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TANNINS FROM SANGUISORBA OFFICINALIS AFFECT IN VITRO RUMEN METHANE PRODUCTION AND FERMENTATION

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ABSTRACT

The present study was conducted to evaluate the effect of different doses of the *Sanguisorba officinalis* tannin extract (SOTE) on *in vitro* methane production, rumen microorganisms and basic rumen fermentation parameters. SOTE contained 451 mg g^{-1} of total tannins was tested at different doses (0, 1, 4, 10, 20, 40 and 100 mg of SOTE per vessel; the input of tannins was 0, 1.3, 5.1, 12.8, 25.6, 51.2, 128.0 g per kg dry matter, respectively) during 24 h fermentation of rumen fluid in batch culture system. The basal substrate consisted of meadow hay and barley meal (60:40 ratio). The rumen fluid was collected, pooled and mixed from three ruminally fistulated, Polish Holstein-Friesian dairy cows. The methane production was decreased significantly at the high doses (40 and 100 mg of SOTE per bottle). This favorable decrease at the highest dose (100 mg) was associated with the reduction of *in vitro* dry matter digestibility (IVDMD), *Entodiniomorpha* sp. and bacteria populations. The population of methanogens was not affected, as compared with the control treatment. These findings suggest that the highest dose of SOTE has a potential to reduce methane production by decreasing the protozoal population and IVDMD. However, the obtained data clearly showed the stimulating effect of the highest dose of tannins on ammonia release, 40 mg of SOTE should be used in further long-term studies. This dose indicated either antimethanogenic effect even when expressed per units of total gas production or volatile fatty acids. 40 mg of SOTE had no negative impact on *in vitro* rumen microbial populations, dry matter digestibility and volatile fatty acid content.

Keywords: methanogens, methane, rumen fermentation, *Sanguisorba officinalis*, tannins.

INTRODUCTION

Scientists are inquiring after the ways of decreasing the negative impact of methane emission on the environment especially from livestock, which represent one of the largest global methane producers (Johnson and Johnson 1995). So far, the only effective way to reduce the rumen methanogenesis was the use of ionophore antibiotics in animal nutrition (Van Nevel and Demeyer 1996). However, today the usage of antibiotic growth stimulators as dietary components in European Union is banned (OJEU 2003). Thus, more focus should be given on finding natural feed additives, which effectively modify the rumen environment and decrease rumen methanogenesis.

Recently, plant extracts containing high concentration of secondary metabolites have become interesting as alternative for antibiotics in animal nutrition. Plants can produce compounds with diverse biological activities that have been exploited in human and veterinary ethno-medicine for thousands of years. These natural plants contain bioactive compounds, such as essential oils, saponins, tannins and organosulphur compounds, and have been shown to improve rumen fermentation and nitrogen metabolism (Patra and Saxena 2009), or to improve animal health and productivity (Rochfort *et al*., 2008). Moreover, some of these active compounds have been shown to modulate volatile fatty acids production or their ratio under *in vitro* and *in vivo* conditions (Cardozo *et al.*, 2001; Sliwinski *et al.*, 2002; Cieslak *et al.*, 2012, Cieslak *et al*., 2014a). In the research by Rira *et al*. (2015) methane production, volatile fatty acid concentration and fermented organic matter decreased with increased proportions of tannin-rich plants. However the mode of action, especially on the microbiology level, remains unknown in most cases (Carreño *et al*., 2015; Rira *et al*., 2015, Wencelová *et al.*, 2015).

One of the most interesting plant, which may affect rumen fermentation, is *Sanguisorba officinalis* L., a native medicinal plant rich in tannins, from northern Europe, Asia, and North America. Shan *et al.* (2007) demonstrated that *S. officinalis* showed one of the strongest inhibitory activities against foodborne pathogens, such as *Listeria monocytogenes* or *Salmonella*

anatum, whereas Kokoska *et al.* (2002) found that *S. officinalis* is one of the most active antimicrobial plants against among other *Escherichia coli* and *Staphylococcus aureus*. Moreover, *S. officinalis* extract inhibited the conidial germination of post harvest pathogens, *i.e.*, *Penicillium digitatum* and *Aspergillus niger in vitro* and reduced brown rot on apricots and nectarines (Gatto *et al.*, 2011). Recently, the herbal drug *Sanguisorbae radix* (*Sanguisorba* root) has been included in European Pharmacopoeia. Despite the known antimicrobial properties of S. *officinalis*, there is a lack of described effects on rumen microbial fermentation, including methane production and changes in microbial population. We hypothesized that tannin from *S. officinalis* could modulate rumen fermentation and methane production by influencing the population of micro-organisms, especially protozoa and methanogens. Thus, the aim of this study was to determine the effect of different doses of the *S. officinalis* root tannin extract (SOTE) on *in vitro* methane production, rumen microorganisms and basic rumen fermentation parameters.

