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RUMEN PROTOZOA POPULATION AND CARBOHYDRATE-DIGESTING ENZYMES IN SHEEP FED A DIET SUPPLEMENTED WITH HYDROLYSABLE TANNINS*

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Abstract

The aim of the study was to compare the effect of adding different sources of hydrolysable tannins to the sheep diet on protozoa population and carbohydrate digestion in the rumen. The study was performed in 3 Polish Lowland ewes fistulated to the rumen in a 3 × 3 Latin square design. Control sheep (CON) received (g/d): meadow hay (600), barley meal (300), soybean meal (100) and vitamin-mineral premix (20). Sheep from the experimental groups were additionally administered 12.6 g/kg DM oak bark extract (OAK) and 3.91 g/kg DM tannic acid (TAN). The net consumption of tannins was approx. 0.4% DM for both additives. Regarding the count of protozoa, a significant interaction between diet and sampling time was documented for all ciliates (P<0.01), with a significant effect of both factors when considered separately. Experimental diets reduced the number of total protozoa and *Entodinium* spp. (before feeding, 2 and 4 h after feeding; P<0.01), while increasing the abundance of *Isotricha* spp. population (4 h after feeding; P<0.01) in the rumen. Interestingly, the count of *Ophryoscolex* spp. after feeding the TAN diet increased before feeding and 2 h after feeding in comparison to the CON and OAK groups, respectively, and subsequently decreased compared to the CON diet (4 and 8 h after feeding, P<0.01). A significant interaction between the diet and sampling time was observed for xylanolytic activity (P<0.01) in the rumen, with a significant effect of sampling time, which decreased its activity in CON (after feeding) and OAK sheep (2 h after feeding; P<0.01). For amylolytic activity (P<0.10), there was a trend towards a significant interaction between experimental factors, with a significant effect on both diet and sampling time. Detailed analysis showed that the TAN diet significantly reduced amylolytic activity 2 h after feeding compared to the CON group (P<0.05). In conclusion, the TAN diet significantly reduced the number of total protozoa and *Entodinium* spp., which consequently reduced amylolytic activity in the rumen, without any significant effect on pH and carbohydrate fermentation in the rumen.

Key words: rumen, polysaccharidase, short-chain fatty acid, oak bark extract, tannic acid

Recently, growing interest has been observed in the use of natural additives in ruminants nutrition, which can have a considerable impact on microbial populations and digestive processes in the rumen (Miltko et al., 2019; Majewska et al., 2021; Colombini et al., 2021). Tannins, as polyphenolic compounds, are examples of such additives. Due to their different chemical structure and biological activity, tannins are divided into hydrolysable (gallotannins and ellagotannins) and condensed types of compounds (Frutos et al., 2004). In general, tannins are considered anti-nutritional substances that limit nutrient digestion by forming complexes mainly with proteins of various origin (dietary, endogenous and microbiological), but also to a lesser extent with carbohydrates and metal ions (Silanikove et al., 2001; Patra and Saxena, 2011). These properties of tannins adversely affect monogastric animals, but may be beneficial for ruminants. Most dietary protein is extensively degraded by ruminal microorganisms to ammonia, and subsequently absorbed and excreted in urine, which has negative consequences for both animal production and the environment (Patra and Saxena, 2011; Haro et al., 2018). Therefore, protection of dietary protein in ruminants is of great importance and allows increasing its flow to the downstream parts of the gastrointestinal tract, and improving animal production with optimal dietary protein level (Mergeduš et al., 2018). Importantly, rumen conditions (especially pH) favour the formation of tannin-nutrients complexes (Mueller-Harvey, 2006). Several studies documented lower ammonia concentrations in the rumen after application of tannin-rich plants, which indicated reduced degradation of dietary protein (Aghamohamadi et al., 2014; Aboagye et al., 2018; Sarnataro and Spanghero, 2020; Wang et al., 2022). Moreover, it was shown that condensed tannins and gallotannins caused higher protein precipitation than ellagotannins (Salminen and Karonen, 2011).

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Tannins may also reduce the digestibility of structural carbohydrates present in the plant cell wall by forming complexes with cellulose, hemicellulose and pectin, as well as enzymes of microbial origin, which may result in changes in short-chain fatty acids levels (SCFA) and rumen pH (Patra and Saxena, 2011; Mergeduš et al., 2018). Additionally, tannins are considered methane inhibitors (Hess et al., 2006; Doce et al., 2009; Cieślak et al., 2014), and potential reasons for reduced methane production in ruminants include lower fibre degradation, increased propionic acid level (known as 'methane antagonist'), as well as reduced abundance of protozoa, closely related to methanogens (Newbold et al., 2015).

