PAPER

Effects of two sources of tannins (*Quercus* L. and *Vaccinium vitis idaea* L.) on rumen microbial fermentation: an *in vitro* study

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Abstract

The aim of the experiment was to determine the effect of different sources of tannins on the *in vitro* rumen fermentation with focus on methane production. In the experiment, a rumen simulation system (RUSITEC) equipped with 4 fermenters (1 L) was used in three replicated runs (6 d of adaptation and 4 d of sampling) to study the effects of *Quercus cortex* extract (QC), *Vaccinium vitis idaea* (VVI) dried leaf extract and a mixture of VVI/QC on rumen microbial fermentation. Fermenters were fed 10.9 g/d of dry matter (DM) of a 600:400 forage:concentrate diet. Treatments were control, QC (2.725 mL), VVI leaves 0.080 g) and mixture of QC/VVI (1.362 mL+0.040 g) and were randomly assigned to fermenters within periods. The equivalent of 2.5 g of tannins/kg dietary DM from three sources of tannins was evaluated. All tannin sources decreased CH_4 and ammonia concentrations, as well as protozoa and methanogen counts (P<0.001). *Vaccinium vitis idaea* and QC/VVI tended (P=0.005) to reduce the acetate to propionate ratio. There were no changes in nutrient digestion. Results suggest that these sources of tannins, especially VVI have the potential to reduce rumen CH_4 production and ammonia concentration without negative effects on *in vitro* DM digestibility, total volatile fatty acids and pH.

Introduction

Tannins are polyphenolic polymers of relatively high molecular weight, which are widely distributed in trees, shrubs and legumes, cereals and grains. Due to their adverse effects on intake and animal performance, tannins were considered as antinutritional compounds (Patra and Saxena, 2011). However, at low concentrations tannins can alter ruminal fermentation and microbial protein synthesis (Bhatta *et al.,* 2009). It has been demonstrated that these compounds reduce ruminal CH_4 production as temperate legumes (Waghorn *et al.,* 2002), as well as purify tannin extracts (Bhatta *et al.,* 2009). The antimethanogenic activity of tannins can be useful in the environmental protection, because ruminants are considered as one of the main source of anthropogenic methane (Goel and Makkar, 2012). Moreover, the reduction of ruminal methanogenesis is desirable for improved efficiency of the digested energy utilisation (Johnson and Johnson, 1995). However, only a few studies identified the plant secondary metabolites, which could decrease the CH_4 emission without altering the basic rumen fermentation parameters (Szumacher-Strabel and Cieslak, 2010). In this case scientists are searching for phytochemicals that have the potential to modulate rumen fermentation without negative effects. Moreover, the climate characteristics of the geographical zone for Europe, perhaps have not abundance in plants which are rich sources of tannins, but some plants such as oak or lingonberry can be a good source of these phytochemicals. Plants such as oak and lingonberry are very often used by the pharmaceutical industry. During the production of dietary supplements for humans, plenty of rich-tannins by-products are also produced. This can be potentially used in animal nutrition. Unfortunately, not much research was carried out using native plants like lingonberry and oak in relation to the modulation of rumen metabolism (Jayanegara *et al.,* 2009). Thus, the aim of this study was to determine the effect of two tannin sources (*Quercus cortex* or *Vaccinium vitis idaea* extracts) on the *in vitro* ruminal fermentation, methanogenesis and microbial populations.

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Materials and methods

Experimental design

In an *in vitro* experiment, four 1 L vessels RUSITEC apparatus (Czerkawski and Breckenridge, 1977) was used to simulate a rumen environment in vitro. The experiment was repeated 3 times and lasted 10 days. To ensure a steady state within the vessels, an adjustment period for the first 6 days was allowed. Measurements were on days 7 to 10. The numbers of samples were duplicated from 4 experimental days from 3 RUSITEC runs (n=24), according to Jalc *et al*. (2009). The equivalent of 2.5 g of tannins/kg dietary dry matter (DM) from three sources of tannins were evaluated. There were four treatment combinations: control (CON; substrate without supplementation), *Quercus cortex* (QC; substrate with 2.725 mL of *Quercus cortex* extract), *Vaccinium vitis idaea* (VVI; substrate with 0.080 g of extract from dried leaves of *Vaccinium vitis idaea*), and a mixture of *Vaccinium vitis idaea* and *Quercus cortex* extracts (VVI/QC, substrate with 0.040 g of extract from dried leaves of *Vaccinium vitis idaea* and 1.362 mL of *Quercus cortex* extract). The tannin supplementation doses were established based on the results of previous experiment by Sliwinski *et al*. (2002).

