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Ruminal degradation of tannin-treated legume meals

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Abstract: The inefficiency of protein utilisation by ruminants fed protein concentrates (based on legume meals) causes serious economic loss and environmental damage owing to their rapid hydrolysis and deamination in the rumen. Thus efforts aimed at slowing the ruminal fermentation of such feeds are needed, and recent studies have observed potentially positive effects of tannins on ruminant nutrition under certain circumstances. Tannins are a complex group of naturally occurring plant polyphenols characterised by their ability to bind with proteins. This property of tannins is considered responsible for the decreased ruminal digestibility of forages both *in vivo* and *in vitro*. Under that perspective, commercial tannic acid was added at three proportions (10, 25 and 50 g kg^{-1} on a dry matter basis) to four different legume meals (horse bean, kidney bean, soybean and pea), and the effect on *in situ* dry matter and crude protein ruminal disappearance was assessed. The results confirmed the dose-dependent (although not persistent after 48 h) slowing of *in situ* digestibility, this effect being significant at the highest tannin treatment when compared with untreated samples. Scanning electron microscopy revealed that soybean seed endosperm cell walls were protected from digestion by the ruminal microbiota, while the digestion of starch granules was relatively unaffected by tannic acid. Electrophoresis of the protein fractions confirmed the lower digestibility of tannin-treated seeds as well as the relative lack of alteration of the electrophoretic profile of individual proteins. Implications for the digestion of concentrates in ruminants are discussed.

Keywords: concentrate feeds; ruminant digestibility; bypass protein; tannins; scanning electron microscopy

INTRODUCTION

Dietary protein ingested by ruminants is extensively degraded by the micro-organisms in their rumen.[1] The rapid conversion of protein to ammonia and its subsequent absorption across the rumen wall before being eventually excreted as urea leads to inefficient nitrogen utilisation in ruminants.[2] Under this perspective the identification and quantification of compounds and/or factors able to reduce ruminal degradation of protein, increasing its outflow to the small intestine, has been a main research topic during the last 25 years.

Great interest is currently being shown in exploring the potential of tannins to protect plant proteins from ruminal degradation, mainly after the banning of meat and bone meals in the EU. Tannins have traditionally been considered as antinutritive factors for ruminant and non-ruminant animals owing to their ability to complex with macromolecules, mainly proteins,[3] this interaction being responsible for a reduced bioavailability of nutrients. In recent years, however, tannins have become a matter of interest in ruminant nutrition because of their potentially useful effects on dietary protein utilisation, mainly related to forages[4,5] but also to concentrates.[6] The chemical bases of the tannin–protein interactions, highly dependent on pH,[7] as well as the particular anatomy and physiology of the ruminant digestive tract, are responsible for this change in the perception of polyphenols in ruminant nutrition.[8] Therefore current research efforts are focused on identification of the factors involved in the slower fermentation of tannin-rich forages and concentrates so that ruminant feeds can be improved.

Scanning electron microscopy (SEM) gives direct evidence of digestive phenomena taking place in the rumen. This technique is a useful complement to *in vivo* and *in vitro* digestibility studies. Although not quantitative, SEM offers qualitative information about the interaction of ruminal micro-organisms and seed structures during digestion processes. Most of the studies carried out using this technique have focused on cereal seeds, and to a lesser extent on legume seeds. On the other hand, SEM studies have been mainly used to assess the effect of formaldehyde and micronisation[9,10] as procedures aimed at manipulating the degradability of seeds in the rumen. Nevertheless, the influence of tannins on the ruminal digestion

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of legume seed structures is still not completely ascertained.

The aims of the present work were (1) the assessment and quantification of the effects of tannic acid on the kinetic parameters of *in sacco* ruminal digestion of some legume meals and (2) the evaluation by SEM of the effects of tannic acid on the different ultrastructures of soybean seed subjected to ruminal digestion.

MATERIALS AND METHODS

Reagents

Acrylamide, *N*,*N*′-methylene-bis-acrylamide, ammonium persulphate, Coomassie Brilliant Blue (BBC R-250), *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED), tannic acid (TA) and standard molecular mass markers for electrophoresis were supplied by Sigma Chemical Co (St Louis, MO, USA). All other reagents were purchased in the purest form available from several commercial sources. Electrophoresis was performed using a Mini Protean II electrophoresis chamber (Bio-Rad, Richmond, CA, USA).

Animals

Four adult, cannulated, non-lactating females of Segureña sheep (local breed) were used. The animals were fed twice daily on a diet based on oat/vetch hay and milled barley grain in a 4:1 dry matter (DM) ratio, vitamin and mineral block supplementation and free access to water. EU requirements related to laboratory animal welfare were met.

