvre n'a pas eu un effet significatif sur la IVDMD, sauf dans le cas des feuilles âgées de* Q. pyrenaica *et* R. canina*, où les valeurs les plus élevées ($P < 0,05$) ont été observées avec la salive de mouton. Pour toutes les espèces arbustives étudiées, la vitesse de production de gaz a été similaire après la pré-incubation dans la salive de mouton et de chèvre, à l'exception des feuilles âgées de* E. australis *et* Q. pyrenaica *où les valeurs les plus faibles ($P < 0,05$) ont été obtenues avec les échantillons pré-incubés dans la salive de chèvre. La production de gaz après 24 h a été significativement plus élevée ($P < 0,05$) avec la salive de chèvre pour la plupart des espèces arbustives. La dégradation théorique estimée dans le rumen a été semblable avec la salive de chèvre et de mouton pour* C. laurifolius *et* Q. pyrenaica*, alors que dans le cas de* R. canina *elle a été significativement plus élevée ($P < 0,05$) avec la salive de chèvre. Ces résultats montrent que l'activité fermentative du rumen* in vitro *pourrait être affectée par la pré-incubation des substrats dans différentes sources de salive.*

Mots-clés : *Salive, ovins, caprins, digestibilité* in vitro*, production de gaz, arbustes.*

Introduction

It is well reported that ruminants have a higher tolerance to tannins than non-ruminants due to extra mastication, secretion of large amounts of saliva and rumen fermentation (Singleton, 1981). For

ruminant species, saliva provides water and alkaline salts and thus is expected to be the key buffering agent against the acids produced in the rumen by the microbial fermentation of feed. Moreover, the high proline content of the salivary proteins of some mammalian herbivores has been considered as a possible pre-digestion mechanism for neutralizing the anti-nutritive effects of tannins. These proteins with a high affinity for condensed tannins (CT) have been found in the saliva of deer, rodents and humans, but not in the saliva of cattle, sheep or goats (Pérez-Maldonado *et al*., 1995), although other studies confirmed their presence in sheep and cow saliva. Given its role in rumen fermentation, any difference in the composition of ruminant saliva could be reflected in differences in the fermentative activity in the rumen and, in particular, in the degradation of tanniniferous substrates. Little information is available about these possible differences between ruminant species in the effects of saliva on rumen fermentation. In view of this scanty data, the aim of the work presented herein was to determine the *in vitro* dry matter digestibility (IVDMD) and gas production kinetics of shrub foliage pre-incubated in sheep and goat saliva.

Material and methods

Samples from four Spanish indigenous browse species were collected from uplands in the province of León (north-west Spain) in different growing seasons in spring and summer. The shrub species were *Erica australis* (Spanish heath), *Cistus laurifolius* (laurel-leaved rock-rose), *Quercus pyrenaica* (hoary oak) and *Rosa canina* (wild dog rose). The plants were clipped with scissors collecting a mixture of leaves and thin stems. In the laboratory, leaves were manually separated from the original samples, immediately freeze-dried and ground in a hammer mill provided with a 1-mm sieve. Chemical composition (CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre and lignin expressed in g/kg DM) and total extractable tannins (TET, tannic acid equivalent, g/kg DM) contents of these browse leaves are presented in Table 1.

Table 1. Chemical composition (g/kg DM) and tannin concentration (tannic acid equivalent, g/kg DM) of the foliage of the browse plants

Species	Season	CP	NDF	ADF	Lignin	TET
E. australis	Spring	52	387	266	164	112
	Autumn	53	393	292	185	121
C. laurifolius	Spring	134	409	212	80	130
	Autumn	72	256	172	67	54
Q. pyrenaica	Spring	163	462	225	62	93
	Autumn	65	573	378	165	51
R. canina	Spring	176	340	117	28	107
	Autumn	59	322	155	53	127

Animals: Saliva and rumen fluid

Four Merino sheep and four Alpine goats housed in individual cages, fitted with a catheter in the parotid duct and fed 1 kg alfalfa hay daily were used. Saliva was collected from both species and stored at –20°C until use. Artificial saliva was prepared according to McDougall (1948) just before use. Rumen fluid was withdrawn before the morning meal from four adult Merino sheep provided with ruminal cannulae and fed 1 kg alfalfa hay daily. Rumen fluid was collected in thermos flasks and taken immediately to the laboratory where was strained through four layers of cheesecloth and kept at 39°C under a CO_2 atmosphere.