In recent years, many *in vitro* studies have focused on the potential of condensed tannins. Nevertheless, research involving animals is an important complement to these studies that allows assessing the influence of biologically active agents on the physiological processes of living organisms. Considering the above, the present study used two sources of hydrolysable tannins – oak bark extract (natural) and tannic acid (synthetic). The core of these molecules is a monosaccharide or branched sugar, where hydroxyl groups are esterified with gallic acid residues (Mueller-Harvey, 2001). More importantly, hydrolysable tannins have a lower molecular weight and lower binding affinity for proteins in comparison to condensed tannins (Aboagye et al., 2018; Vasta et al., 2019; Wang et al., 2022). This in turn makes them susceptible to cleavage by several microorganisms inhabiting the rumen to intermediates (gallic acid, pyrogallol, resorcinol), and ultimately to acetic and butyric acids (Singh et al., 2001). Such metabolites can be easily absorbed in the digestive tract (Aboagye et al., 2018; Wang et al., 2022). Therefore, their proportion in a diet should be carefully monitored due to possible toxic effects to ruminants when administered in high doses (Barbehenn and Constabel, 2011).

It was hypothesized that tannin-rich additives used in the present study could directly modify protozoa population and consequently influence fermentation in the rumen. Thus, the aim of the study was to compare the addition of different sources of hydrolysable tannins to sheep diets on the number of protozoa, carbohydrate-degrading enzymes and SCFA levels in the rumen.

Material and methods

The present study is part of a previously published work of Majewska et al. (2022), which presented the effect of hydrolysable tannins on the rumen fatty acid profile with a detailed description of the experimental design. All procedures in the present study were accepted by the Local Animal Care Ethics Committee for Animal Experiments in Warsaw (Poland); permission No. 79/2015.

Table 1. Composition of sheep diets (adopted from Majewska et al. (2022) as part of the same experiment)

Item	CON	OAK	TAN
Components (g/kg DM)			
meadow hay	588	581	586
barley meal	294	290	293
soybean meal	98.0	96.8	97.7
Polfamix O-K[1]	19.6	19.4	19.5
oak bark extract	–	12.6	–
tannic acid	–	–	3.91
Chemical composition (g/kg DM)			
dry matter	887	887	887
crude protein[2]	110	109	109
crude fat	21.8	21.8	21.7
starch	222	219	221
NDF	442	436	440
ADF	371	366	369
ADL	35.7	35.4	35.6
crude ash	34.4	34.2	34.3
Nutrient intake (g/d)			
dry matter	905	917	908
crude protein	112	112	112
crude fat	22.2	22.5	22.2
starch	226	226	226
NDF	451	451	451
ADF	378	378	378
ADL	36.4	36.5	36.4
crude ash	35.1	35.3	35.1

CON, control diet; OAK, diet with oak bark extract addition; TAN, diet with tannic acid addition; DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin.

[1]Content in g/kg: Ca 240, Na 60, P 120, Mg 65, Zn 2.5, Mn 3.0, vit. E 1.5, Se 0.003, Co 0.015; IU: vit. A 300.000, vit. D_3 30.000.

[2]expressed as N × 6.25.

Animals and diets

The study was carried out in 3 Polish Lowland ewes (48.7±4.3 kg live weight), fitted with rumen cannulas in a 3 × 3 Latin square design. The experiment consisted of three experimental periods lasting 37 days each, comprising gradual transition to a diet (14 days), adaptation to a specific diet (21 days), and finally, sampling (2 days). The control diet (CON group) was formulated in accordance with the recommendations for ruminants (Table 1, IZ PIB-INRA, 2009) and consisted of (g/d): meadow hay (600), barley meal (300), soybean meal (100) and vitamin-mineral premix (20, Polfamix O-K, Trouw Nutrition Polska, Grodzisk Mazowiecki, Poland). The experimental groups additionally received 12.6 g/kg DM oak bark extract (*Quercus alba* L., Chem&Pol; OAK group), containing 30% tannins; or 3.91 g/kg DM tannic acid (16201, Sigma-Aldrich Co., St. Louis, MO, USA; TAN group). Both tannin-rich additives (powder form) were administered to the animals with the concentrate during morning feeding. More importantly, the doses of OAK and TAN supplementation were balanced according to tannins content. Thus, the net intake of tannins for both additives was 3.91 g/kg DM per day (approximately 0.4% DM, Table 1). The animals were fed twice a day at 7:00 am and 3:00 pm. During the experiment and sampling time, sheep were housed in individual pens, equipped with separate facilities for forage and concentrate, with *ad libitum* access to water and salt licks.

Feed samples were collected during the experiment for further analyses. The chemical composition of sheep diets (meadow hay and concentrate) was determined (AOAC, 2011; Table 1).

Rumen fluid and digesta sampling

Ruminal fluid was collected with a copper tube connected to a syringe from the middle and ventral sacs of the rumen. The end of the copper tube had numerous holes with a diameter of 0.2 cm, allowing rumen fluid collection. Subsequently, rumen fluid was precisely mixed and filtered through a surgical gauze to remove all possible residues of feed particles. The samples of ruminal fluid were used to determine pH values, protozoa count as well as SCFA levels. The samples of rumen digesta (solid and liquid fractions) were collected manually from different locations in the rumen (dorsal and ventral regions) to obtain representative material. Ruminal digesta were thoroughly mixed, collected to plastic containers (approximately 150 g) and stored at –20°C for enzymatic activity determination. The remaining digesta were immediately returned to the rumen. Both types of samples (ruminal fluid and digesta) were collected directly before morning feeding (0 h) and 2, 4 and 8 h after feeding to detect even minor changes in all parameters over the time.

