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ORIGINAL PAPER

Effect of dietary plant tannin supplementation on rumen fermentation and enteric methane production

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ABSTRACT. Although protozoa play a crucial role in ruminal microbial networks, their contribution to rumen fermentation and methane emissions remains controversial. Tannins, polyphenolic compounds derived from various plants, can affect microbial activity, fermentation processes, protein degradation, and methane production in the rumen. This study investigated the impact of dietary supplementation with chestnut, quebracho, and seaweed tannins on rumen fermentation parameters, protozoan populations, and enteric methane production. Rumen fluid (RF) samples were collected from four cannulated Simmental cows assigned to four dietary treatments: a control diet (0TAN) and three tannin-enriched diets: 150 g/day (150TAN), 200 g/day (200TAN), and 250 g/day (250TAN) in a 4 × 4 Latin-square design. The basal diet contained 64.3% meadow hay, 13.1% grass silage, 9% maize silage, 11.5% cereal concentrate, and 0.7% mineral concentrate. Most rumen fermentation parameters, including pH, total volatile fatty acids, acetate, propionate, acetate:propionate ratio, and isovalerate, were significantly influenced ($P < 0.005$) by the tannin-enriched diet, except for ammonia and butyrate concentrations. Tannin supplementation at 200 g/day, the concentrations of acetate, isovalerate, and the acetate ratio significantly increased ($P < 0.001$), whereas propionate production decreased. Significant effects ($P < 0.001$) were also observed with respect to the total number of protozoa and the relative abundance of protozoan genera. These findings suggest that dietary tannins modulate protozoan composition and the rumen microbial community, demonstrating antimethanogenic potential. The results confirm that tannins suppress rumen methanogenesis by reducing the size of protozoan population and enteric methane production.

Introduction

Rumen microbiologists and nutritionists aim to optimize the rumen microbial environment to improve metabolic efficiency and animal production. Several studies have investigated the effect of various chemical and organic feed additives (e.g., essential oils, ionophores, defaunation agents, and methane inhibitors) on animal growth, feed utilisation, and enteric methane production (Patra and Yu, 2012; Kaya et al., 2022).

Plant bioactive compounds (e.g., tannins, saponins, and flavonoids) and plant extracts have gained significant attention due to their ability to improve rumen metabolism. Their beneficial effects include reduced methanogenesis and protein degradation

in the rumen, increased microbial protein synthesis and protein flow to the duodenum by targeting specific groups of rumen microbial populations (Kumar and Vaithiyanathan, 1990; Wallace, 2004; Mueller-Harvey, 2006; Kamra et al., 2008; Waghorn, 2008; Patra and Saxena, 2009a,b). Tannins, a class of polyphenolic polymers, are particularly effective due to their ability to form complexes, primarily with proteins thanks to the presence of a large number of phenolic hydroxyl groups. By forming tanninprotein complexes, tannins prevent protein degradation in the rumen. These compounds may also inhibit the growth of proteolytic bacteria. Depending on the type of tannins, diets with moderate tannin levels can increase body weight, wool development, milk production, and reproductive efficiency, although these effects have not been consistently observed (Patra and Yu, 2012). The objective of the present study was to evaluate the effect of dietary supplementation with plant and seaweed tannins on rumen fermentation parameters and enteric methane production. Numerous studies have reported a positive association between enteric CH_4 emissions and the role of rumen protozoa in methanogenesis (Dai et al., 2022); however, the relationship between enteric methane emissions and the abundance of ciliate protozoa remains poorly understood. Furthermore, limited information is available on how factors such as diet composition, ruminal fermentation, and nutrient digestibility influence protozoan populations. Therefore, the current study focused on the effects of tannin supplementation on the distribution of rumen protozoan populations and the effect of tannin supplementation on the abundance of potentially methanogenic protozoan genera.

Material and methods

Experimental design and animal diet

The experiment was conducted to determine the effect of chestnut (*Castanea sativa*), quebracho (*Schinopsis balansae*), and seaweed (*Ascophyllum nodosum*) tannins on ruminal fermentation. Four Simmental cows, surgically fitted with ruminal cannulas, were used as experimental animals in a 4 × 4 Latine Square design. The cows were housed in the experimental facility at the Research Institution Agrovyzkum Rapotin (Czech Republic) in individual pens (6.7 m^2) and fed only their individual rations. The animals had *ad libitum* access to water, and the experimental diets met their energy and nutrient requirements according to NRC (2016) recommendations. The animal procedures were reviewed and approved by the Ministry of Agriculture (14608/2021-MZE-18134) of the Czech Republic. Each experimental period lasted 24 days and included 10 days of adaptation to the diets and 14 days of measurements. All animals were individually fed twice daily, at 07:00 and 14:00, with approximately 60% and 40% of the total daily ration, respectively. The basal diets, provided as a total mixed ration, consisted of meadow hay, grass silage, maize silage, and concentrates (Tables 1 and 2).