Extract characterisation

In order to determine the content of tannins in crude extract of *Vaccinium vitis-idaea* leaves and in alcohol extract (500 mL methanol/L) of *Quercus cortex*, the 50 mg dry sample of VVI crude extract was diluted in water, while 1 mL of QC was evaporated to dryness and re-dissolved in distilled water. Both these water solutions were fractionated on a C18 Sep-Pack cartridges (Waters Corporation, Milford, MA, USA) preconditioned with water. Cartridges were washed with water and then with methanol (600 mL methanol/L) to elute tannins. These fractions were evapourated and redissolved in 0.5 mL of methanol (600 mL methanol/L) for analyses. The modified vanillin-HCl method of Broadhurst and Jones (1978) was applied for tannin analysis. Samples (0.25 mL) were placed in a glass vials and 1.5 mL of vanillin reagent (4 g in 100 mL methanol) and 0.75 mL HCl (370 mL HCl/L) were added. The reagents were stirred and the measurement by spectrophotometer Hewlett Packard 6453A (Hewlett Packard, Palo Alto, CA, USA) at 500 nm wavelength was immediately completed. The (+)-catechin was used as a standard.

Rumen inoculum and artificial saliva

Vessel inoculum was obtained from three ruminally cannulated Polish Holstein-Friesian dairy cows (age 3 yrs, body weight 600±25 kg) fed 20 kg/d of DM of a 600:400 forage:concentrate diet. The fermentation inoculum (solid and liquid) was collected before morning feeding, and samples from each cow were combined and thoroughly mixed to provide a single 4 L sample of rumen fluid containing about 400 g of digesta solids. This was transported aerobically immediately to the laboratory at 39°C and transferred to a water bath with continual CO_2 purging until the RUSITEC was ready to receive it.

Artificial saliva was created by dissolving 9.80 g Na_2HCO_3 and 4.68 g Na_2HPO_4/L of double distilled water ddH_2O. To this, 10 mL/L of salt mix was added, which contained 47 g NaCl, 57 g KCl, 5.40 g $CaCl_2$ and 12.80 g $MgCl_2$ dissolved in 1 L of ddH_2O. For better growth of protozoa, an addition of 3 mL of the following was added to the mixture: 0.64 g $ZnSO_4$, 0.34 g $MnSO_4$ and 0.02 g $CoSO_4$ dissolved in 1 L of ddH_2O.

Rumen simulation system

The RUSITEC apparatus consisted of 4 air-tight 1 L vessels immersed in a water bath maintained at 39°C. Each vessel was filled with 820 mL of strained rumen fluid, 100 mL of artificial saliva, one nylon bag (100 µm pore size) containing 11 g DM of digesta solids and another containing the experimental diet (10.9 g DM/bag; Table 1). The incubation vessel was then sealed and saturated with N_2 to obtain anaerobic conditions. Bags were moved up and down by the motor-driven arms continuously. Artificial saliva was continuously infused using a peristaltic pump adjusted to attain a liquid dilution rate of 0.035/h (500 mL of saliva/d). Displaced effluent and fermentation gases were collected in an effluent vessel and gas collection bag respectively. To stop fermentation in the effluent vessels, 10 mL of 6N HCl was added to each. After 24 h of incubation, each vessel was opened and bags with rumen digesta solids were removed, squeezed and washed with artificial saliva. The new bag, containing the experimental diet, was inserted into the incubation vessels. On subsequent days, the bag which had been incubated for 48 h was withdrawn and replaced with a new one in a similar way.