In vitro digestibility

Procedures described by ANKOM (1998) were followed. About 250 mg dry weight of each sample were weighed into polyester bags (size 5 cm × 5 cm; pore size 20 μm). Bags were placed in 5-l

fermentation glass jars, that can be closed with a plastic lid provided with a single-way valve which avoids the accumulation of fermentation gases. Three jars were used, and 18 bags (two per sample plus two emptied bags) were placed in each jar. Eight hundred ml of each source of saliva (artificial, sheep or goat saliva) were dispensed in each jar. Then the jars were placed in an incubator (DAISY, ANKOM) at 39°C with continuous rotation to facilitate the effective immersion of the bags in saliva. After 4 h of soaking the samples in saliva, the jars were withdrawn from the incubator, opened and filled with diluted rumen fluid up to 2 l. Diluted rumen fluid had been prepared according to Goering and van Soest (1970) by diluting under anaerobiosis and at 39°C sheep rumen liquor in the culture medium (buffer, mineral and reductive solution) in the proportion 1:2 (v/v). After the addition of buffered rumen fluid, jars were placed again in the incubator at 39°C with continuous rotation. After 48 h of incubation, the jars were opened and the bags were gently rinsed first under cold tap water and then in a washing machine (short washing cycle – 10 min – with cold water). Bags were dried at 60°C for 48 h and then wash out in a neutral detergent solution at 100°C for 1 h. The dry residue was weighed out and considered as the truly indigestible matter to calculate the IVDMD (Goering and van Soest, 1970). Two runs of incubation were performed in two consecutive weeks (four replicates per treatment).

In vitro gas production kinetics

About 400 mg of each sample were weighed into serum bottles and pre-incubated with 20 ml of one of the saliva sources. Six bottles were used for each substrate, two for each saliva source. All the bottles were placed in the incubator at 39°C. After soaking the samples in saliva for 4 h, 30 ml of diluted rumen fluid (in the proportion 1:2 v:v) prepared as described before were dispensed in each bottle at 39°C and under anaerobiosis. All the bottles were crimped and replaced in the incubator at 39°C, being shaken at every time of reading the gas. Volume of gas produced in each bottle was recorded at 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 y 144 h after inoculation time, using a pressure transducer (Theodorou *et al*., 1994). At the end of the incubation period, the contents of each serum bottle were filtered using glass crucibles to calculate DM loss after 144 h incubation. Cumulative gas production data were fitted to an exponential model to estimate kinetic parameters, and then estimate the extent of degradation of the feed in the rumen (France *et al*., 2000). Two runs of incubations were performed in two different weeks (four replicates per treatment).

By using the same inoculum (sheep rumen liquid) and the same buffer, mineral and reductive solution, it is expected that within each substrate the differences between experimental treatment can be attributed to the effects of the pre-incubation in the different saliva sources. Differences among artificial, sheep and goat saliva were examined by one-way ANOVA.

Results and discussion

There was a significant ($P < 0.01$) and negative correlation between IVDMD and the lignin content.

The pre-incubation in sheep saliva tended to result in higher IVDMD than with artificial or goat saliva, although differences were not always significant (Table 2). The IVDMD values after pre-incubation in sheep saliva were significantly higher than those observed with artificial saliva for the foliage of *E. australis* collected in spring and that of *Q. pyrenaica* and *R. canina* collected in autumn. The pre-incubation in goat saliva only resulted in higher IVDMD than artificial saliva with foliage of *C. laurifolius* collected in spring. Differences between sheep and goat saliva were only significant for foliage of *Q. pyrenaica* and *R. canina* collected in autumn, corresponding the higher values to the sheep saliva. Irrespective of the saliva type, IVDMD showed significant differences between the studied species (Table 2). *R. canina* foliage showed the highest values, and mature leaves of *E. australis* and *Q. pyrenaica* the lowest IVDMD coefficients. The low degradability of these substrates could be attributed, partly, to their high NDF and lignin contents.

The extent of degradation (dg), rate of gas production (c) and gas production after 24 h incubation time (G24) are presented in Table 3. The gas production rate of mature leaves of *E. australis* and Q. *pyrenaica* was higher ($P < 0.05$) with the pre-incubation in sheep saliva than either in goat or in artificial saliva, but for mature leaves of *R. canina* the highest rate was observed with the pre-incubation in goat saliva. Gas production at 24 h of *in vitro* incubation was higher after soaking in

sheep than in goat saliva for young foliage of *C. laurifolius*, *Q. pyrenaica* and *R. canina*, and for mature leaves of *C. laurifolius*, *Q. pyrenaica* and *E. australis*. For most of the studied species dg was lowest when substrates were pre-incubated in artificial saliva and highest when drenched in sheep saliva, though the differences were not statistically significant, except in the case of *R. canina,* where the highest dg values ($P < 0.05$) were found with goats saliva. The extent of degradation in the rumen was affected by the pre-treatment with different saliva sources to a lesser degree than gas production at 24 h of incubation. The estimation of the extent of rumen degradation is highly dependent on the potentially degradable fraction of the substrate that is assumed to be equal to the DM disappearance after 144 h of incubation. It is possible that the adverse effect of tannins on the fermentative activity were overcome with time.