Sample preparation

Tannic acid-treated legume meals (horse bean, kidney bean, soybean and pea; Table 1) were prepared as follows. Legume seeds were milled and passed through a 2 mm mesh to get a finely ground meal. Tannic acid was diluted in aqueous solution ($100\,g\,l^{-1}$) and then added to the meals in order to reach final proportions of 10, 25 and $50\,g\,kg^{-1}$ on a DM basis (from 28 to $202\,g\,kg^{-1}$ on a crude protein (CP) basis; Table 1). Different amounts of distilled water were added during the mixing of TA with the meals in order to achieve complete homogenisation, being later removed by air-forced drying at 45 °C. The product obtained was milled again and passed through a 2 mm mesh to grind lumps before being used in the assays. In all cases, controls were prepared as described above but using only distilled water instead of TA solution.

Ruminal digestibility

Legume meals treated with TA were incubated *in situ* according to the procedure described by Mehrez and Ørskov.[11] Approximately 3 g of each meal was placed in a 9 cm × 5 cm, 50 μm pore size polyester bag (Ankom Technology Co, Fairport, NY, USA). Twelve bags were introduced simultaneously into each animal, corresponding to a duplicate per meal and concentration of TA, and incubated for each of the time intervals assessed (0, 2, 4, 8, 12, 24 and 48 h). The incubations were performed in duplicate (four bags per legume, time and TA concentration). Once recovered from the rumen, the bags were extensively washed with cold tap water until the water ran clear, and immediately placed in an air-forced dryer at 50 °C. After drying, the contents of the duplicate bags were mixed and dry matter and crude protein (Kjeldahl N × 6.25) were determined.[12] Results were expressed as the percentage of crude protein on a DM basis of the samples remaining in the nylon bags. The percentage disappearance of DM and CP from the bags at each incubation time was calculated from the proportion remaining after incubation in the rumen. The disappearance rate was fitted to the equation described by Ørskov and McDonald,[13]

$$D = a + b(1 - e^{-ct})$$

where D is the loss from the bag after t hours, a is the immediately soluble fraction, b is the insoluble component that is degradable by rumen micro-organisms in time t, and c is the rate of degradation of fraction b (h^{-1}). The immediately soluble fraction was estimated by stirring the bags in $0.2\,mol\,l^{-1}$ phosphate buffer, pH 6.8, at 39 °C for 60 min.

The non-linear parameters a, b and c were estimated by an iterative least squares procedure, and best fit values were chosen as the smallest sum of squares after convergence, using a specific application (Table Curve 2D, Jandel Scientific, AISN Software, San Rafael, CA, USA). The effective degradabilities of DM (DMED) and CP (CPED) were calculated by the equation described by Ørskov and McDonald,[13]

$$ED = a + bc/(c + k)$$

Table 1. Chemical composition of legume meals

		% on DM basis					% TA on CP basis*		
Legume	% dry matter	Crude protein	Ether extract	Crude fibre	N-free extract	Ash	A	B	C
Horse bean	87.5	26.2	2.9	9.4	57.6	3.9	3.8	9.5	19.1
Kidney bean	89.3	25.2	2.1	5.3	62.6	4.8	4.0	9.9	19.8
Soybean	90.2	35.6	19.7	5.9	33.5	5.3	2.8	7.0	14.0
Pea	88.6	24.7	1.8	6.6	63.2	3.7	4.0	10.1	20.2

Data were obtained according to the procedures described in Ref 12.

* Legume meals were treated with tannic acid at 10 (A), 25 (B) and 50 (C) $g\,kg^{-1}$ on a DM basis according to 'Materials and methods' section.

where k is the estimated rate of outflow from the rumen. ED was estimated at rumen outflow rates of 2, 5 and 8% h^{-1}, which were considered to represent slow, medium and fast rumen turnover rates respectively.[14]

Scanning electron microscopy

Untreated and TA-treated (50 g kg^{-1}) soybean meals were chosen for SEM studies. Residuals of the nylon bags after the *in situ* incubation times were fixed in 50 ml l^{-1} glutaraldehyde, pH 7.2, for 3 h. Then samples were washed five times with 0.1 mol l^{-1} cacodylate buffer, pH 7.2, in order to eliminate excess glutaraldehyde. Samples were finally resuspended in the same buffer and stored at 4 °C until their dehydration. This procedure was carried out using a series scale in ethanol, and samples were dried to critical point under CO_2 atmosphere. Samples were fixed on aluminium stubs with coal paste and metallised with gold (Polaron E5000, Quorum Technologies Ltd, Watford, UK). Specimens were viewed using a Zeiss DSM 950 scanning electron microscope (Carl Zeiss AG, Jena, Germany).