Determination of protozoa counts

The samples of filtered ruminal fluid were fixed with a 4% formaldehyde solution and stored in tightly closed containers at 4°C until analysis. Protozoa numbers were counted and classified according to Dehority (1993), and determined using a light microscope according to Miltko et al. (2015). Briefly, the counts of total protozoa as well as protozoa from the families *Ophryoscolecidae* (*Entodinium* spp., *Diplodinium* spp., *Ophryoscolex* spp.) and *Isotrichidae* (*Isotricha* spp., *Dasytricha* spp.) were documented in ruminal fluid.

Extraction and enzymatic analyses

Enzymes were extracted from rumen digesta (2 g, wet weight) with 20 ml of 1% phosphate buffer (pH 6.0), 2.5 ml of carbon tetrachloride (CCl_4) and lysozyme (Miltko et al., 2016 a). Briefly, the suspension was incubated for 3 h at 37°C and then liquid fraction was centrifuged at 11,000 g at 4°C for 25 min. The supernatant was collected and served as a source of enzymes. The incubation process was conducted in the presence of the enzyme fraction from each sample, phosphate buffer (pH 6.0) and purified substrates at 40°C for 1 h. Low-viscosity carboxymethylcellulose (C5678), beechwood xylan (X4252), potato starch (S2004) and pectin from citrus (P9125) were used as substrates for the determination of cellulolytic, xylanolytic, amylolytic and pectinolytic activity in rumen digesta. All substrates for enzymatic assays were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The addition of the dinitrosalicylic acid reagent (D0550, Sigma-Aldrich Co., St. Louis, MO, USA) and heating at 100°C for 5 min stopped the enzymatic reaction. Reducing sugars were quantified spectrophotometrically at 560 nm and hydrolytic activity of the tested enzymes was determined on this basis.

Determination of SCFA concentration

Filtered ruminal fluid samples were treated with 85% formic acid, centrifuged (11,000 × g for 25 min) and stored in vials at 4°C until analysis. SCFA levels were determined according to Miltko et al. (2016 b) using a gas chromatograph (Shimadzu GC-2010, Tokyo, Japan) equipped with a ZB-WAX capillary column (30 m length, 0.25 mm i.d. × 0.25 μm film thickness, Zebron, Phenomenex) and a flame ionization detector (FID); helium served as a carrier gas. Additionally, 4-methylvaleric acid was used as an internal standard (277827, Sigma-Aldrich Co., St. Louis MO, USA). The temperature of FID and injector was kept constant at 280°C and 250°C, respectively. During the analysis, the temperature programme of the column was as follows: starting with 80°C, maintaining for 1 min, increasing to 220°C (15°C/min), and maintaining it for 4 min. FA peaks were identified according to the standards provided, including acetic acid (695092), propionic acid (402907), isobutyric (I1754) and butyric acids (B103500), as well as isovaleric (129542) and valeric acids (240370) (Sigma-Aldrich Co., St. Louis MO, USA). Subsequently, they were integrated using the GC software 112 (LabSolutions, Shimadzu, Tokyo, Japan).

Statistical analysis

The results are presented as means with standard deviations. Data obtained from 2 days of sampling were averaged. The normality of data was checked using the Shapiro–Wilk test, and the homogeneity of variances using the Levene test. Data that did not show a normal distribution were converted to natural logarithms (number of protozoa, except for *Diplodinium* spp. and *Dasytricha* spp.; amylolytic activity, total SCFA and valerate concentrations). The results were subjected to repeated measures analysis of variance (ANOVA), with diet, sampling time and their interactions as main effects, using the following general linear model:

$$Y_{jkl} = \mu + A_j + D_k + T_l + (D + T)_{kl} + \mathcal{E}_{jkl}$$

where Y_{jkl} – mean of observations; μ – overall mean; A_j – animal effect (n = 3); D_k – diet effect (CON, OAK, TAN); T_l – sampling time effect (0, 2, 4 and 8 h); $(D + T)_{kl}$ – interaction between diet and sampling time; and $\mathcal{E}_{jkl}$ – random error.

Differences between means were determined by a Tukey test. The effects of animal (3 sheep) and period (I, II and III) on the obtained results were also verified. Significance between means was declared at $P \leq 0.05$ (StatSoft®, Kraków, Poland). The trends at $0.05 < P < 0.10$ were also described and discussed. Data presented in Tables 2, 3 and 4 are raw results before logarithmic transformations.

Results

Animal diet

Dry matter (DM) intake for CON, OAK and TAN animals was 905, 917 and 908 g/d, respectively (Table 1, according to Majewska et al., 2022). No orts were observed, because the sheep were fed limited amounts and the daily feed consumption represented 2.1% of the ewe's live body weight.