MATERIALS AND METHODS

Preparation of S. officinalis root extract: Roots of *S. officinalis* were harvested in fall (October 2010), from the Botanical Garden of Medicinal Plants, at the Medical University of Wroclaw, Poland. The plant material has been thoroughly cleaned and shredded into pieces of ca. 5 mm size. Afterwards they were air-dried at room temperature in the dark.

For extraction, 450 g of roots were defatted by maceration with 600 ml of petroleum ether. The roots were extracted three times using 3 x 600 ml of 70/100 ml acetone [containing 0.1/100 ascorbic acid for preventing tannin oxidation]. Extraction was carried out for 2 h at 30°C in the ultrasonic bath (InterSonicIS-27, Olsztyn, Poland), followed by filtration of the crude extracts. All filtrates were pooled and the solvent was evaporated in the rotary vacuum evaporator (Unipan type 350P, Warsaw, Poland) at 40°C. The crude extract (146 g) was dissolved in 400 ml 5/100 methanol in deionized water and partitioned with 95/100 n-butanol (10 x 200 ml). The aqueous and butanol (BuOH) phases were separately concentrated under reduced pressure until dry water (40.8 g) and butanol (98 g) extracts were obtained.

The BuOH extract was suspended in deionized water (at 1:10 w/v proportion) and thoroughly mixed with the polymeric resin - Amberlite XAD-16 (Rohm&Haas – Dow Chemical, U.S.A.). After 2 h, the whole mixture was transferred to the Schott funnel and eluted sequentially with deionized water, 50/100 aqueous methanol and 100/100 methanol. Polymeric adsorbent was washed until tannins disappeared from elute. The solvent was then evaporated in a vacuum and the dry fractions were weighed. As a result, the tannin-enriched fraction was obtained from the 50/100 methanol fraction (SOTE) with an extraction efficiency of 61.35/100.

Characteristics of extract: The phytochemical screening for various polyphenol compounds was performed using spectrophotometric methods during the subsequent extraction steps and in the final product SOTE. All assays were performed in six repetitions and repeated twice.

Total polyphenols and tannins: A modified Folin-Ciocalteu assay was used for analysis of total content of reducing phenolic compounds according to Singleton and Rossi (1965). Tannin compounds were measured by parallel experiments with extracts vortexed for 1 h with 10 mg ml^{-1} using hide powder (Sigma, Poznan, Poland). The results were expressed as gallic acid equivalents according to the standard gallic acid (Fluka, Switzerland) calibration curve. Total tannins were calculated by subtraction of results for polyphenols non-absorbed by hide powder, from the total phenol content. The proportion between hydrolysable and condensed tannins was estimated by acid hydrolysis of the extract and repeating of the assay for hydrolyzed samples.

Rhodanine assay for gallotanin content: The assay was performed according to Inoue and Hagermann (1988). The assay uses oxygen-free acid hydrolysis followed by specific staining of gallic acid by rhodamine (Sigma-Aldrich, U.S.A.). The gallic acid concentration was calculated from gallic acid authentic standard calibration curve. The gallotannin content was calculated by subtraction of the results of non-hydrolyzed from hydrolyzed samples.

Flavan-3-ols determination: For measuring the content of total flavan-3-ols (sum of monomeric catechins and oligomeric proanthocyanidins) we used the specific assay with dimethylaminecinnamic aldehyde (DMACA) according to Li *et al.* (1996). The results were expressed as epicatechin equivalents calculated from standard (-)-epicatechin (Fluka, Poznan, Poland) calibration curve.

Total proanthocyanidins acid butanol assay: The protocol by Porter *et al.* (1986) that used hydrolysis/oxidation of oligomericproanthocyanidins resulting in colored red products was utilized. The results are expressed as procyanidin B1 equivalents according to standard calibration curve.

Total carbohydrates content: To determine total carbohydrates content the spectrophotometric sulfuric acid/phenol method of Dubois *et al.*, as modified by Taylor (1995) was used. The content of total carbohydrates in the extracts were calculated using glucose standard (analytical grade, POCh, Gliwice, Poland) curve.