Sampling

The experiment ran for 10 d with sampling of rumen fluid for rumen fermentation parameters on d 7, 8, 9 and 10 at 3 h before the addition of the new feed bag. The pH was measured immediately after collection using a pH meter. Rumen fluid after incubation (3.6 mL) was immediately analysed for NH_3-N. Volatile fatty acids (VFA) was determined in 3.6 mL of collected rumen fluid mixed with 0.4 mL of 46 mM $HgCl_2$. Samples for VFA were stored at -20°C prior to analysis. For estimation of microorganism populations, 1.5 mL of rumen fluid was collected. Fermentation gases were collected over 24 h in gas-tight bags (TECOBAG 81; Tessaraux Container GmbH, Bürstadt, Germany) connected to the effluent vessels by flushing the RUSITEC system with gaseous N_2 before removing the bags.

Analytical methods

Substrates and substrate residues after 48 h of incubation were dried at 70°C and analysed for the amount of DM (no. 930.15), ash (no. 942.05), ether extract (no.920.39) and N (no. 954.01), according to AOAC (2007). The VFA profile was determined by high performance liquid chromatography (Waters 2690; Waters Corporation) equipped with: a Waters 2487 detector (Waters Corporation), an automatic injector (no. 1079), and an Aminex HPX-87 300 x 7.8 m (Bio-Rad, Hercules, CA, USA) column. As mobile phase, 0.004 M H_2SO_4 was used, a 10 µL sample volume was injected into the column. Quantitative and qualitative identification of individual peaks was made using the method based on external standard prepared by mixing individual VFA purchased from Supelco (Sigma Aldrich, St. Louis, MO, USA) using Millenium® 32 software (2001). The population of bacteria was obtained with Thoma counting chamber (Blau Brand, Wertheim, Germany; Ericsson *et al.,* 2000). Counts of protozoa (*i.e.,* entodiniomorphs and holotrichs) were determined according to Michalowski *et al*. (1986). Briefly, 1 mL of rumen fluid was fixed with 6 mL of formaldehyde (40 mL formaldehyde/L). Fixed protozoa were counted in the drop of rumen fluid with defined volume (100 µL) under a light microscope (Primo Star no. 5; Zeiss, Oberkochen, Germany). The ammonia concentration was checked by the colourimetric Nessler's methods as modified by Szumacher-Strabel *et al.* (2002). Fermentation gases were analysed for the concentration of CH_4 by a SRI310 gas chromatograph (SRI Instruments, Torrance, CA, USA) equipped with a thermal conductivity detector and Carboxen-1000 column (mesh size 60/80; Sigma Aldrich) according to Szumacher-Strabel *et al.* (2011). The volume of collected fermentation gases was obtained by pressing the gas into a closed tube filled with water and measuring the amount of displaced water. Quantification of methanogens was carried out with the fluorescence *in situ* hybridisation technique described by Pers-Kamczyc *et al.* (2011). Briefly, samples of rumen fluid were

Table 1. Chemical composition of the feeds (n=3) used in the *in vitro* studies.

	Diet, g/kg DM	DM, g/kg	OM, g/kg DM	aNDF, g/kg DM
Corn silage	206	449	424	202
Lucerne silage	165	260	231	118
Meadow hay, chopped	225	924	888	603
Wheat grain, ground	142	894	887	142
Corn grain, ground	103	896	881	211
Rapeseed meal	158	920	856	184
Mineral components	1	980	-	-

DM, dry matter; OM, organic matter; aNDF, neutral detergent fibre.

washed by phosphate buffered saline (PBS) solution and incubated with buffer containing NaCl, Tris-HCl and sodium dodecyl sulfate for 3 h. After that, samples were washed with PBS again and stained with oligonucleotide probe (S-S-GTGCTCCCCCGCCAATTCCT-a-A-20) overnight at 40°C. Next, samples were viewed with a fluorescence microscope (Nikon E600 Eclipse; Nikon Corp., Tokyo, Japan). Images of the fluorescent signals were taken with a cooled digital charge-coupled device camera, driven by a computer-aided software Lucia (Laboratory Imaging, Praha, Czech Republic) and counted manually.