Table 2. Ciliates counts (× 10^4/ml) in sheep rumen fluid

Item	Diet (D)	Sampling time (T)				P-value		
		0 h	2 h	4 h	8 h	D	T	D × T
Total ciliate	CON	97.8±5.62 a x	54.7±1.75 b x	59.9±1.99 b x	53.9±8.75 b x	<0.001	<0.001	<0.001
	OAK	45.7±4.41 a y	34.9±2.98 b y	32.7±2.63 b y	32.5±1.40 b y			
	TAN	57.2±1.30 a y	41.1±3.92 b y	37.7±1.88 b y	59.6±8.27 a x			
Entodinium	CON	85.9±4.85 a x	48.4±0.21 b x	49.3±5.33 b x	47.1±5.83 b x	<0.001	<0.001	<0.001
	OAK	34.7±3.36 a y	29.6±2.32 ab y	28.3±1.76 ab y	25.0±2.58 b y			
	TAN	47.7±1.41 a z	31.0±2.71 b y	31.9±1.60 b y	40.8±9.14 ab x			
Diplodinium	CON	1.74±0.262 a	1.04±0.040 b	1.35±0.136 ab	1.63±0.531 a	0.085	<0.001	<0.001
	OAK	1.31±0.306 a	0.89±0.110 ab	0.75±0.117 b	0.82±0.060 ab			
	TAN	2.29±0.219 a	0.98±0.246 b	0.69±0.359 b	0.99±0.397 b			
Ophryoscolex	CON	0.91±0.181 a x	0.20±0.020 bc xy	0.17±0.023 b x	0.39±0.023 c x	0.001	<0.001	<0.001
	OAK	0.79±0.061 a x	0.17±0.042 b x	0.11±0.023 b xy	0.12±0.020 b y			
	TAN	2.13±0.502 a y	0.38±0.092 b y	0.07±0.031 c y	0.15±0.012 d y			
Isotricha	CON	2.37±0.552 a	0.83±0.303 b	0.79±0.216 b x	0.92±0.144 b x	0.001	<0.001	<0.001
	OAK	1.41±0.103	1.29±0.136	1.30±0.072 y	1.44±0.104 x			
	TAN	2.29±0.081 ac	1.20±0.151 b	1.36±0.139 bc y	2.67±0.474 a y			
Dasytricha	CON	5.36±0.837 a	3.63±0.751 b	3.52±0.356 b	4.46±0.106 ab	0.063	<0.001	<0.001
	OAK	2.56±0.087 a	5.52±1.180 b	3.01±0.375 a	2.37±0.569 a			
	TAN	4.01±0.603 a	3.56±0.092 ab	2.31±0.324 b	4.49±0.456 a			

CON, control diet; OAK, diet with oak bark extract addition; TAN, diet with tannic acid addition; D, main effect of diet; T, main effect of sampling time; D × T, diet and sampling time interaction effect; a, b, c, d – means with different letters in a row differ significantly at P≤0.05 between sampling time (0, 2, 4, 8 h).

x, y – means with different letters in a column differ significantly at P≤0.05 between diet (CON, OAK, TAN).

Table 3. Activity of polysaccharidases in rumen digesta of sheep (μM released product/g DM/min)

Enzyme activity	Diet (D)	Sampling time (T)				P-value		
		0 h	2 h	4 h	8 h	D	T	D × T
Cellulolytic[1]	CON	32.9±1.60 a	22.4±3.58 b	26.4±5.44 ab	29.6±6.28 ab	0.970	0.015	0.217
	OAK	29.8±2.30	26.8±1.92	26.3±5.30	30.6±3.97			
	TAN	26.9±2.03	25.1±6.32	30.6±7.78	31.4±4.09			
Xylanolytic[2]	CON	94.6±6.16 a	58.7±6.04 b	61.9±6.14 b	68.6±8.60 b	0.689	< 0.001	0.001
	OAK	83.2±3.14 a	50.5±9.56 b	68.4±1.70 a	70.1±3.41 a			
	TAN	70.9±7.10	62.3±6.46	68.5±10.8	66.9±8.48			
Amylolytic[3]	CON	7.21±0.696 a	10.2±1.38 b x	9.10±0.456 ab	7.54±1.135 a	0.014	0.001	0.088
	OAK	7.04±0.764	8.02±0.878 xy	9.20±0.936	7.89±0.165			
	TAN	6.81±0.716	7.13±0.323 y	7.64±0.534	7.22±0.205			
Pectinolytic[4]	CON	11.4±1.12	10.3±1.15	11.3±1.45	12.2±3.26	0.545	0.012	0.700
	OAK	8.91±1.394	9.59±1.724	11.2±2.80	11.4±3.16			
	TAN	8.15±1.306	9.25±0.692	10.9±1.80	11.1±2.04			

CON, control diet; OAK, diet with oak bark extract addition; TAN, diet with tannic acid addition; D, main effect of diet, T, main effect of sampling time; D × T, diet and sampling time interaction effect;
[1]expressed as μM glucose released/g DM/min;
[2]expressed as μM xylose released/g DM/min;
[3]expressed as μM glucose released/g DM/min;
[4]expressed as μM glucuronic acid released/g DM/min.
a, b – means with different letters in a row differ significantly at P≤0.05 between sampling time (0, 2, 4, 8 h).
x, y – means with different letters in a column differ significantly at P≤0.05 between diet (CON, OAK, TAN).