Experimental design: *S. officinalis* tannin extract containing 451 mg g^{-1} of total tannins was tested in two

repetitions at seven doses (0, 1, 4, 10, 20, 40 and 100 mg of SOTE per vessel; the input of tannins was 0, 1.3, 5.1, 12.8, 25.6, 51.2, 128.0 g per kg dry matter, respectively) in four replicates for each repetition. Each repetition comprised of a four vessels for each dose of extract (n= 2 x 4 = 8 true replicates), a control without extract and four vessels as blanks (without substrate). The experiment was repeated within two consecutive days (two repetitions). As a basal substrate (control) mixture of meadow hay and barley meal in the ratio 60:40 was used.

Preparation of inoculum: The rumen fluid was collected from three ruminally fistulated, Polish Holstein-Friesian dairy cows (age 3 years, mean body weight 600 ± 25 kg) fed with the diet (kg · day–1) containing lucerne silage, 46.0; meadow hay, 1.80; maize meal, 0.90; dry brewer's grains, 0.60; protein concentrate (35% crude protein), 1.50; wheat bran, 0.60; and commercial concentrate (19% crude protein), 5.50. The rumen fluid was sampled before morning feeding, squeezed through 4-layers cheesecloth into a Schott Duran® bottle (one liter) with an O_2-free headspace, transported under anaerobic conditions to the laboratory at 39°C and used as a source of inoculum. Before starting to prepare batch culture study rumen fluid from the three animals was pooled and mixed.

Batch culture: The batch culture method was adopted from Szumacher-Strabel *et al.*, (2004). Briefly, rumen fluid was mixed with the buffer solution (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4x7H_2O$, 64 mg $CaCl_2x2H_2O$, 4 mg Na_2CO_3 and 600 mg cysteine hydrochloride per 1 liter of double distilled water (ddH_2O) in the 1:4 (v/v) ratio. Incubations were run at 39°C under CO_2 in 40 ml buffered rumen fluid added to pre-warmed 125 ml serum flasks. The basal substrate consisted of 240 mg of meadow hay (dry matter – 897 g kg^{-1}, crude protein – 164 g kg^{-1} DM, crude fiber – 256 g kg^{-1} DM, ether extract – 19 g kg^{-1} DM) and 160 mg of barley meal (dry matter – 856 g kg^{-1}, crude protein – 138 g kg^{-1}DM, crude fiber – 44 g kg^{-1} DM, ether extract – 19 g kg^{-1} DM), both grounded to 1 mm. The basal substrate was supplemented by various doses of SOTE. The incubation flasks, sealed with rubber stoppers and aluminum caps, were placed in an incubator for 24 h and periodically mixed.

Gas and methane production analysis: After 24 h of incubation, gas production was determined by the displacement of syringe piston, which was connected to the serum flasks. The gas produced due to fermentation of substrate was calculated by subtracting gas produced in blank vessels (without substrate) from total gas produced in the vessels containing buffered rumen fluid and substrate. For methane determination, 500 µl gas was sampled from the headspace of incubation vessels in an gastight syringe (GASTIGHT® Syringes, Hamilton Bonaduz AG, Switzerland) into SRI310 gas chromatograph equipped with a thermal conductivity detector (TCD) and Carboxen – 1000 column (mesh side 60/80, 15 FT x 1,8 INS.S, SUPELCO). Nitrogen was used as the carrier gas at a constant flow of 30.0 ml min^{-1}. The oven temperature was programmed as follows: initially 180°C for 1.5 min, then increasing at 20°C min^{-1} to 220°C. 500 µl gas samples were injected. Observed peaks were identified and methane concentration was calculated by comparison of retention times with appropriate gases standards (mix gases 5.63/100 CO_2, 5.56/100 CH_4, 5.10/100 H_2 and N_2 remains, Multa S.C. Poland) using PeakSimple ver. 3.29.

Ammonia concentration determination: Quantitative analysis of ammonia concentration was carried out using Nessler's method. Briefly, after 24 h of incubation, 3.6 ml of buffered rumen fluid was sampled from the serum flasks and centrifuged at 12000 rpm for 5 min. A 100 µl of supernatant was then transferred to the tube contained 200 µl of 1/100 polyvinyl alcohol, 200 µl of 20/100 potassium sodium tartrate, 200 µl of Nessler reagent and 19.3 ml of ddH_2O and incubated 10 min at the room temperature. After incubation, the samples were checked spectrophotometrically and compared to the blank sample (without rumen fluid).