Table 1. Ingredient composition of the experimental diets, % of DM

Ingredients	Diets			
	0TAN	150TAN	200TAN	250TAN
Meadow hay	65.7	64.6	64.2	63.5
Grass chop	13.1	13.1	13.1	13.1
Maize chop	9.0	9.0	9.0	9.0
Mineral concentrate[1]	0.7	0.7	0.7	0.7
Granulated feed mixture[2]	11.5	11.5	11.5	11.5
Supplement MIXTANE[3]	0	1.1	1.5	2.2
Total	100	100	100	100

0TAN – control diet without tannin and seaweed enriched supplement, 150TAN – diet with 150 g of MIXTANE, 200TAN – diet with 200 g of MIXTANE, 250TAN – diet with 250 g of MIXTANE; [1] provided per kg of product: IU: vit. A 130 000, vit. D_3 150 000; mg: vit. E (alpha-tocopherol acetate) 1500; g: Cu (from copper sulphate) 0.65, Zn (from zinc oxide) 1.6, Mn (from manganate oxide) 1.5, Co (from cobalt sulphate) 0.88, I (from potassium iodide) 0.041, Se (from sodium selenite) 0.018; [2] BIOSTAN (Biokron, s.r.o., Czech Republic), contained per kg of product: %: barley 15, oat mill feed 65, wheat 15, malt flower 10, sunflower expellers 5, extracted soybean meal 3; [3] ingredients used as carriers (calcium carbonate, wheat middling, seaweed extracts): mg: vit. B_1 (thiamine) 1000 ; active ingredients: %: chestnut (*Castanea sativa*) tannin extract 30, seaweed (*Ascophyllum nodosum*) tannin extract 45, and quebracho tannin extract 2; DM – dry matter

Table 2. Chemical characteristics of the main feed ingredients, % of DM

Nutrient composition	Ingredients					
	meadow hay	maize chop	grass chop	mineral concentrate	GFM	MIXTANE[1]
DM, %	89.98	91.58	89.83	99.79	88	90.21
Crude protein, %	4.15	6.93	16.34	0.01	11.41	14.82
Starch, %	0	38.07	1.07	0	38.73	29.4
Ash, %	4.06	3.01	8.45	98.92	8.47	10.44
Crude fibre, %	30.96	40.43	21.51	2.74	5.62	4.08
Fat, %	1.55	2.74	4.5	0.09	2.68	3.91
NDF, %	56.47	31.3	42.59	0.22	19.47	16.67
ADF, %	0	38.07	1.07	0	38.73	29.4

GFM – granulated feed mixture, DM – dry matter, NDF – neutral detergent fibre, ADF – acid detergent fibre; [1] ingredients used as carriers (calcium carbonate, wheat middling, seaweed extracts): mg: vit. B_1 (thiamine) 1000; active ingredients: %: chestnut (*Castanea sativa*) tannin extract 30, seaweed (*Ascophyllum nodosum*) tannin extract 45, and quebracho tannin extract 25

Dietary treatments contained increasing levels of tannins extract (0, 150, 200, 250 g/cow/day), prepared using the MIXTANE product (MG2MIX, Château Bourg, France), which contained tannin extracts from quebracho (*Schinopsis balansae*), chestnut (*Castanea sativa*) and seaweed (*Ascophyllum nodosum*). The diets were fed as total mixed rations, with the tannin-rich supplement manually mixed before each feeding.

Sampling, data collection, and chemical analyses

Chopped meadow hay, maize, grass silages, and concentrates were sampled weekly to determine dry matter DM and nutrient composition. The chemical composition of dietary ingredients (Table 2) was analysed for DM, crude protein, crude fat, crude fibre, and ash contents according to the EC Commission Regulation No 152/2009 (2009). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents were determined sequentially using an ANKOM A200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to the methodology supplied by the company, based on the methods described by Van Soest et al. (1991). Starch content was determined using the Ewers polarimetric method, which involved partial acid hydrolysis of starch, followed by measurement of the optical rotation of the resulting solution according to STN EN ISO 10520 (1997). Rumen fluid (RF) samples from each cow were collected 2 h after the morning feeding once a week throughout the experiment. Samples were collected via a rumen cannula with a probe connected to a vacuum pump and transported to the laboratory in a pre-warmed thermos flask filled with CO_2 for further analysis. Tests of RF included measurements of pH, physical characteristics, concentrations of nitrogenous compounds (NC) and ammonia (NH_3-N), determination of the total number of protozoal ciliates (TPC), composition of ciliate protozoan genera, molar proportions of volatile fatty acids (VFA), and total VFA.

The pH was measured directly after sample collection using a portable pH meter (EUTECH CyberScan PC510 pH/Conductivity Bench Meter, Columbus, OH, USA). Aliquots of 20 ml of ruminal fluid were stored at −20 °C for subsequent analysis of VFA, NC, and NH_3-N. Samples for the analysis of rumen protozoa were preserved in 1 ml of 10% formaldehyde solution and stained overnight with Brilliant Green Dye. The total numbers of rumen protozoa per ml was counted using a Bürker chamber and a fluorescent optical microscope (INTRACO FL200, INTRACO MICRO, s.r.o., Tachlovice, Czech Republic) at 40× magnification according to the procedure described by Dehority (2004). Protozoan genera present in each sample were identified according to the phenotypical criteria described by Ogimoto and Imai (1981), Baraka (2012), and Dehority (2018). VFA were determined by gas chromatography (Agilent 6820 Gas Chromatograph System; Agilent Technologies, Santa Clara, CA, USA) using the method described by Filípek and Dvořák (2009). Nitrogenous compounds and ammonia were determined using The Kjeldahl method, as described by Chen et al. (1987).