Electrophoresis of bag residues

Samples of untreated and TA-treated soybean meal of the bag residues were suspended (50 g l^{-1}) after the *in situ* incubation times (0, 2, 4, 8, 12, 24 and 48 h) in 0.1 mol l^{-1} Tris-HCl, 0.01 mol l^{-1} NaCl and 20 g l^{-1} SDS buffer, pH 9.0, and then homogenised by sonication (Microson XL200, Misonix Inc, Farmingdale, NY, USA). Mixtures were centrifuged (15 min, 20 000 × g) and the supernatants were diluted (1:1) in sample buffer (0.125 mol l^{-1} Tris-HCl, 200 ml l^{-1} glycerol, 0.04 g l^{-1} bromophenol blue, 20 g l^{-1} SDS and 200 ml l^{-1} β-mercaptoethanol) and boiled for 5 min. SDS-PAGE separation of protein fractions was done according to Laemmli,[15] using 40 g l^{-1} polyacrylamide stacking gels and 120 g l^{-1} separating gels. Aliquots (10 µl) of the mixtures were loaded in each well and 5 µl of molecular mass markers were added to each gel. After electrophoretic separation, gels were stained overnight using 1 g l^{-1} Coomassie Brilliant Blue (BBC R-250) in methanol/acetic acid/water (40:10:50) solution and then destained in the same solution without the stain.

Statistics

Percentages of CP and DM digestibility were normalised by $\sin^{-1}$ transformation of their square root prior to statistical analysis.[16] Data were analysed by one-way ANOVA followed by a comparison of means (Fisher's LSD procedure) in order to assess the statistical significance of the differences among TA treatments and outflow rates within each legume meal. The contribution of the different factors studied (TA treatment, legume meal and rumen outflow rate) to the effective degradability was assessed by multifactor ANOVA. In this case the contribution of each factor was determined after removing the effects of the other factors. All statistical analyses were conducted using Statgraphics Plus 4.0 software (Statistical Graphics Corp, Rockville, MD, USA).

RESULTS

Dry matter and nitrogen disappearance

The *in situ* DM and CP disappearance curves of untreated controls and TA-treated legume meals (horse bean, kidney bean, soybean and pea) are shown in Fig 1. The non-linear kinetic parameters a, b and c fitted after *in situ* DM and CP disappearance[13] are given in Table 2.

The results indicate that untreated legume meals had a high degradability after being incubated in the rumen for periods longer than 24 h, and less than 20% of the DM and CP in untreated meals remained undegraded (Fig 1). The addition of TA to the different meals was responsible for decreases in DM and CP ruminal disappearance. The lower DM and CP degradation was based both on a marked decrease in the DM and CP immediately degradable fraction (a) as well as on a lower rate of degradation (c) for all the meals and doses studied.

The rate of DM and CP ruminal disappearance (c) was affected by TA treatment in a dose-dependent manner when compared with untreated controls ($P < 0.05$). This effect was more evident for CP than for DM (Table 2), indicating a certain selective effect of TA on the protein component of the legume seeds ($P < 0.05$). TA treatment also decreased the extent of DM and CP ruminal disappearance, but had less influence on this parameter than on the rate of degradation, and differences were significant ($P < 0.05$) only at the highest TA concentration assayed after 48 h of *in situ* incubation (Fig 1).

Differences in DM and CP *in situ* disappearance due to TA treatment were more noticeable in the initial stages of the assay (less than 24 h), indicating a time-dependent effect. This was confirmed when DMED and CPED values (Table 2) were compared for the three rumen outflow rates considered ($k = 0.02$, 0.05 and 0.08 h^{-1}), showing a most evident decrease for the highest rumen outflow rate ($k = 0.08\ h^{-1}$). This dependence of effective degradability on changes in rumen outflow rate was confirmed by multifactor ANOVA, which indicated that the main factor contributing to DMED was the rumen outflow rate ($F = 444.9$), followed by the TA treatment ($F = 134.4$), while the weakest (although significant, $P < 0.05$) influence was produced by the legume species ($F = 11.4$). Similar results were obtained when CPED data were studied (rumen outflow rate, $F = 264.6$; TA treatment, $F = 114.8$; legume species, $F = 14.5$).