Table 4. pH and short-chain fatty acids concentrations in sheep rumen (mM/100 ml)

Item	Diet (D)	Sampling time (T)				P-value		
		0 h	2 h	4 h	8 h	D	T	D x T
pH	CON	6.75±0.164 a	6.26±0.198 b	6.31±0.234 b	6.58±0.201 ab	0.789	<0.001	0.297
	OAK	6.71±0.147 a	6.26±0.126 b	6.36±0.187 b	6.54±0.208 ab			
	TAN	6.77±0.076 a	6.37±0.181 b	6.34±0.172 b	6.63±0.126 a			
Total SCFA	CON	8.98±1.359 a	11.4±1.88 b	11.0±1.57 b	9.33±1.532 a	0.578	<0.001	0.167
	OAK	9.38±0.308 a	11.2±0.37 b	10.6±0.37 bc	9.75±0.428 ac			
	TAN	8.59±0.683 a	10.5±0.95 b	10.5±0.95 b	9.02±1.151 a			
Acetate	CON	6.08±1.067 a	7.52±1.428 b	7.34±1.156 b	6.35±1.162 a	0.543	<0.001	0.196
	OAK	6.40±0.261 a	7.52±0.300 b	7.20±0.248 bc	6.76±0.456 ac			
	TAN	5.76±0.575 a	6.96±0.799 b	7.11±0.749 b	6.17±0.938 a			
Propionate	CON	1.38±0.148 a	2.06±0.274 b	1.87±0.305 b	1.49±0.198 a	0.605	<0.001	0.048
	OAK	1.43±0.065 a	1.88±0.071 b	1.72±0.082 b	1.50±0.091 a			
	TAN	1.35±0.122 a	1.85±0.176 b	1.74±0.220 b	1.43±0.200 a			
Butyrate	CON	1.09±0.165 a	1.36±0.241 b	1.31±0.214 b	1.11±0.190 a	0.734	<0.001	0.927
	OAK	1.11±0.098 a	1.37±0.190 b	1.33±0.182 b	1.15±0.093 a			
	TAN	1.04±0.055 a	1.29±0.104 b	1.30±0.162 b	1.06±0.108 a			
Valerate	CON	0.10±0.006 a	0.16±0.012 b	0.16±0.026 b	0.11±0.007 a	0.191	<0.001	0.532
	OAK	0.10±0.009 ab	0.13±0.015 a	0.12±0.012 a	0.10±0.007 b			
	TAN	0.10±0.010 ab	0.14±0.010 a	0.13±0.019 a	0.11±0.017 b			
Isoacids[1]	CON	0.33±0.027 a	0.30±0.018 b	0.28±0.010 bc	0.27±0.026 c	0.347	<0.001	0.039
	OAK	0.34±0.034 a	0.27±0.028 b	0.24±0.022 bc	0.24±0.028 c			
	TAN	0.34±0.045 a	0.28±0.033 b	0.26±0.043 b	0.25±0.036 b			

CON, control diet; OAK, diet with oak bark extract addition; TAN, diet with tannic acid addition; D, main effect of diet; T, main effect of sampling time; D × T, diet and sampling time interaction effect;
[1]Sum of isobutyric and isovaleric acids; a, b – means with different letters in a row differ significantly at P≤0.05 between sampling time (0, 2, 4, 8 h).
x, y – means with different letters in a column differ significantly at P≤0.05 between diet (CON, OAK, TAN).

Protozoan population

Significant interactions between dietary treatment and sampling time were observed for the total number of ciliate and their genera (P<0.001; Table 2). Detailed analysis also showed a significant effect of sampling time for all protozoa (P<0.001). In most cases, the abundance of protozoa decreased after feeding, regardless of dietary treatment (except for *Dasytricha* in the OAK group 2 h after feeding). In relation to diet, a decreased number of total protozoa and *Entodinium* spp. was observed in OAK and TAN sheep in comparison to CON animals before feeding and 2 and 4 h after feeding (P<0.001). Interestingly, a lower count of total protozoa and *Entodinium* spp. was recorded 8 h after feeding in OAK sheep (P<0.001) compared to other groups. A downward trend was observed for *Diplodinium* spp. population in OAK sheep before feeding (P=0.085) in comparison to the TAN group. In the latter, an increased number of *Ophryoscolex* spp. was found before feeding compared to CON and OAK (P=0.001), and 2 h after feeding in relation to OAK sheep (P=0.001). Interestingly, the abundance of *Ophryoscolex* spp. population decreased in the TAN (4 and 8 h after feeding, P=0.001) and OAK groups (8 h, P=0.001) in comparison to CON sheep. An increased number of *Isotricha* spp. was noted in sheep fed the diets with OAK (4 h after feeding) and TAN addition (4 and 8 h after feeding) when compared to the CON group (P=0.001). On the other hand, the number of *Dasytricha* spp. tended to decrease in OAK sheep before and 8 h after feeding in comparison to the CON group and 8 h after feeding in relation to the TAN group (P=0.063).

Carbohydrate-digesting enzymes

A significant interaction of diet and sampling time was recorded for xylanolytic activity in the rumen (P=0.001; Table 3). Detailed analysis showed that only the sampling time affected this parameter and decreased the activity in both CON (after feeding) and OAK groups (2 h after feeding) (P<0.001). For amylolytic activity, there was a trend towards a significant interaction between experimental factors (P=0.088) and the effect of diet and sampling time. An increased activity of amylase was observed 2 h after feeding in the CON group (P=0.001) compared to its activity before and 8 h after feeding. With regard to diet, it was found that TAN significantly reduced amylolytic activity 2 h after feeding compared to the CON group (P=0.014). No significant interaction between experimental factors was observed for both cellulolytic (P=0.217) and pectinolytic (P=0.700) activities in the rumen.

pH and SCFA

The pH value ranged from 6.26 to 6.77 and was not dependent on the diet and time interaction. However, its value significantly decreased 2 and 4 h after feeding irrespective of the diet (Table 4).