Rumen fluid for CH_4 and CO_2 production measurements was collected at the end of the experiment, two hours after the morning feeding from each cow. RF was immediately strained through two layers of cheesecloth to separate the liquid RF from the residual solids. Subsequently, 500 ml of RF was transported to the laboratory in sealed thermoses. Samples of total mixed ration (TMR) feed were collected on the same day as rumen samples, freeze-dried and sieved to obtained 1 mm feed particles to serve as the substrate in subsequent *in vitro* fermentation. Kansas State Buffer (pH 6.8) was used at a 1:3 ratio (Tunkala et al., 2022). RF (10 ml) from each sample was subsequently added to the substrate-buffer mixture. The bottles were capped with pressure sensor modules of the ANKOM system, flushed with carbon dioxide, placed in a water bath (20-L Analogue Water bath, WB20; Ratek Instruments Pty Ltd., Boronia, Australia), and pre-warmed to 39 °C. A blank bottle with buffer and RF was also included as a control. All *in vitro* incubation procedures were conducted following the manufacturer's manual (ANKOM, 2018).

After 48 h of incubation, 10 ml of headspace gas from each bottle was collected by displacement into a tube prefilled with 25% sodium chloride solution for gas analysis.

CH_4 and CO_2 concentrations were determined by injecting 100 µl of the collected gas into a gas chromatograph (Agilent 6820 Gas Chromatograph System; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionisation detector (FID) and stainless steel column.

Correlation analysis

Correlation analysis was performed using STATISTICA 14 (StatSoft CR s.r.o., Prague, Czech Republic), and the Heatmap was generated using

Microsoft Excel. Pearson's correlation coefficient was employed to assess the strength of associations between variables (ruminal fermentation parameters and protozoan community members at the genus level). The correlation coefficient (r) ranged from −1 to 1, with positive correlation indicated by $r > 0$ and negative correlation by $r < 0$.

Statistical analysis

Statistical analysis was performed using Statistica 14.0.0. (TIBCO, Palo Alto, CA, USA). Data were analysed using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Normality of the distribution of variables was tested using the Shapiro-Wilk test, and all variables were found to be normally distributed. Diets were treated as independent variables, with diet effects considered as response variables. The statistical model for the analysis of variance in a 4 × 4 Latine Square design was as follows:

$$Yijk = \mu + Hi + Cj + T(k) + Eijk,$$

where: Yijk – response variable in row i, column j, treatment k; μ – general mean; Hi – row effect; Cj – column effect; and Tk – treatment effect; Eijk – random error.

Variability in the data was expressed as the standard error of the mean (SEM), and a probability level of $P < 0.05$ was considered statistically significant. Pearson's correlation coefficients were calculated using Statistica 14.0.0 to examine correlations between the relative abundance of protozoan genera (PG) and rumen fermentation parameters (RFP). A heat map was generated using Pearson's correlation values in MS Excel to visualize the relationship between PG and RFP. The correlation was considered significant at $P < 0.05$.

Results

Effect on rumen fermentation variables

The results of the RFP are presented in Table 3, showing significant differences between the treatment groups ($P < 0.05$). Tannin supplementation had a significant effect on the molar proportions of total VFA ($P = 0.002$), acetate ($P = 0.001$), propionate ($P = 0.009$), isovalerate ($P = 0.001$), and the acetatetopropionate ratio ($P = 0.001$). Total VFA increased at a dose of 150 g compared to the diet containing 200 g of tannins. Acetate concentration was significantly higher with the 200 g dose compared to all other groups (0TAN, 150TAN, 250TAN).

Table 3. Fermentation parameters in the rumen of TMR-fed cows with different tannin supplementation levels

Item	Diets				SEM	*P*-value
	0TAN	150TAN	200TAN	250TAN		
pH	6.47	6.5	6.56	6.46	0.06	0.064
Nitrogenous compounds, g/kg	2.84^{a}	3.81^{b}	2.02^{a}	2.66^{a}	0.22	0.001
NH_3-N, mg/dl	3.88	3.78	4.17	3.64	0.44	0.33
VFA_{tot}, mmol/l	104.3	113.47^{a}	97.9^{b}	106	2.57	0.002
Acetate, %	69.27^{a}	70^{a}	71^{b}	69.27^{a}	0.99	0.001
Propionate, %	16.43^{a}	15.95	15.24^{b}	16.31^{ac}	0.81	0.009
Butyrate, %	12.88	12.68	12.59	13.27	0.52	0.535
Isovalerate, %	1.25^{a}	1.23^{a}	1.49^{b}	1.16^{a}	0.19	0.001
Acetate:propionate	4.26^{a}	4.36^{a}	4.68^{b}	4.25^{a}	0.28	0.001

TAN – tannins, N-NH_3 – ammonia nitrogen, VFA_{tot} – total volatile fatty acids, SEM – standard error of the mean; 0TAN – control diet without tannin and seaweed enriched supplement, 150TAN – diet with 150 g of MIXTANE, 200TAN – diet with 200 g of MIXTANE, 250TAN – diet with 250 g of MIXTANE; ^{abc} – means with different superscripts in the same row are significantly different at $P < 0.05$

Propionate levels decreased in the rumen fluid obtained from animals supplemented with 200 g and 250 g of tannins compared to the control. The concentration of isovalerate and the acetate/propionate ratio were significantly higher in animals supplemented with 200 g of tannins compared to all other groups (0TAN, 150TAN, 250TAN). The pH value was not affected by dietary tannin administration ($P > 0.05$). The amount of nitrogen compounds (NC) was significantly higher ($P < 0.001$) in the animals supplemented with 150TAN compared to all other diets. Regarding total VFA concentrations, tannin supplementation at a dose of 150 g (150TAN) resulted in higher VFA levels compared to animals fed 200 g of tannins per day (200TAN). However, none of the treatments exerted a significant impact on NH_3N concentration. In contrast, acetate, propionate, and isovalerate concentrations, as well as the acetate:propionate ratio, were significantly affected by the dietary treatments.