Scanning electron microscopy of soybean

The SEM results for untreated and TA-treated soybean meal (SBM) after 4, 8 and 12 h of *in situ* incubation are shown in Fig 2. After 4 h of incubation, rumen microbes preferentially attacked

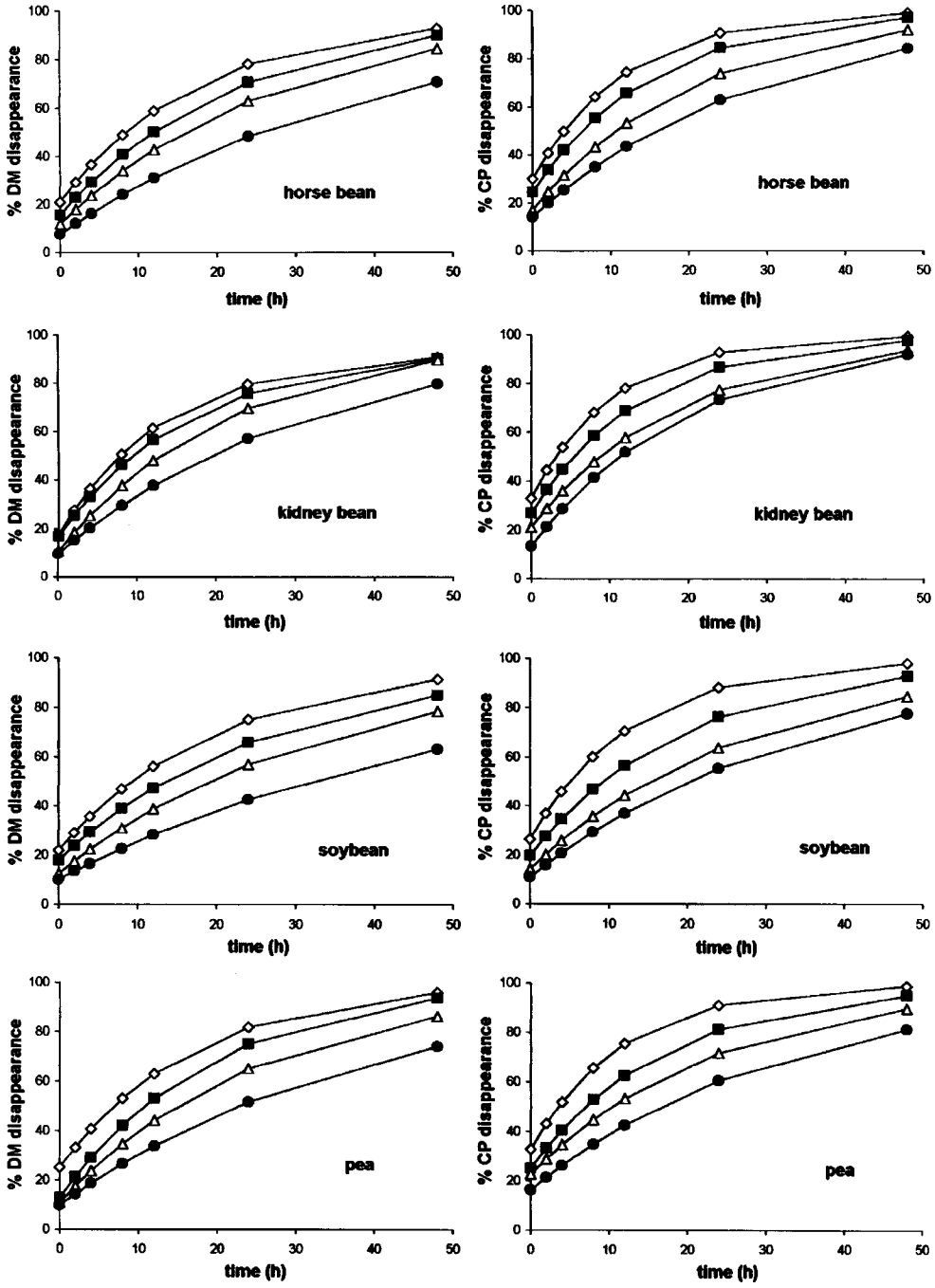

Figure 1. *In situ* dry matter (left) and nitrogen (right) disappearance from untreated (diamonds) and tannic-acid treated legumes (horse bean, kidney bean, soybean and pea) at three concentrations, 10 (squares), 25 (triangles) and 50 (circles) g tannic acid kg^{-1} DM.

the protein matrix of untreated SBM endosperm cells, as evidenced by the increased porosity of this structure, while there was no evidence of digestion on the surface of starch granules (Fig 2-1). After 8 h of incubation, endosperm cells were colonised, their walls were progressively digested and starch granules were released with no detectable damage (Fig 2-3). Surprisingly, even after 12 h of incubation, intact starch granules were released with no sign of hydrolysis by the ruminal microbiota (Fig 2-5).