***In vitro* dry matter digestibility:** For the *in vitro* dry matter digestibility (IVDMD) the same experimental design was used as during the batch culture. The buffered rumen fluid was incubated with 400 ± 1 mg of substrate for 24 h at 39°C. After incubation, the incubation vessels content was transferred to the previously weighed crucible. The residues of incubation were washed with 50 ml distilled water and dried at 105°C for 3 days. The percent loss in weight of dry matter (DM) of the feed was determined and presented as IVDMD.

Protozoa and bacteria counts: After termination of incubation, the contents of the serum flasks were mixed properly and 1 ml sample was mixed with 6 ml of 4/100 formaldehyde for protozoa determination and 20 µl was added to 6980 µl of Hayem solution (2.5 g $HgCl_2$, 25 g Na_2SO_4 and 5.0 g NaCl per 1 liter of double distilled water) to analyze the total number of bacteria. The protozoa were counted microscopically (150x) in the drop of rumen fluid with the defined volume, with the discrimination on the Isotrichid (*Isotricha spp.*) and *Entodiniomorphid* (*Entodinium spp.*, *Epidinium caudatum*, *Metadinium medium*, *Eudiplodiniu mmaggii*, and *Ostracodinium gracile)* ciliates. The bacteria were obtained with Thoma chamber (0.02 mm depth, Blau Brand, Wertheim, Germany).

Quantification of methanogens: The determination of methanogens population was carried out with the fluorescence *in situ* hybridization technique, according to the laboratory manual of Stahl *et al.*, (1995) with some

modification described by Pers-Kamczyc *et al.,* (2011). A domain-specific oligonucleotide probe targeting all methanogens (S-S-Arc-0915-a-A-20) were used and the temperature of hybridization were 56°C. Samples were viewed with a fluorescence microscope (Zeiss Axiovert 2000) with AxioImage Observer Software and filters specific for DAPI and Rhodamine (magnification x100). Images were taken with a video camera (AxioCamMRm Rev. 3 Fire Wire) and counted manually.

Volatile fatty acids (VFA) determination: At the end of incubation, 3.6 ml of fermented rumen fluid was stabilized with 0.4 ml of a 46 mM $HgCl_2$ solution and frozen until analyses by HPLC. After thawing the mixture was centrifuged at 12000 rpm for 10 min, filtered through 0.22 µm and 10 µl of clear supernatant was injected to the High Performance Liquid Chromatograph (Waters 2690 equipped with Waters 2487 Dual λ detector and Aminex HPX-87H column (300 mm x 7,8 mm)). As a 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 of individual VFA (acetate, propionate and butyrate acids) purchased from Supelco and using the Millenium 2001 software (version 2.15).

Statistical analyses: Batch culture experiments (24 h incubation) were performed in two repetitions and four replications (4 incubation vessels) for each of the seven SOTE doses. Data were analyzed by ANOVA using the General Linear Model (GLM) procedures of SAS (Version 6.0; SAS Inst. Inc. Carry, NC, 1989) with day and treatment as a factor. No statistical influence of day was observed, therefore, this factor has been deleted from the model. Treatment means were calculated using the Least Square Means (LSMEANS) option of SAS. When *F*-tests were significant, single degree of freedom orthogonal contrasts were used to determine linear and quadratic effects of increasing SOTE supplementation. Since SOTE supplementation level increments was not equal in diet (*i.e.,* 1, 4, 10, 20, 40 and 100 mg), coefficients for orthogonal polynomials were generated using IML (Interactive Matrix Language) procedures of SAS for unequal spacing (Version 6.0; SAS Inst. Inc. Carry, NC, 1989). Moreover the Pearson correlation coefficient was calculated for number of total protozoa, bacteria and groups of protozoa and digestibility of food to explain relationships between quantified parameters. Data were statistically different when $P<0.05$.

RESULTS

S. officinalis tannin extract composition: The extraction achieved efficiency (Table 1) of over 62.5 g tannin-rich extract from 450 g of roots. The successive purification steps lead to the significant enrichment in polyphenol and tannin content. The absorption on the polymer resin – Amberlite XAD16 followed by elution with increased concentration of methanol permitted the selective enrichment of the fraction eluted with 50% methanol in gallic acid, total tannins and especially in gallotannins, while the content of condensed tannins remained on a similar level (Table 1). Also, the amount of non-phenolic compounds decreased markedly. The carbohydrates content was reduced about five times during the purification procedure, from 230.24 (± 19.05) mg glucose equivalent per g of dry crude acetone extract, 74.12 (± 1.11) mg/g in the butanol fraction, to 47.74 (± 6.09) mg/g glucose in SOTE.