Effect on rumen protozoan communities and enteric methane production

The impact of tannin supplementation on the protozoan community in the rumen is summarised in Table 4. The 200TAN diet significantly reduced the total protozoan count ($P = 0.001$), as well as the abundance of individual protozoan genera and main protozoan groups (Holotrichs and Entodiniomorphids) in the rumen ($P < 0.05$). The abundance of Holotrichs significantly increased ($P < 0.001$) in animals supplemented with tannins at all doses

Table 4. Effect of tannin supplementation on the relative abundance of ciliate protozoa in the rumen, %

Item	Diets				SEM	*P*-value
	0TAN	150TAN	200TAN	250TAN		
Total rumen protozoa, 10^4 cells/ml	29.93^{a}	26.88	22.9^{b}	29^{a}	4.83	0.01
HP	14.33^{a}	30.65^{b}	26.12^{c}	28.99^{d}	1.8	$P < 0.01$
EP	8.1^{a}	3.13^{b}	1.84^{c}	3.25^{bd}	0.46	$P < 0.01$
Isotricha	5.14^{a}	2.47^{b}	2.66^{bc}	5.37^{a}	0.09	$P < 0.01$
Dasytricha	3.45^{a}	24^{bd}	15.93^{c}	17.7^{bcd}	0.7	$P < 0.01$
Charonina	3.47^{a}	7.28^{b}	5.91^{c}	2.11^{d}	0.07	$P < 0.01$
Buetschlia	1.74^{a}	0.92^{b}	1.97^{a}	3.37^{c}	0.08	$P < 0.01$
Entodinium	66.18^{a}	65.26^{a}	66.8^{a}	59.9^{b}	3.14	$P < 0.01$
Diplodinium	7.77^{a}	0.9^{b}	3.65^{c}	4.77^{d}	0.03	$P < 0.01$
Epidinium	3.82^{a}	0.85^{b}	3.63^{a}	5.08^{c}	0.14	$P < 0.01$
Ophryoscolex	n/d	0.8^{a}	0.89^{a}	0.46^{b}	0.02	$P < 0.01$
Ostracodinium	8.46^{a}	2.19^{b}	0.94^{c}	2.55^{d}	0.01	$P < 0.01$
Polyplastron	n/d	n/d	n/d	0.43	0.01	$P < 0.01$

TAN – tannins, SEM – standard error of the mean, TRP – total rumen protozoa; abundance of major groups – Entodiniomorphid protozoa (EP) and Holotrich protozoa (HP), and at the genera level; 0TAN – control diet without tannin and seaweed enriched supplement, 150TAN – diet with 150 g of MIXTANE, 200TAN – diet with 200 g of MIXTANE, 250TAN – diet with 250 g of MIXTANE; $^{a–d}$ means with different superscripts in the same row are significantly different $P < 0.05$

tested (150, 200, and 250 g/day/animal) compared to the control group. On the contrary, the number of Entodiniomorphids was significantly lower ($P < 0.001$) in animals supplemented with tannins compared to the control.

The most abundant protozoan genera identified in this study are shown in Figure 1, while Figure 2 illustrates their relative abundance across the dietary treatments (0TAN, 150TAN, 200TAN, 250TAN). A decrease in methane production was observed in the rumen of animals supplemented with tannins in comparison to the control group (Figure 3).

Correlation between ruminal fermentation parameters and protozoan abundance

Pearson's correlation analysis revealed significant associations between most protozoan genera (Figure 4). For ciliate protozoa, positive correlations were observed between the abundance of *Isotricha* spp. and *Diplodinium* spp. ($r = 0.81$; $P = 0.02$), *Isotricha* spp. and *Epidinium* spp. ($r = 0.82$; $P = 0.02$), *Isotricha* spp. and *Buetschlia* spp. ($r = 0.74$; $P = 0.09$), *Dasytricha* spp. and *Ophryoscolex* spp. ($r = 0.83$; $P = 0.001$), *Charonina* spp. with *Ophryoscolex* spp. ($r = 0.67$; $P = 0.02$) and *Entodinium* spp. ($r = 0.61$; $P = 0.04$), *Buetschlia* and *Diplodinium* ($r = 0.87$, $P < 0.01$), as well as between the genera *Diplodinium* and *Epidinium* and *Ostracodinium* ($r = 0.71$, $P = 0.01$ and 0.80, $P = 0.003$). Conversely, strong negative correlations were recorded between *Ophryoscolex*, *Ostracodinium and Diplodinium* ($r = -0.89$ and $r = -0.93$; $P < 0.001$), *Dasytricha* spp., *Diplodinium* spp. and *Charonina* spp. ($r = -0.91$ and $r = -0,95$; $P < 0.001$), and *Isotricha* spp. and *Charonina* spp. ($r = -0.95$; $P < 0.001$).