A significant interaction between diet and time was found for propionate (P=0.048) and isoacid (P=0.039) concentrations in the ruminal fluid (Table 4). Only the sampling time significantly affected the parameters tested, but this effect depended on the type of acid. Regardless of the diet, an increased concentration of propionic acid 2 and 4 h after feeding was observed in comparison to its levels before feeding and 8 h after feeding (P<0.001). The situation was opposite for isoacids, where their concentrations were significantly lower 2, 4 and 8 h after feeding as compared to samples collected before feeding (P<0.001).

Discussion

The difficulty in planning the experiments with the use of biologically active compounds (in this case tannins) results primarily from the selection of an appropriate dose that would be effective in inducing a biological effect in animals. According to the literature, different quantities of the additives are administered, depending on the type of tannins. For example, high concentrations of condensed tannins (60–120 g/kg DM) may significantly reduce feed intake and digestion, while low concentrations (20–40 g/kg DM) may exert beneficial effects on ruminants, e.g. improving animal performance, preventing flatulence and parasitic larval invasions (Min et al., 2005; Hoste et al., 2015; Huyen et al., 2016). However, no detrimental effects on animal production at ≤20 g/kg DM were observed for hydrolysable tannins (Frutos et al., 2004; Jayanegara et al., 2012, 2015).

According to the literature, tannic acid is considered a hepatoxin (Seigler, 1998). Furthermore, Wang et al. (2022) showed that the addition of various doses of tannic acid (0.3, 0.6 and 0.9% DM) to goat diets increased the activity of alanine aminotransferase, IL-1ß and IL-6 in blood plasma, which could indicate a negative influence of tannins on physiological and immune functions. The present study used a low dose of tannins (approximately 0.4% DM) to avoid their potential toxicity. Indeed, Rojas-Román et al. (2017) showed that among various doses used in the study (0, 2, 4 or 6 g/ kg DM), 4 g/kg DM tannin extract (a mix of hydrolysable and condensed tannins) positively affected growth performance of finishing lambs.

Protozoan population

It is well known that the digestion process in the rumen occurs with the participation of microorganisms (so-called alloenzymatic digestion), which produce hydrolytic enzymes that digest plant carbohydrates, including cellulose, xylan, pectin, inulin and starch (Miltko et al., 2016 a). Thus, ruminant animals utilise the energy stored in plant carbohydrates to meet their needs.

Tannins are generally considered inhibitors of microorganism development (McSweeney et al., 2001). Tannins have been shown to act through two mechanisms, i.e. i) forming complexes with the cell wall and hindering micronutrient inside the cell, and/or ii) reducing their at-

tachment to feed particles by showing high affinity for secreted extracellular enzymes (Daglia, 2012). Unfortunately, the effect of tannins on protozoa population does not seem to be as pronounced as observed in bacteria. Some studies demonstrated a negative effect of these compounds on protozoa counts (Carulla et al., 2005; Aghamohamadi et al., 2014; Sarnataro and Spanghero, 2020), but some showed their positive influence (Chiquette et al., 1989; Terrill et al., 1992; Majewska et al., 2021); however, the mechanisms of tannin action on protozoa remain largely unknown.

Regardless of the dietary treatment, the genus *Entodinium* was the most abundant group of protozoa in the present study (approximately 80%). Less numerous were ciliates from the genera *Dasytricha* spp., *Isotricha* spp., *Diplodinium* spp. and *Ophryoscolex* spp., respectively. Tannin-rich additives influenced all genera of protozoa, but due to the large number of *Entodinium* spp. (dominant) in the rumen, the impact of tannins on their population seemed to be the most pronounced. Interestingly, such protozoa are involved in the breakdown of easily digestible carbohydrates, including starch as the main source of energy. Both additives significantly reduced the number of total ciliate and *Entodinium* protozoa, however the effect of OAK appeared to be stronger and lasted even 8 h after feeding. The reason for this could be the variation in the content of various types of tannins in the OAK diet. According to Smeriglio et al. (2017), apart from hydrolysable tannins (gallic and ellagic acids), oak bark also contains small amounts of condensed tannins (proanthocyanidins). The results of the present study are consistent with findings of Cieślak et al. (2014), who reported a reduced number of total protozoa after adding both oak bark (hydrolysable tannins) and lingonberry leaves (condensed tannins) extracts. Similarly, Salami et al. (2018) observed a lower abundance of protozoa in sheep supplemented with hydrolysable tannins from tara. On the other hand, Abarghuei et al. (2011) showed decreased counts of total protozoa, as well as *Isotricha*, *Diplodinium* and *Eudiplodinium* populations in sheep fed a diet with oak leaves (*Quercus libani* Oliv., hydrolysable tannins), while these authors found no effect on the genera *Entodinium*, *Dasytricha*, *Metadinium* and *Ophryoscolex*. Differences in response of protozoan populations to tannins could be mainly dependent on the diet composition. The present study used oak bark extract, while in the aforementioned research, alfalfa was completely replaced with oak leaves. When studying the effect of tannins on ruminants, it is extremely important to know whether tannins were added in the form of an extract, a pure supplement or a tannin-rich forage. The origin of tannins is also of great importance. Different parts of individual plants may differ in the type and amount of biologically active substances (Gherman et al., 2000). In our previous study, we reported an increased abundance of *Entodinium* protozoa after 0.3% DM oak bark addition to the sheep diet (Majewska et al., 2021). Such differences in the results could be due to lower tannins content in pure bark used in the previous study compared to the extract, and possibly the presence of other biologically active substances in oak bark.