Addition of TA to soybean meal resulted in no morphological differences detectable by SEM. Nevertheless, the pattern of degradation was clearly altered. The TA-treated cell wall matrix was markedly less digested at the different incubation times when compared with untreated SBM. This effect was noticeable for at least the first 12 h of incubation (Figs 2-2, 2-4 and 2-6). However, the most surprising effect was observed in starch granules. After 4 h of incubation, granules in TA-treated SBM were extensively colonised by ruminal microbes (Fig 2-2), which contrasts with the above-mentioned lack of starch hydrolysis in untreated SBM (Fig 2-1). This effect was also observed after 12 h of incubation (Figs 2-5 and 2-6). In short, TA interfered with the digestion of endosperm cell walls, while the treatment

Table 2. Estimated non-linear parameters of digestibility and effective degradability of dry matter and crude protein disappearance according to Ørskov and McDonald[13]

Legume	TA ($g\,kg^{-1}$)	% DMED*						% CPED*					
		a	*b*	*c*	$k = 0.02\,h^{-1}$	$k = 0.05\,h^{-1}$	$k = 0.08\,h^{-1}$	*a*	*b*	*c*	$k = 0.02\,h^{-1}$	$k = 0.05\,h^{-1}$	$k = 0.08\,h^{-1}$
Horse bean	0	21.00	76.75	0.057^{a}	$77.81^{a,I}$	$61.89^{a,II}$	$52.93^{a,II}$	30.03	69.97	0.084^{a}	$86.54^{a,I}$	$73.89^{a,II}$	$65.87^{a,II}$
	10	15.72	84.28	0.044^{ab}	$73.66^{a,I}$	$55.17^{ab,II}$	$45.63^{b,II}$	24.52	75.48	0.066^{b}	$82.45^{ab,I}$	$67.47^{ab,II}$	$58.64^{b,II}$
	25	11.91	88.09	0.036^{bc}	$68.54^{ab,I}$	$48.78^{b,II}$	$39.25^{b,II}$	16.99	83.01	0.048^{c}	$75.59^{bc,I}$	$57.65^{bc,II}$	$48.12^{c,II}$
	50	7.67	90.33	0.025^{c}	$57.85^{b,I}$	$37.78^{c,II}$	$29.18^{c,III}$	14.02	85.98	0.035^{d}	$68.73^{c,I}$	$49.42^{c,II}$	$40.19^{c,II}$
Kidney bean	0	17.64	75.47	0.072^{a}	$76.70^{a,I}$	$62.18^{a,II}$	$53.39^{a,II}$	33.15	66.85	0.093^{a}	$88.17^{a,I}$	$76.63^{a,II}$	$69.09^{a,II}$
	10	16.88	77.86	0.059^{ab}	$75.03^{ab,I}$	$59.02^{a,II}$	$49.93^{b,II}$	26.89	73.11	0.071^{b}	$83.93^{ab,I}$	$69.79^{ab,II}$	$61.27^{b,II}$
	25	10.78	89.22	0.045^{bc}	$72.55^{bc,I}$	$53.04^{b,II}$	$42.90^{c,II}$	21.26	78.74	0.052^{c}	$78.13^{ab,I}$	$61.40^{bc,II}$	$52.28^{bc,II}$
	50	9.71	90.29	0.031^{c}	$64.59^{c,I}$	$44.27^{c,II}$	$34.93^{d,III}$	13.37	86.63	0.049^{c}	$74.89^{b,I}$	$56.25^{c,II}$	$46.28^{c,II}$
Soybean	0	22.01	76.80	0.049^{a}	$76.55^{a,I}$	$60.02^{a,II}$	$51.18^{a,II}$	26.39	73.61	0.077^{a}	$84.82^{a,I}$	$71.02^{a,II}$	$62.49^{a,II}$
	10	17.93	80.07	0.038^{ab}	$70.39^{ab,I}$	$52.51^{b,II}$	$43.72^{b,II}$	19.79	80.21	0.051^{b}	$77.41^{ab,I}$	$60.29^{b,II}$	$51.02^{b,II}$
	25	12.56	86.44	0.030^{b}	$64.42^{bc,I}$	$44.98^{b,II}$	$36.13^{c,II}$	14.26	85.74	0.036^{c}	$69.38^{bc,I}$	$50.15^{bc,II}$	$40.87^{c,II}$
	50	10.14	88.86	0.019^{b}	$53.43^{c,I}$	$34.61^{c,II}$	$27.19^{d,II}$	10.89	89.11	0.029^{c}	$63.63^{c,I}$	$43.60^{c,II}$	$34.60^{c,II}$
Pea	0	25.16	75.47	0.058^{a}	$81.28^{a,I}$	$65.69^{a,II}$	$56.88^{a,II}$	32.85	67.11	0.084^{a}	$87.05^{a,I}$	$74.92^{a,II}$	$67.22^{a,II}$
	10	13.02	88.80	0.050^{ab}	$76.45^{ab,I}$	$57.42^{b,II}$	$47.17^{b,II}$	25.01	74.25	0.059^{b}	$80.46^{ab,I}$	$65.20^{b,II}$	$56.53^{b,II}$
	25	10.88	89.16	0.039^{bc}	$69.82^{bc,I}$	$49.95^{c,II}$	$40.10^{c,II}$	22.74	77.24	0.042^{c}	$75.06^{c,I}$	$58.00^{bc,II}$	$49.33^{b,II}$
	50	9.80	90.20	0.026^{c}	$60.78^{c,I}$	$40.66^{d,II}$	$31.92^{d,III}$	16.35	82.59	0.032^{d}	$67.17^{c,I}$	$48.58^{c,II}$	$39.95^{c,II}$