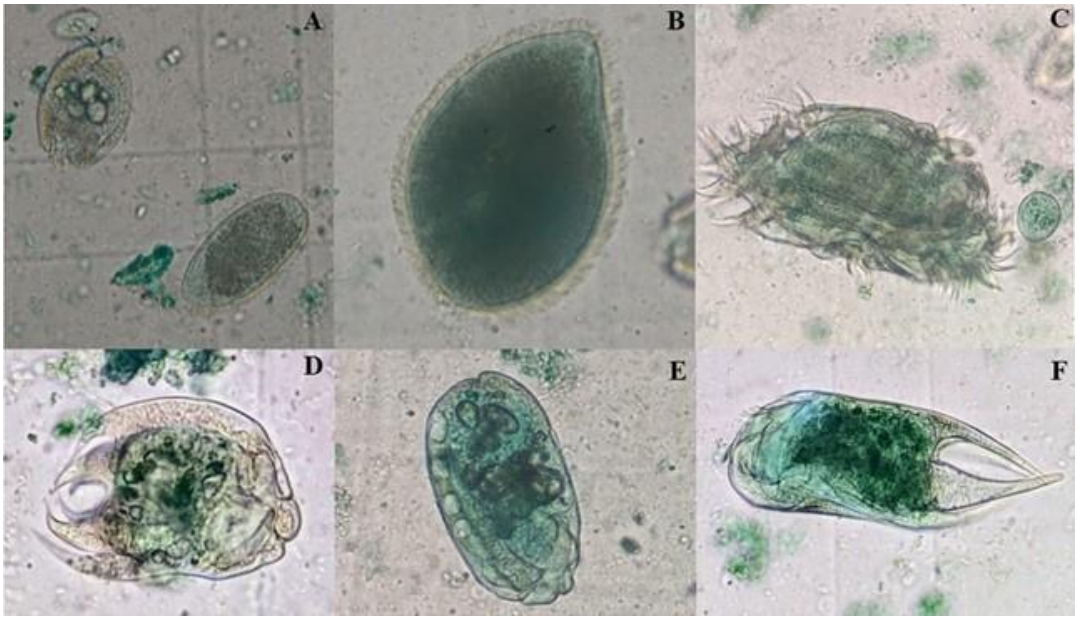

Figure 1. Photomicrographs of protozoan genera (40× magnification). (A) *Entodinium* spp. (left corner), *Dasytricha* spp. (right corner), (B) *Isotricha* spp., (C) *Ophryoscolex* spp., (D) *Diplodinium* spp. (*Diplodinium dentatum*), (E) *Ostracodinium* spp., (F) *Epidinium* spp. Author: MSc. Svetlana Malyugina. For better visualisation, rumen fluid samples were stained with a Brilliant Green Dye solution

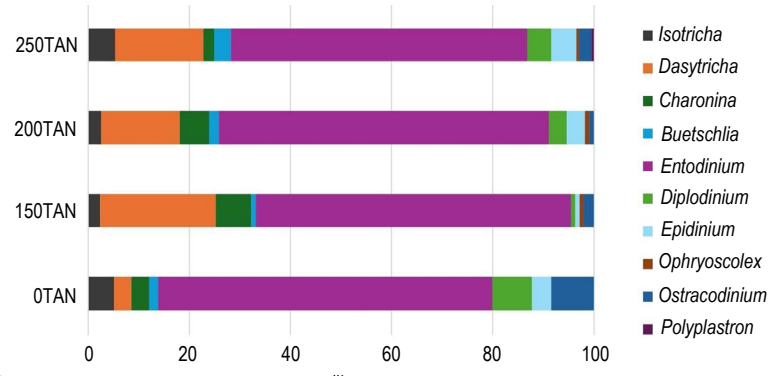

Figure 2. Relative abundance of observed protozoan genera in the rumen of cows fed with 0TAN, 150TAN, 200TAN, and 250TAN diets

0TAN – control diet without tannin and seaweed enriched supplement, 150TAN – diet with 150 g of MIXTANE, 200TAN – diet with 200 g of MIXTANE, 250TAN – diet with 250 g of MIXTANE

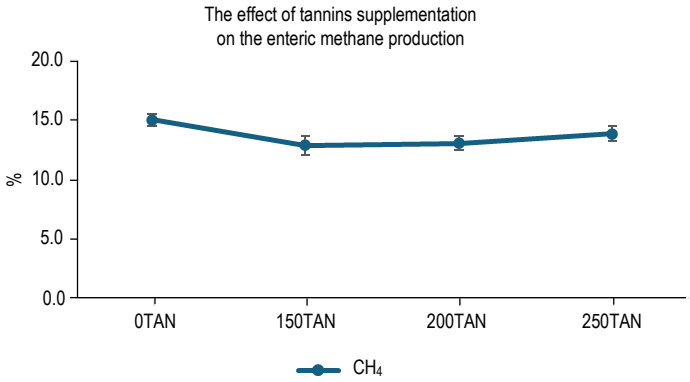

Figure 3. Effect of tannin supplementation on methane production in the rumen

CH_4 – methane; 0TAN – control diet without tannin and seaweed enriched supplement, 150TAN – diet with 150 g of MIXTANE, 200TAN – diet with 200 g of MIXTANE, 250TAN – diet with 250 g of MIXTANE