Table 2. *In vitro* dry matter digestibility of browse leaves pre-incubated in different sources of saliva

Species	Season	Saliva			sed†
		Artificial	Sheep	Goat	
E. australis	Spring	67.2^{b}	71.3^{a}	69.0^{ab}	1.31
	Autumn	62.7	64.2	63.2	1.08
C. laurifolius	Spring	70.3^{b}	72.5^{ab}	73.2^{a}	1.21
	Autumn	75.6	77.5	76.0	1.15
Q. pyrenaica	Spring	76.7	77.7	74.0	2.03
	Autumn	59.7^{b}	62.4^{a}	58.7^{b}	1.06
R. canina	Spring	88.2	89.8	90.0	1.32
	Autumn	86.7^{b}	91.8^{a}	86.4^{b}	1.26

†Standard error of the difference.
^{a,b}In the same row, values with different superscripts differ significantly ($P < 0.05$).

Table 3. Effect of saliva source on parameters of gas production kinetics of the foliage of some shrub species sampled at two different maturity stages

Species	Parameters	Spring				Autumn			
		Artificial	Sheep	Goats	sed†	Artificial	Sheep	Goats	sed†
E. australis	c (%/h)	4.3	4.6	4.4	0.11	4.3^{b}	4.9^{a}	4.2^{b}	0.22
	dg (%)	26.0^{b}	28.3^{a}	26.4^{b}	1.19	23.6	25.4	24.7	1.21
	G24 (ml/g)	75	75	72	2.7	65^{a}	65^{a}	57^{b}	3.0
C. laurifolius	c (%/h)	4.0	4.4	4.3	0.22	3.7	4.0	3.7	0.13
	dg (%)	30.0	32.4	29.7	2.66	32.4^{b}	34.3^{a}	33.1^{ab}	0.55
	G24 (ml/g)	97^{b}	110^{a}	95^{b}	5.7	110^{b}	122^{a}	110^{b}	2.7
Q. pyrenaica	c (%/h)	5.2	5.3	5.3	0.08	2.8^{b}	3.2^{a}	2.9^{b}	0.10
	dg (%)	42.8^{b}	46.6^{a}	44.7^{ab}	1.39	22.4^{b}	26.3^{a}	25.6^{a}	0.80
	G24 (ml/g)	140^{ab}	147^{a}	139^{b}	3.5	65^{b}	77^{a}	67^{b}	3.5
R. canina	c (%/h)	5.4^{b}	5.7^{a}	5.7^{a}	0.14	3.4^{b}	3.5^{b}	4.0^{a}	0.20
	dg (%)	50.9^{c}	55.4^{b}	57.6^{a}	0.99	35.8^{c}	38.9^{b}	41.8^{a}	0.61
	G24 (ml/g)	170^{b}	185^{a}	177^{b}	3.2	115^{b}	132^{a}	135^{a}	3.0

†Standard error of the difference.
^{a,b}Within the same row and sampling season, values with different superscripts differ significantly ($P < 0.05$).

With respect to the seasonal variation, the IVDMD and gas production parameters of all studied species, except *C. laurifolius*, were significantly lower in autumn than in spring. This would be the consequence of the increase of cell wall and lignin contents (Table 1) associated with a higher maturity stage.

IVDMD values reported seem unexpectedly high given the high concentrations of TET in some plant species (*R. canina*) that could depress their digestibility. Extraction with neutral detergent solution can remove some indigestible material (tannins), thus overestimating the *in vitro* digestibility (Hanley *et al*., 1992) determined by the technique of Goering and van Soest (1970). However, this cannot be the only reason as *R. canina* foliage showed higher G24 and dg values than other browse species in spite of its high TET content. This could be ascribed to the fact that biological activity of tannins depends widely on their origin and structure and not only on their concentrations in the browse.

Our results support the hypothesis that sheep may secrete saliva with certain capacity to attenuate the undesirable effect of tannins on ruminal fermentation, whereas goat saliva would be less active in neutralizing plant tannins. The capacity of sheep and goat saliva to enhance rumen fermentation *in vitro* would be substrate dependent, as it could not be confirmed in all the browse plants studied. It has been suggested that such capacity can be attributed to the presence of salivary proteins rich in proline with a high affinity for tannin compounds (Austin *et al.,* 1989; Hagerman and Robbins, 1993). The concentration of these proteins could be slightly higher in sheep than in goat saliva. However, these proteins can offset the depressing effect of tannin up to a certain level corresponding to their tannin-binding capacity. Beyond that level the negative effects of tannins on fermentative activity would become evident. Furthermore, tannins are not the only factor that depresses fermentative activity and other secondary compounds should be taken into account.

Conclusion

The differences observed *in vitro* in digestibility and gas production kinetics after the pre-incubation in artificial, sheep and goat saliva of the foliage of some browse species with different tannin concentrations support the hypothesis that sheep saliva may be a bit superior to neutralize the detrimental effects of the condensed tannins on ruminal fermentative activity. This ability was no detected in goat saliva. Our results draw attention to the biological significance of saliva secretion as an adaptive mechanism of ruminants to the consumption of tanniniferous feeds.

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