* Effective degradability of dry matter (DMED) and crude protein (CPED) according to 'Materials and methods' section. Parameter *k* is the rumen outflow rate (% h^{-1}) considering low ($k = 0.02$), medium ($k = 0.05$) and high ($k = 0.08$) intakes.[14]

Values obtained within each legume at different concentrations of TA (letters) for the different outflow rates (Roman numerals) sharing a superscript are not significantly different ($P < 0.05$).

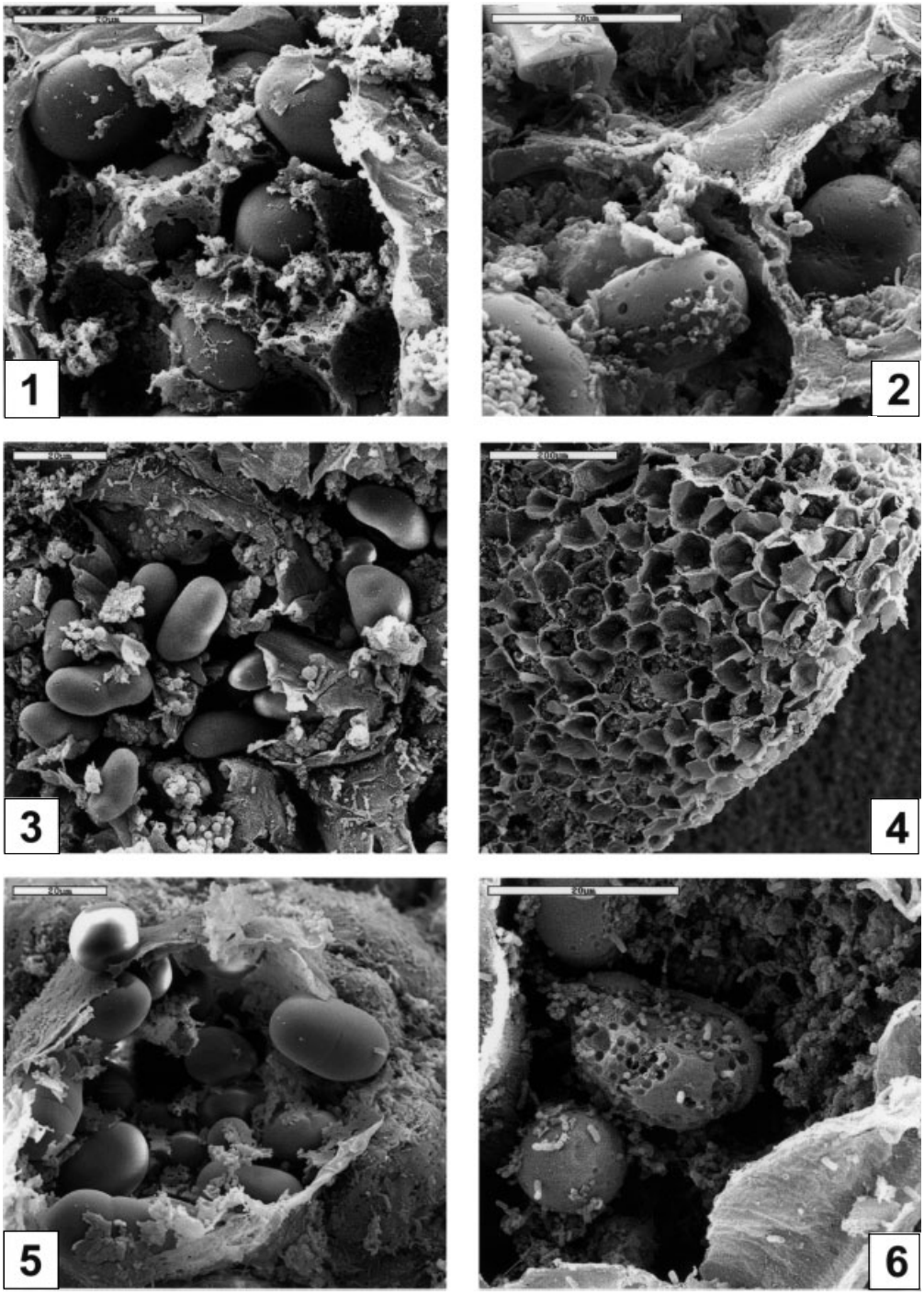

Figure 2. Untreated (1, 3, 5) and tannic acid-treated (2, 4, 6) soybean after 4 (1, 2), 8 (3, 4) and 12 (5, 6) h of ruminal *in situ* incubation. Rumen micro-organisms digest preferentially the walls of endosperm cells, releasing the entrapped starch granules of untreated soybean, this occurring both in the early stages of incubation (1) and after 12 h (5). Noticeable is the lack of microbial hydrolysis in starch granules of untreated meal. On the contrary, tannic acid-treated soybean showed a persistence of the endosperm cell walls (2, 4), while entrapped starch granules were actively colonised and hydrolysed (2, 6) from the initial stages of incubation when compared with untreated meal.