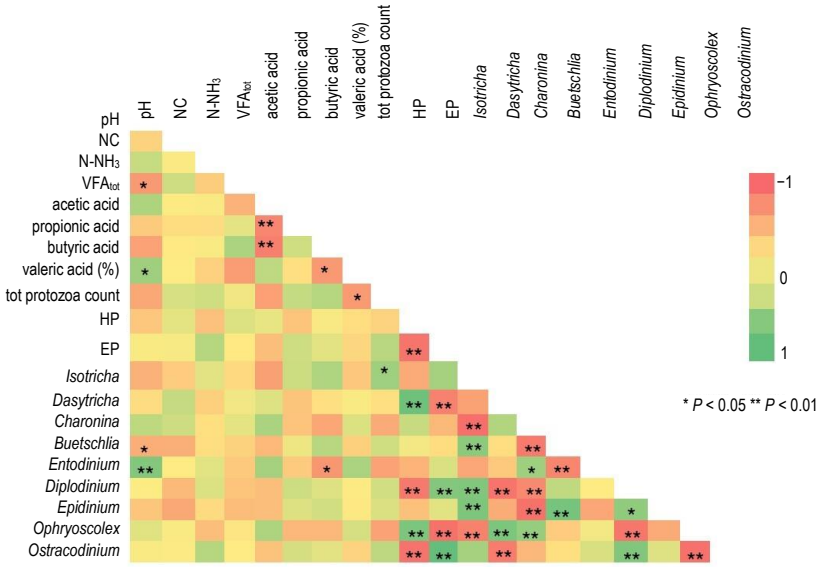

Figure 4. Heatmap based on Pearson's correlation coefficients between the relative abundance of rumen protozoan genera and rumen fermentation parameters. Asterisks (* and **) indicate a significant correlation at $P < 0.05$ and 0.01. Red and green titles indicate positive and negative correlations, respectively

NC – nitrogen compounds, $N\text{-}NH_3$ – ammonia nitrogen, VFA_{tot} – total volatile fatty acids, HP – Holotrich protozoa, EP – Entodiniomorphid protozoa

Discussion

Previous *in vivo* and *in vitro* studies investigating the impact of various plant tannins on rumen fermentation and gas production have not always been consistent. An *in vitro* study conducted by Makkar et al. (1997) demonstrated a decrease in VFA production when using 0.8 mg/ml medium prepared from leaves of tannin-rich plant (*Ichostachys cinerea, Cassia sieberiana*, *Robinia pseudoacacia*, and *Acioa barteri*). In the latter study, condensed tannins demonstrated a greater suppressive effect on VFA production compared to hydrolysed tannins. In another *in vitro* study (Reyes, 2019), saponins and tannin treatments decreased molar proportions of acetic acid 12 h postincubation, compared to the control. However, 18 h after fermentation, total VFA levels, and molar proportions of butyric and valeric acids were higher than those in the control group. An *in vivo* experiment conducted on sheep fed a basal hay diet supplemented with two different doses of *Elaeis guineense* (0.5 and 0.9 g/kg DM) demonstrated an increase in total VFA concentration at the 0.5 g/kg DM supplementation level compared to the control, along with an increase in acetate concentration. However, butyrate concentration increased only at the higher dose of 0.9 g/kg DM (Osakwe et al., 2004). Similarly, Besharati and Taghizadeh (2009) reported that the presence of tannins in the plant material reduced fermentation, resulting in significant differences in VFA and NH_3N concentrations. In contrast, Sadarman et al. (2019) found that adding tannins from acacia and chestnuts had no significant influence on DM degradation, total VFA, NH_3N, or pH. Additionally, in beef cattle fed a forage-based diet, Beauchemin et al. (2007) found that supplementation with quebracho tannin (10 or 20 g/kg DM) decreased the molar proportion of acetate, acetate:propionate ratio, and ruminal ammonia compared to control cattle not receiving tannins.

VFA production is the main source of energy for ruminants, and any reduction could have adverse nutritional consequences (Benchaar et al., 2007). VFA, formed during fermentation of organic matter in the rumen, can significantly affect animal production performance and product composition in ruminants (Dijkstra, 1994; Seymour et al., 2005).

In the current study, the concentrations of supplemented tannins were 12.5, 16.6, and 20.8 g/kg DM, which was very similar to the levels used by Beauchemin et al. (2007). However, our results showed a significant increase ($P < 0.05$) in acetate, acetate:propionate ratio, and isovalerate concentrations following tannin administration at 16.6 g/kg DM (200TAN), accompanied by a concurrent decrease in propionate and total VFA levels. Bhatta et al. (2009) also reported in their *in vitro* study that tannins increased pH and reduced total VFA concentrations and protozoan counts, but increased propionate production. The *in vivo* results of the present work are partially consistent with the latter study, but here, as opposed to the *in vitro* tests, tannins caused a decrease in propionate molar proportions. According to Moss et al. (2000), propionate formation can be considered a competitive pathway for CH_4 production. Szumacher-Strabel and Cieślak (2012) noted that the suppression of methanogenesis could be due to the transformation of readily digestible carbohydrates, such as starch, to propionic acid. This process may affect hydrogen transfer, potentially limiting the rate of methanogenesis. The hypothesis regarding the competition between propionate production and methanogenesis in the rumen may explain our results, showing a dose-dependent increase in methane in the ruminal fluid of animals supplemented with tannins (Figure 3).

In the present study, tannin supplementation did not significantly affect NH_3-N concentrations ($P = 0.33$), which could be attributed to factors such as variations in tannin sources, or differences in rumen fluid sampling procedures and protocols.

However, dietary tannins at a dosage of 200 g (16.6 g/kg DM) significantly reduced total VFA concentrations compared to animals fed a lower dosage (12.5 g/kg DM). In addition, tannin supplementation at the dosage of 200 g significantly increased the concentration of acetate, isovalerate, and the acetate:propionate ratio in the rumen compared to all treatments (0TAN, 150, and 250 TAN).