In the present study, the influence of tannin-rich additives on the abundance of *Ophryoscolex* spp. protozoa varied during the sampling period. These protozoa are responsible for the digestion of structural carbohydrates, including cellulose and hemicellulose. The decreased number of these protozoa after TAN (4 and 8 h after feeding) and OAK addition (8 h after feeding), in comparison to CON, could be the result of tannin action, especially considering the results obtained before feeding for the TAN group. It was presumed that tannins present in the tested additives could form complexes with cellulose and hemicellulose, which in consequence could result in a deficiency of substrates required for growth and development of these protozoa. However, it is difficult to explain differential effects of both tannin sources on protozoa from the family *Isotrichidae* (*Isotricha* spp. and *Dasytricha ruminantium*), which are able to utilise the same type of carbohydrates. There is a high possibility that the presence of different types of tannins in the OAK diet could determine the obtained results.

Any changes in the number of protozoa over time should be also considered in relation to the bacterial population, because protozoa feed on bacteria (Williams and Coleman, 1997). Certainly, knowing the influence of tannins on bacteria could facilitate the interpretation of the current results. Therefore, further studies should include an extended analysis regarding the effects of tannins on the rumen microbial population.

Polysaccharidases activity

According to the literature, the mechanism of tannin action on the reduced digestion of carbohydrates in the rumen is not fully understood and may result from several factors, including substrate deprivation (cellulose, hemicellulose, starch and pectin), enzymes inhibition (cellulases, amylases and galactosidases), as well as direct effect on microorganisms (Frutos et al., 2004; He et al., 2006; Mergeduš et al., 2018). Furthermore, the formation of tannin-protein (enzymes) complexes may be influenced by a number of factors related to polyphenols (molecular weight, degree of polymerization), protein (molecular weight, proline content, hydrophobicity, flexibility), as well as the environment, in which the complexes are formed (Gonçalves et al., 2011; Amoako and Awika, 2016; Barrett et al., 2018).

Interestingly, a reduced amylolytic activity in rumen digesta was noted in the present study only in TAN sheep 2 h after feeding, with no significant effect on other examined enzymes, including cellulase, xylanase and pectinase. A laboratory study of Tong et al. (2014) showed that some fractions of *Eugenia jambolana* bark extract (hydrolysable tannins), containing both ellago- and gallotannins, decreased α-amylase activity. Similar to the present study, Björck and Nyman (1987) reported reduced activity of starch-degrading enzymes

after TAN addition. Moreover, the latter authors also observed lower activity of cellulase and hemicellulase. An *in vitro* study of Ozkose et al. (2011) also demonstrated decreased activity of fibrolytic enzymes after the addition of different concentrations of tannic acid. It seems that differences in enzymes response to tannins or lack thereof could depend on the presence of various components (as in OAK), chemical structure of tannins (especially the degree of polymerization), and their affinity for these enzymes (Seigler, 1998). Modification of enzymatic activity might also be attributed to the interactions between tannins and substrates. When tannins are added in high concentrations in the diet, free tannins (not bound to proteins) may also form complexes with carbohydrates (Patra and Saxena, 2011). The nature of the formation of such complexes is not fully elucidated, but depends rather on the type of tannins (molecular weight, flexibility, water solubility) contained in the diet, and seems to be independent of pH (Seigler, 1998). Interestingly, the formation of complexes with carbohydrates is not uniform. According to Amoako and Awika (2016), tannins can bind to starch more efficiently than to cellulose and xyloglucan (hemicellulose), which could partially explain the present results. Importantly, the amount of amylose present in starch may affect its binding efficiency, while the formation of complexes between tannins and amylopectin appears to be non-specific and less important (Amoako and Awika, 2016).