did not prevent microbial attachment and hydrolysis of starch granules. Despite this hydrolysis, the overall effect of TA on starch seems to be an indirect protection by a reduced susceptibility of the protein matrix in which starch granules are enclosed.

Electrophoretic separation of protein fractions

The electrophoretic separations of untreated and TA-treated SBM protein fractions of bag residues after the different incubation times are shown in Fig 3. The reversibility of tannin–protein interactions allowed the solubilisation needed prior to the electrophoretic separation. Gels showed a higher persistence of separated protein fractions in those bags containing soybean meal previously treated with TA (Fig 3A), compared with the untreated meals (Fig 3B). Moreover, it was noticeable that both the amount and the mobility of electrophoretically separated bands were scarcely affected by previous interaction with TA, indicating reversible, non-denaturing TA–protein binding.

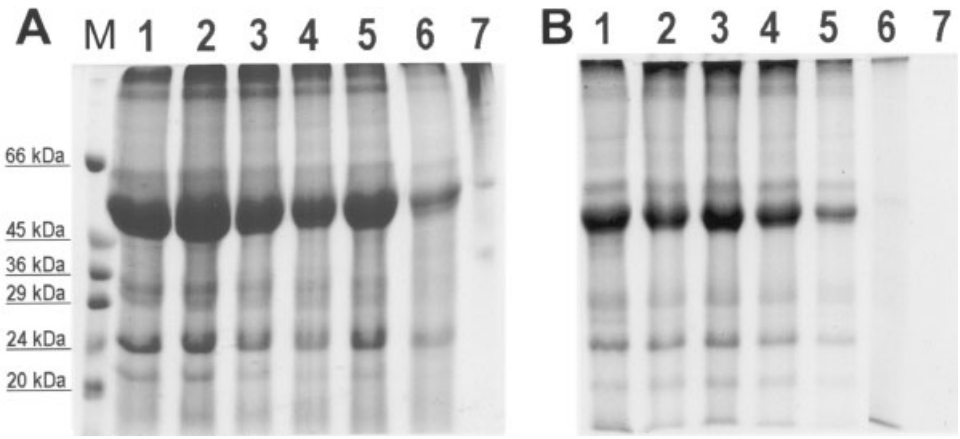

Figure 3. Electrophoretic separation of soybean meal protein fractions after *in situ* incubation: A, 50 g kg^{-1} tannic acid-treated soybean; B, untreated soybean. Lane M: standard molecular mass markers. Lanes 1–7: protein fractions of bag residues after 0, 2, 4, 8, 12, 24 and 48 h of incubation respectively.