Statistical analysis

For all measurements (rumen microbial population, parameters of rumen fermentation, concentrations of individual VFA) repeated on different days (7-10), data were combined across days for each fermenter vessel. Data were analysed by two-way ANOVA, with diet and experimental runs included as a main effect. Experimental runs have been removed from the model due to lack of influence. Differences among treatments (between diets) were tested using the Tukey post hock test. Data were accepted as statistically different if $P<0.05$. Tables 2 and 3 show group means and standard errors of means. All statistical analyses used SAS (1996).

Results and discussion

Extract characteristics

The 1 g extract of *Vaccinium vitis idaea* contained 37.1 mg of tannins, whereas 1 mL *Quercus cortex* extract contained 0.96 mg of tannins, which yielded 31.0 mg/g DM of QC.

Ruminal fermentation

Incubation fluid pH did not differ among treatments (Table 2). Compared with CON, all tannin containing diets had lower ($P=0.05$) ammonia concentrations by 18.3, 22.9 and 21.9%, respectively. In the presence of tannins, ciliate protozoal and methanogen counts were less ($P<0.001$) than in CON, whereas no differences occurred in bacterial counts. Enteric gas production increased when extracts of *Quercus cortex* extract and *Vaccinium vitis idaea* leaves were supplemented (Table 2). The highest CH_4 decrease of 21.9% was with VVI, and both other diets containing *Quercus cortex* extract and mixture QC+VVI decreased CH_4 production by 18.0 and 18.6%, respectively. There was no difference in total VFA concentration (Table 3). However, a higher propionate concentration occurred in VVI than in the other treatments and higher isobutyrate in VVI compared with QC. Differences in molar proportions of butyrate and isovalerate also occurred among treatments. QC+VVI decreased butyrate and increased isovalerate concentrations compared with CON and QC groups. The acetate (A):propionate (P) ratio was lower in comparison to CON in groups with addition of VVI and QC+VVI. Simultaneously, apparent digestibility of DM, organic matter (OM) and aNDF did not differ among treatments (Table 2).

General remarks

The concentration of secondary metabolites in plants depends on factors such as the part of plant, the parameters of growth, as well as biotic and abiotic environmental stresses (Gherman *et al.,* 2000). According to the characteristics of extracts from *Quercus cortex* and *Vaccinium vitis-idaea*, in our studies the tannins content was relatively high, especially in VVI extract. Jayanegara *et al*. (2009) demonstrated that 72/100 g of total phenols in *Vaccinium vitis idaea* was condensed tannins but there is no information about the type of tannins in *Quercus cortex*.

Effects of tannins on rumen protozoa, bacteria, fungi and methanogens are variable and mostly depend on the type of tannins, their origin and supplementation levels (Patra and Saxena, 2011). Results obtained *in vivo* with sheep (Salem *et al.,* 1997) showed that tannins increased protozoal numbers, although they did not affect the ciliate population *in vitro* (Khiaosa-Ard *et al.,* 2009). However, Makkar *et al*. (1995) demonstrated inhibitory effects of quebracho tannins (0.4 mg/1 mL of medium) on total protozoa, entodiniomorphs and holotrichs. These results are consistent with Animut *et al.* (2008) where increasing levels of tannins (50, 101 and 151 g/kg DM) in diets reduced protozoa numbers in goat rumens. These results are consistent with ours in which inhibitory effects of tannins from QC, VVI, as well as a mixture of QC and VVI have been observed. Previously, condensed tannins have been showed to be more

Table 2. Effects of 2.5 g of tannin supplementation from *Quercus cortex*, *Vaccinium vitis idaea* and their combination on the *in vitro* rumen microbial population and rumen fermentation parameters.