Beauchemin et al. (2007) evaluated the inclusion of condensed quebracho tannin extract in the basal diet of Angus cattle and observed no effects on body weight, nutrient intake or methane emissions. In another study, when sheep were fed a diet containing a mixture of fresh-cut *Lotus corniculates* and ryegrass/clover as a source of condensed tannins, the authors observed no effect on VFA concentrations or ammonia (Waghorn and Shelton, 1997). In contrast, the current study demonstrated that dietary inclusion of tannins derived from quebracho, chestnut, and seaweed significantly affected rumen fermentation parameters and reduced methane emissions. This discrepancy could be attributed to different dosages, chemical

composition, and source of tannins used in individual studies. Additionally, relatively short experimental periods may also be responsible for the lack of significant effects on rumen fermentation variables. Numerous studies have demonstrated that tannins reduce methane production in ruminants (Min el., 2006; Abdalla et al., 2007). Changes in CH_4 generation are typically associated with alterations in the VFA profile due to the involvement of alternative electron sinks that help dispose of reducing power (Ungerfeld et al., 2003). In the current study, tannin supplementation at doses of 150 g, 200 g, and 250 g reduced methane production (Figure 3), but the decreases were relatively modest, at 2.18% (150TAN), 1.98% (200TAN), and 1.19% (250TAN) in comparison to the control.

The percentage of enteric methane in the rumen in cows supplemented with the highest dose of tannins (250TAN) tended to increase compared to the other experimental diets (150TAN, 200TAN), which may be explained by the adaptation of the rumen microbial ecosystem to the presence of tannins in the diet. Ruminal microorganisms may have certain adaptive mechanism that allow them to degrade tannins more efficiently over time (Brooker et al., 1999; Odenyo et al., 1999; Pell et al., 1999). In nature, ruminants typically consume tannin-rich feeds, and as a result, they appear to develop defensive mechanisms against these substances (Makkar, 1997).

Microorganisms resistant to high levels of condensed tannins have been reported in ruminants consuming tannin-rich feeds (Brooker et al., 1999).

In this study, methane production was reduced by 2.3% by the 150TAN diet, by 2.21% with the 200TAN diet, and only by 1.16% with the 250TAN diet compared to the control. Interestingly, the lowest tannin dose had the greatest impact on enteric methane production. However, the short experimental period should be considered when interpreting these findings, as the effect on methane emission could be different with a longer trial period due to a possible adaptation of rumen microorganisms to a tannin-rich diet. Our results are consistent with those of Tan et al. (2011), who observed a similar decrease in CH_4 production using a comparable tannin supplementation.

Animut et al. (2008) observed a linear decrease in CH_4 emissions in goats fed increasing levels of *Kobe lespedeza*, which were correlated with tannin levels in the diet. Similarly, Abdalla et al. (2007) reported a reduction in enteric methane when using *Mimosa caesalpiniifolia* as a tannin source. However, in the study of Beauchemin et al. (2007), tannin addition did not affect CH_4 production. These discrepancies can possibly be attributed to different types of tannins and their origin, as well as the length of the experiment and tannin feeding.

Tannins are generally recognized as inhibitors of microorganism development (McSweeney et al., 2001). Tavendale et al. (2005) proposed two main mechanisms through which tannins exert their antimethanogenic effects: i) direct inhibition of rumen microorganism development; ii) and interference with fibre digestion, leading to a decrease in hydrogen production, which is the main substrate for methanogens. In addition, Hess et al. (2003) suggested that the inhibitory effect of tannins on rumen methanogenesis might be linked to the suppression of protozoa-associated CH_4 production.

In the present study, dietary tannins significantly impacted the total protozoan counts ($P = 0.001$). Additionally, the results showed a concurrent reduction in CH_4 production and the overall number of protozoa in the rumen after tannin administration. This is consistent with previous research indicating that the absence of protozoa in defaunated animals was shown to decrease enteric CH_4 production.

The relationship between lower enteric CH_4 production and the absence of protozoa in defaunated animals remains unclear, with several conflicting explanations proposed in the literature. Morgavi et al. (2012) hypothesised that defaunation leads to a decrease in the number of methanogenic bacteria, which are considered the sole producers of methane in the rumen. Alternatively, some researchers proposed that defaunation leads to the elimination of protozoan-associated methanogens, such as *Methanobrevibacter* sp. and *Methanomicrobium* sp., thereby lowering enteric methane production (Finlay et al., 1994: Zhou et al., 2009). These hypotheses require a more detailed investigation of the microorganisms involved in methane production in the rumen as well as their interactions with protozoa. Several studies have reported an inhibitory effect of tannins on the protozoan population in the rumen (Salami et al., 2018; Lima et al., 2019; Sarnataro et al., 2020). Consistent with these findings, the present study also observed a reduction in the total protozoan population. Patra and Yu (2012) attributed the inhibitory effects of tannins to both direct actions on protozoa and methanogenic bacteria, as well as indirect effects that reduce fibre digestibility in the rumen. Reduced fibre degradation leads to a decrease in hydrogen availability, which consequently decreases methane production in the

rumen (Piluzza et al., 2014). A depressive effect of tannin supplementation on rumen protozoa was also observed in the present study. This contrasts with the findings of Vasta et al. (2010), who observed an increase in the abundance of rumen protozoa in lambs supplemented with quebracho tannins at 9.57% DM (Vasta et al., 2010). On the other hand, Benchaar et al. (2008) did not observe changes in the total number of rumen protozoa when supplementing the cow's diet with quebracho tannins at a dose of 150 g/day/cow. However, as suggested by Morgavi et al. (2010), the relationship between protozoa and methane production is highly complex and cannot be reduced to a simple cause-and-effect relationship.