DISCUSSION

It is widely known that, from a metabolic point of view, the utilisation of nitrogen and starch contained in concentrate feeds is much more efficient in the intestine than in the rumen,[1,17] and, under this perspective, some studies have shown beneficial effects of tannins on nitrogen retention and on productive parameters.[18,19]

Plant tannins are divided into two main categories, hydrolysable and condensed tannins. Most tannins belong to the latter group, and there is a limited presence of hydrolysable tannins such as TA in natural feeds. This fact has led to the assumption of a weak effect of hydrolysable tannins when they are used in digestibility assays. However, the biochemical mechanisms involved in tannin–protein binding are relatively simple, based mainly on the formation of hydrogen bonds in an optimal pH environment. Tannin–protein interactions do not depend essentially on the hydrolysable or condensed nature of the tannin, but on the presence of a sufficient amount of hydroxyl groups capable of interacting with susceptible radicals of the proteins.[7,20] In this sense, previous studies have shown that TA is able to precipitate the proteins of legume meals.[21]

The decrease in the immediately degradable fraction (*a*) and the lower rate of degradation (*c*) described for TA were also observed for *in situ* ruminal disappearance of legume meals after heat treatment,[22] as well as for soybean meal treated with TA[23] (the only legume in common with the present study), although quantitative differences were found between the results of the latter study and those of the present work. A possible explanation for such discrepancies could lie in the different sources of TA used. Although it is supposed to be a single molecular entity, considerable differences in the composition of commercial sources of TA have been reported,[24] this heterogeneity being reflected in significant differences in their ability to bind and precipitate a given protein source.[25]

The hydrolysis of TA in the rumen by the microbiota was also considered to be responsible in those studies that observed weak, non-persistent effects of TA on DM and CP ruminal disappearance,[23] since there is some evidence for the hydrolysis of tannins by ruminal micro-organisms.[26,27] Nevertheless, the experimental conditions in those studies were quite far from rumen physiology, and, given the relatively quick outflow of ground meals across the rumen,[28] it is highly questionable that bacterial acyl-hydrolase (tannase) activity could be decisive in the breakdown of TA in TA–protein complexes during the *in situ* assay.

There is a risk of overprotection (and decreased further intestinal bioavailability of amino acids) when feed proteins are treated by chemical procedures, as described for formaldehyde.[29,30] However, this effect seems to be quite small in the case of TA. In fact, the solubilisation of proteins from TA–protein complexes is a prerequisite for the electrophoretic separation of protein fractions, and the lack of alteration in the electrophoretic pattern of bands observed in Fig 3 (and in previous studies[21]) suggests a lack of overprotection of the legume seed proteins as a result of this treatment. This phenomenom could also explain the previously reported beneficial effects of tannins in the improvement of productive parameters of sheep fed tannin-rich forages.[31,32]

The results obtained by SEM are in agreement with those findings pointing to seed structures other than starch granules (mainly cell wall proteins) as the main determinant of the extent of seed digestion[33,34] in both untreated and TA-treated soybean meal. However, these results are highly contradictory with the extensive and quick *in vivo* digestion of starches taking place in the rumen.[35] The most feasible explanation could be the limitation imposed by the *in situ* bag technique,[36] taking into account the impossibility of having knowledge about the fate of released starch granules from untreated soybean endosperm cells. As revealed by SEM, the medium size of starch granules (Fig 2) is smaller than the bag pore size (about 50 μm); thus granules can escape from bags and, probably, be extensively hydrolysed in the rumen.

Although *in sacco* assays together with SEM of bag residues give valuable knowledge about physiological aspects of the ruminal hydrolysis of legume meals, their limitations in predicting the real utilisation of nutrients in the whole animal make necessary further research using *in vivo* assays. In this sense it must be pointed that some studies[37] found that heat treatment of beans strongly decreased the disappearance of nitrogen from nylon bags incubated in the rumen, although such treatment failed to influence the amino acid nitrogen flow into the duodenum. Therefore results from *in situ* incubations should be treated with caution and should ideally be supported by evidence based on measurements of nitrogen flow in animals fitted with duodenal cannulae.

The results obtained here suggest that tannins could be promising agents in achieving less-degradable concentrate feeds for ruminants. In contrast to other chemicals, tannins are natural compounds normally present in many plant species, and livestock producers and consumers might better accept their use. From a practical point of view the main interest of tannins probably does not lie in their external addition to feeds but in the fact that their expression is genetically regulated.[38] The selection of tannin-containing legumes and the genetic modification of tannin-free varieties are possible alternatives to improve plant protein and starch utilisation by ruminants.

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