	Control	QC	VVI	QC+VVI	SEM
pH	6.95	6.86	6.90	6.90	0.018
Ammonia, mmol/L	12.6^{a}	10.3^{b}	9.72^{b}	9.84^{b}	0.254
Methane, mM	3.11^{a}	2.55^{b}	2.43^{b}	2.53^{b}	0.059
Gas production, 10^3 mL TGP	2.95^{b}	3.67^{a}	3.70^{a}	3.31^{ab}	0.127
Methane/TGP, x10^{-3}/mL	1.10^{a}	0.72^{b}	0.70^{b}	0.80^{b}	0.040
Protozoa, x10^3/mL	9.54^{a}	6.29^{b}	6.40^{b}	6.47^{b}	0.350
Bacteria, x10^3/mL	28.8	35.7	47.1	38.3	3.33
Methanogens, x10/mL	58.4^{a}	44.1^{b}	30.1^{c}	39.2^{b}	1.37
Apparent digestibility					
DM	0.525	0.511	0.520	0.535	0.770
OM	0.525	0.511	0.521	0.536	0.796
aNDF	0.219	0.181	0.205	0.242	1.17

QC, *Quercus cortex;* VVI, *Vaccinium vitis idaea;* TGP, total gas production; DM, dry matter; OM, organic matter; aNDF, neutral detergent fibre. ^{a,b}Different superscripts indicate differences between means in the same row ($P<0.05$).

Table 3. *In vitro* volatile fatty acid production by mixed rumen microbes as an effect of *Quercus cortex, Vaccinium vitis idaea* and their combined supplementations.

	Control	QC	VVI	QC+VVI	SEM
Total VFA	82.2	78.7	81.4	76.8	1.29
A	44.38	41.55	41.59	39.36	0.820
P	11.87^{b}	12.72^{a,b}	14.25^{a}	12.70^{a,b}	0.278
Isobutyrate	2.34^{a,b}	1.94^{b}	2.40^{a}	1.95^{a,b}	0.059
Butyrate	13.39^{a}	11.97^{a}	10.94^{a,b}	9.76^{b}	0.373
Isovalerate	4.77^{b}	5.34^{b}	6.97^{a,b}	8.11^{a}	0.404
Valerate	5.48	5.17	5.27	4.90	0.188
A/P	3.78^{a}	3.30^{a,b}	2.98^{b}	3.11^{b}	0.099

QC, *Quercus cortex*; VVI, *Vaccinium vitis idaea*; VFA, volatile fatty acids; A, acetate; P, propionate. ^{a,b}Different superscripts indicate differences between means in the same row ($P<0.05$).

toxic for protozoa compared to hydrolysing tannins (Bhatta *et al.,* 2009). On the other side, it should be also noted that, rumen protozoa had different metabolic responses to the tested form and concentration of analysed experimental factors what was showed before (Kisidayova *et al*., 2006; Cieslak *et al.,* 2009). Moreover, Tan *et al*. (2011) reported that a tannin extract from *Leucaena* linearly decreased the protozoa population. Additionally, our results showed also that tannins from QC, VVI and QC+VVI did not have any effect on total bacteria populations *in vitro*. It is also observed that numerical increase in the number of bacteria was associated with reduction of the population of protozoa. However, it has not been determined how analysed tannins affect particular species of rumen bacteria. Some authors evaluated effects of tannins on particular bacteria species. For example, Molan *et al*. (2001) demonstrated the toxic effect of condensed tannins from *Lotus* sp. on proteolytic rumen bacteria: *Streptococcus bovis*, *Eubacterium* sp., *Prevotella bryantii*, *Butyrivibrio fibrisolvens*, *Clostridium proteoclasticum*. Moreover, they observed that the effect of CT depends on, among others, the molecular weight and chemical structure of tannins. Similarly, addition of phlorotannins to rumen bacterial cultures inhibited growth of *Fibrobacter succinogenes*, but stimulated growth of *Streptococcus bovis* and *Prevotella bryantii* (Wang *et al.,* 2009). The lack of effect of tannins in these studies may be due to inhibitory effects on some bacterial species and stimulatory effects on others. Moreover, due to the long period of exposure to tannins, rumen bacteria could acquire resistance. Patra and Saxena (2011) suggested mechanisms of bacterial tolerance to dietary tannins such as synthesis of tannin-complexing polymers, formation of extracellular glycocalyx from tannins and cell wall/membrane, tannin degradation and synthesis of siderophores which chelate tannins and cations.