Dietary inclusion of tannins caused a significant effect on all protozoa at the genus level, as well as on the protozoan groups Holotrichs, and Entodiniomorphids (Table 4). The abundance of Holotrich protozoa (HP) increased ($P < 0.001$), whereas the number of Entodiniomorphid protozoa (CEP) decreased ($P < 0.001$) in animals supplemented with tannins compared to the control. Belanche et al. (2014) reported that *HP* inoculation increased both the counts of methanogens (+0.41 $\log_{10}$) and rate of methanogenesis (+54%). Currently, little information exists on the effects of HP and EP on CH_4 emissions under varying experimental conditions. There is also a significant lack of knowledge regarding how the relationship between CH_4 emissions and the rumen protozoan community (including total rumen protozoa (TRP), EP, HP, and individual protozoan genera) can be quantitatively affected by other factors such as dietary components, ruminal fermentation, and nutrient digestibility. It has been suggested that HP may have a more significant effect on ruminal methanogenesis than EP due to HP's association with different endosymbiotic methanogens (Belanche et al., 2014). The primary mechanisms through which protozoa could enhance methanogenesis is based on their ability to produce H_2 in hydrogenosomes (a mitochondria-like organelle) and to host epi- and endosymbiotic methanogens, thereby protecting them from oxygen toxicity (Fenchel and Finlay, 2006). Hydrogenosomes have been identified in species of the genera *Epidinium*, *Isotricha,* and *Dasytricha*, but not in *Entodinium caudatum* and *Diploplastron affine* (Ellis et al., 1994). In our study, *w*e hypothesised that a decrease in HP would be concomitant with a reduction in enteric CH_4; however, our results did not confirm this hypothesis. On the contrary, they showed increased CH_4 production (Figure 1) and decreased abundance of HP following tannin supplementation. Newbold et al. (2015) have suggested that HP plays a disproportionate large role in supporting methanogenesis, which may explain the unexpected increase in CH_4 production observed in our study despite lower abundance of HP.

Regardless of dietary treatment, the genus *Entodinium* was the predominant group of protozoa in the current study (approximately 60–66%), which is consistent with other studies on cattle and sheep (Kittelmann et al., 2013, 2015). Less abundant were ciliates from the genera *Isotricha*, *Dasytricha*, *Charonina*, *Buetschlia*, *Diplodinium*, *Epidinium*, *Ophryoscolex*, *Ostracodinium*, and *Polyplastron*. In addition, the genus *Polyplastron* spp. was detected only in cows supplemented fed the 250TAN diet, while *Ophryoscolex* spp. were entirely absent in the control group (0TAN) and occurred at very low percentages in the other dietary groups. Protozoa of the genus *Ophryoscolex* are typically involved in the digestion of structural carbohydrates, including cellulose and hemicellulose. Fibre content is an important factor in shaping the protozoan community structure in the rumen, with some genera, such as *Ophryoscolex* and *Polyplastron*, being more likely present in high-grain conditions (Majewska et al., 2023). The composition of the basal diet in the present study may not have provided an adequate amount of substrates for these species. Tannins present in the feed could form complexes with cellulose and hemicellulose, which, in consequence, potentially reduced the availability of substrates required for the growth and development of these protozoa (Majewska et al., 2023). *Cosmopolitan* species such as *Entodinium*, *Isotricha*, and *Dasytricha* are commonly present in cattle fed hay and silage/grain diets (Tymensen et al., 2012), which aligns with the findings of this study where these protozoan genera were most abundant. The genus *Isotricha* includes intracellular methanogens, and thus has been associated with methanogenesis in the rumen (Irbis and Ushida, 2004). However, dietary tannins at dosages of 150 g and 200 g significantly reduced ($P < 0.001$) the abundance of this genus compared to the control group (Table 4), while simultaneously increasing the population of *Dasytricha* spp. in the rumen at 150 g, 200 g, and 250 g *(*$P < 0.05$). Both genera taxonomically belong to the group of HP, known for its role in facilitating methanogenesis in the rumen.

However, this contrasting result may be offset, e.g., by an increase in the abundance of methanogenic bacteria or other alterations in the rumen microbial community, which were not assessed in this study. The genus *Ostracodinium* was less

abundant in cows fed tannin-enriched diets (TAN150, TAN200, TAN250) compared to the control (0TAN), while the genus *Polyplastron* was observed only in cows offered TAN250. The counts of *Charonina spp.* were significantly higher in the animals fed the 150TAN and 200TAN diets, whereas the abundance of *Buetschlia* spp. increased progressively with incrementing dietary tannin doses. These genera are typically present in relatively low numbers, and the relationship between their occurrence and tannin supplementation or other dietary modifications remains unexplored.

In summary, changes in protozoan communities should also be considered in relation to bacterial populations, as protozoa feed on bacteria (Williams et al., 1992). Understanding the impact of tannins on bacteria would undoubtedly facilitate the interpretation of the current results. Therefore, further studies should include a more comprehensive analysis regarding the effects of tannins on the rumen microbial population.

Conclusions

In summary, tannin supplementation appears to be a promising strategy for mitigating CH_4 emissions from cattle. Our results demonstrated a suppressive effect of dietary tannins on rumen methanogenesis by reducing the abundance of total protozoa and enteric methane production. However, based on these results, it is not possible to unequivocally recommend tannin dosage that would simultaneously reduce methane emission, as well as improve animal productivity and rumen fermentation. Therefore, further *in vivo* research involving larger animal groups, preferably dairy cows, is needed to assess the economic implications of incorporating tannins in diets on milk production and methane emissions.

In addition, further research is needed to explore the complex rumen-microbial adaptations resulting from changes in protozoan populations and their impact on methane generation. Efforts should also focus on strengthening the understanding of dietmicrobe interactions to better modulate the rumen microbiome, improving feed digestibility, reducing energy loss through gas emissions, and simultaneously mitigating methane emissions by livestock.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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