As previously mentioned, ruminal digestion is possible thanks to the participation of microorganisms (bacteria, protozoa, fungi). Thus, the reduced amylolytic activity of rumen digesta in sheep fed the TAN diet in the present study was likely closely related to a lower number of *Entodinium* spp. These protozoa possess hydrolytic enzymes that are able to digest starch-rich feed (Miltko et al., 2016 a). However, the influence of amylolytic bacteria on the obtained results cannot be excluded. An *in vitro* experiment of Sarnataro and Spanghero (2020) demonstrated that the addition of 1.5% chestnut extract (hydrolysable tannins) significantly decreased *Ruminobacter amylophilus*. These bacteria utilise starch as an energy source, which may confirm our speculations. Interestingly, Ozkose et al. (2011) showed that the response of different groups of bacteria to the presence of tannic acid varied. This could be related to the adaptation mechanisms of some microorganisms to the presence of hydrolysable tannins (Singh et al., 2001). However, an *in vitro* study of Cipriano-Salazar et al. (2018) revealed that only 20% of bacteria isolated from the ruminal fluid of sheep showed tolerance to 0.63 and 1.25 mg of tannic acid per litre, which corresponded to 2% of tannins in the diet. Additionally, Cieslak et al. (2014) noted that the addition of both oak bark (hydrolysable tannins) and lingonberry leaf (condensed tannins) extracts significantly decreased the abundance of methanogens. Unfortunately, we did not examine the effect of these additives on bacteria and methanogens. Hence, a more detailed research on the relationships of bacteria and protozoa, and methanogens and protozoa in the rumen after tannins addition would be a valuable aid to fully define their mechanism of action on polysaccharidases.

Until recently, it was believed that tannins could only adversely affect the activity of enzymes. An important issue was addressed in the studies of Adamczyk et al. (2017 a, b), as these authors showed that in some cases, a low amount of tannins could increase enzymatic activity and modulate enzyme activities. This fact was confirmed in our previous study on sheep fed a diet with a low dose of lingonberry leaves or oak bark (0.3% DM), containing condensed and hydrolysable tannins, respectively, where increased activity of lipase, amylase and trypsin (as trends) in pancreatic-biliary juice was documented (Majewska et al., 2017). Additionally, a biochemical work of Adamczyk et al. (2017 b) revealed that tannin-enzymes complexes still exhibited low activities. According to the latter authors, tannins could act as non-competitive inhibitors, which meant that they did not bind to the catalytic site of the enzyme, but to other regions.

SCFA production

The products of carbohydrate digestion in ruminants are primarily SCFAs, which cover about 80% of the energy requirements of animals (Gäbel and Sehested, 1997). Despite the changes in the protozoan population and reduced amylolytic activity after tannins addition, we did not observe any significant effect on pH and SCFA production in the rumen. Michałowski (1987) considered protozoa the main producers of butyric acid, thus, it could be assumed that a fraction of protozoan species would also reduce the content of butyric acid in the rumen. However, the amount of butyric acid produced was similar in all experimental groups, regardless of the sampling time point; similar relationships were also observed in a study of Salami et al. (2018). Despite changes in the microbial population, including reduced counts of protozoa and methanogens, hydrolysable (*Castanea sativa* and *Caesalpinia spinosa*) and condensed tannins extracts (*Acacia negra* and *Uncaria gambir*) did not affect SCFA production. Interestingly, higher total SCFA levels were observed in lambs fed a diet with hydrolysable extract addition compared to condensed tannins (Salami et al., 2018). As the authors suggested, hydrolysable tannins were susceptible to degradation by microbial enzymes in the rumen (acetate and butyrate as end products), which could increase the SCFA pool (Salami et al., 2018). Perhaps, part of butyric acid in this study originated from the degradation of hydrolysable tannins, thus losses in its production were not observed, despite visible changes in the *Entodinium* abundance. Moreover, both dietary additives did not significantly affect the concentration of total SCFA in the ruminal fluid. The current results were consistent with findings of Śliwiński et al. (2002), who demonstrated that low and high doses of *Castanea sativa* wood extract (hydrolysable tannins, 1 and 2 g/kg DM) did not affect SCFA concentrations. Similar findings were reported by Sarnataro and Spanghero (2020),

who applied low dietary doses of chestnut tannins (1.5% DM maize). Interestingly, in an *in vitro* study of Cieslak et al. (2014), the effect of different types of tannins (hydrolysable and condensed tannins, equivalent to 2.5 g tannins/kg DM) on the SCFA profile was variable. The latter authors showed that oak bark extract (hydrolysable tannins) did not significantly change the concentration of SCFA, similarly to the results acquired in the present study. However, the cited authors documented that the addition of lingonberry leaf extract (condensed tannins) increased propionate levels, which may have contributed to the reduction of methane production (Cieslak et al., 2014). On the other hand, Lavrenčič and Pirman (2021) showed that low concentrations of chestnut wood extracts (15 g/kg soybean meal, hydrolysable tannins) increased total SCFA levels, without significant changes in individual SCFA, which could suggest a stimulating effect of tannins on microorganisms. On the other hand, an increased concentration of acetic acid was observed at higher tannin levels (30 and 60 g/kg soybean meal), which confirmed that tannins could also act in a dose-dependent manner.

Conclusions

The current study demonstrated that both tannin-rich supplements reduced the total number of protozoa and *Entodinium* spp. (dominant genus) in sheep. However, only the TAN diet significantly decreased amylolytic activity of ruminal digesta 2 h after feeding, without any significant effect on pH and SCFA concentrations. The differences observed in the response of enzymes to the presence of OAK and TAN could result from the chemistry of the tested additives, their different affinities for proteins of microbial origin and carbohydrates. It can be concluded that the low tannin content in the sheep diet (3.91 g/kg DM) did not interfere with carbohydrate fermentation in the rumen.

However, further studies are required, especially microbiological analyses, to identify all possible mechanisms of TAN and OAK action on carbohydrate-digesting enzymes.

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