The rumen microorganisms are involved in the fermentation and thus the changes in their metabolism could alter the basic parameters of ruminal process, such as pH, ammonia concentration or VFA profile as well as digestibility. In our study, digestibility of DM, OM and aNDF, and pH, was not affected by tannins addition, which is consistent with Tan *et al*. (2011), where condensed tannins from *Leucaena* sp. affected IVDMD only by 7% and did not change ruminal pH. On the other hand, ammonia concentration in the present study was lower in all experimental groups. Similarly, Grainger *et al*. (2009) reported that ruminal ammonia concentration was lower in the rumen of dairy cows supplemented with *Acacia mearnsii* tannins, which may lead to i) reduction of the protein degradation rate in the rumen (Patra and Saxena, 2011); ii) improved efficiency of microbial protein synthesis (Bhatta *et al.,* 2009); and iii) reduced urea N excretion in urine (Grainger *et al.,* 2009). Presumably, in presented study protein-tannin complexes were formatted, that are minimally degraded by ruminal microbes (McSweeney *et al.,* 1999) and this resulted in reducing the concentration of ammonia in rumen fluid. Moreover, the previously described anti-protozoal activity of used plant extracts is also favourable as it may improve efficiency of microbial protein synthesis due to suppression of the bacteriolytic activity of ruminal protozoa and this could escalate protein flow to the duodenum to enrich protein by-passing the rumen. Our results on CH_4 production by tannin supplementation are consistent with Tan *et al*. (2011), who showed reduction in CH_4 production by tannin inclusion of 10 mg CT/500 mg DM. Also, Animut *et al*. (2008) observed that goats fed increasing levels of *Kobe lespedeza* had linearly decreased CH_4 emissions which were correlated with the condensed tannin (CT) level. Similarly, supplementation of forage diets with tannins from *Acacia mearnsii* decreased methanogenesis in sheep (Carulla *et al.,* 2005) and dairy cows (Grainger *et al.,* 2009). *In vitro* CH_4 production was also 90% mitigated by inclusion of *Terminalia chebula* extract (Patra *et al.,* 2006). However, CH_4 production was not affected in Beauchemin *et al*. (2007) and Min *et al*. (2003). These discrepancies might be caused by different types of tannins (*e.g.,* hydrolysable *vs* condensed) and their origin. It is generally agreed that phytochemicals can affect the methanogenesis by: i) a direct effect on ruminal methanogenic bacteria and archaea; and an ii) indirect effect on fibre digestion to decrease production of hydrogen, which is a substrate for these microorganisms (Tavendale *et al.,* 2005). Additionally, it has been suggested that an inhibitory effect of tannins on rumen methanogenesis was due to protozoa associated CH_4 production (Hess *et al.,* 2003). In our study, the decrease of CH_4 production was not related with decreased digestibility of DM, but corresponded to reduction of protozoa and methanogen populations. This indicates that the tannins contained in the analysed plants can suppress ruminal methanogenesis either directly or indirectly. Directly, by reducing the population of protozoa in *in vitro* condition by reducing the concentration of H_2, a necessary substrates for the formation of methane. And indirectly, by suppressing methanogens population - major methane producing microorganisms in rumen ecosystem.

The reduction of methane production for diets supplemented with *Vaccinium vitis idaea* and *Vaccinium vitis idaea* mixed with *Quercus cortex*, corresponded with A:P ratio decrease and propionate increase (but only for *Vaccinium vitis idaea*) without decreasing of total VFA or pH. No limitation of total VFA or pH values and decreased A:P ratio reflects the reduced production of CH_4 throughout the changed utilisation of hydrogen used in P pathway, without adversely affecting the fermentation (Demeyer and Van Nevel, 1975). These results suggest that the fermentation responses patterns in *Vaccinium vitis idaea* are similar to CH_4 reduction by ionophores (Goodrich *et al.,* 1984), but without negative influence on the animal organism.

Conclusions

Tannins from *Vaccinium vitis idaea* and *Quercus cortex* have antimethanogenic activities caused by direct inhibition of methanogens and protozoans but without a decrease of DM digestibility. Results suggest that tannin extracts have potential to reduce rumen methanogenesis; still, the mechanism of action needs evaluation in *in vivo* conditions.

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