Table 1. The characteristics of extracts from *Radix Sanguisorbae*

	Dried roots	Crude acetone extract	n-butanol extract	Amberlite + 50% MeOH (SOTE)
Total polyphenols (GAE[1] mg/g)	111.0	433.7±2.9	612.4±29.6	855.9±11.6
Total tannins (GAE mg/g)	68.8	268.9±6.9	301.1±11.7	451.1±23.1
Free gallic acid (mg/g)	2.9	11.3±0.5	11.9±0.4	59.3±0.4
Gallotannins (GAE mg/g)	21.3	83.0±0.4	105.9±0.9	250.9±1.0
Flavan-3-ols (EE[2] mg/g)	1.6	6.4±0.2	10.7±0.1	17.8±0.2
Proanthocyanidins (PCB1[3] mg/g)	4.0	15.7 ±0.2	21.0 ±0.1	17.7±0.5
Hydrolyzable/condensed tannin ratio	-	1.7	2.26	15.3
Extraction efficiency [%]	-	32.4	70.0	61.4

± standard deviation (n=6),
[1]GAE – gallic acid equivalents,
[2]EE – (-)-epicatechin equivalents,
[3]PCB1 – procyanidin B1 equivalents,

Effect of S. officinalis on rumen fermentation parameters and microbial populations: The effect of SOTE on basic parameters of ruminal fermentation is presented in table 2. Inclusion of SOTE at different doses decreased values of analyzed basic parameters (pH, ammonia concentration, methane production, TPG and

IVDMD) of ruminal fermentation (linear $P<0.01$) as well as ammonia concentration and TGP (quadratic $P<0.01$). As the amount of SOTE supplements increased to 40 and 100 mg the methane expressed per units of total gas production and volatile fatty acids decreased in a linear and quadratic manner ($P<0.001$). No changes occur when methane was calculated per gram of *in vitro* dry matter digestibility. The response of basic ruminal parameters corresponded with reduction in microbial populations of total bacteria, *Entodiniomorpha*, *Holotricha* and methanogens (linear $P<0.01$; Table 2). Also quadratic contrast ($P<0.01$) was observed in microbial counts, except for methanogens population. Positive correlations have been observed among the number of total protozoa ($r=0.65$, $P=0.0001$), number of the Entodiniomorpha ($r=0.66$, $P=0.0002$) and total bacteria ($r=0.59$, $P=0.001$). Moreover, SOTE addition caused a significant decrease in propionate (linear $P<0.001$ and quadratic $P<0.05$; Table 2) and total VFA (linear $P<0.01$).

Table 2. Effect of SOTE on the *in vitro* rumen fermentation parameters, microbial population and volatile fatty acid production.

	Adding levels of *S. officinalis* tannin extract (mg)								Contrasts	
	Control	**1.0**	**4.0**	**10.0**	**20.0**	**40.0**	**100.0**	**SEM[6]**	**linear**	**Quadratic**
Basic parameters										
pH	7.7	7.7	7.8	7.8	7.8	7.9	8.0	0.02	<0.001	0.204
Ammonia (mmol/l)	16.8	18.1	18.3	16.8	15.3	15.7	23.6	0.71	0.003	0.01
Methane (mM)	12.0	12.1	12.3	12.6	11.3	8.9	4.9	0.54	<0.001	0.793
TGP[1] (ml)	39.8	40.8	41.8	40.5	35.3	31.5	26.3	1.09	<0.001	0.002
IVDMD[2] (%)	37.9	31.6	33.1	29.9	28.0	28.6	12.2	1.66	<0.001	0.813
Methane (mM)/g IVDMD	9.1	10.8	10.6	11.9	11.2	9.0	12.1	0.48	0.378	0.519
Methane (mM)/ml TGP	0.18	0.18	0.26	0.29	0.23	0.14	0.09	0.01	<0.001	<0.001
Methane (mM)/mM VFA	0.30	0.30	0.30	0.31	0.32	0.28	0.19	0.01	<0.001	<0.001
Microbial population										
Total bacteria (10^8 cells/ml)	17.6	19.9	23.4	25.4	19.7	19.0	10.2	0.88	<0.001	<0.001
Entodiniomorpha (10^3 cells/ml)	39.3	35.3	47.6	42.1	39.7	40.1	25.8	1.28	<0.001	0.001
Holotricha (10^3 cells/ml)	1.4	1.5	1.4	1.6	1.8	1.7	1.2	0.04	0.01	<0.001
Methanogens[3]	0.20	0.25	0.30	0.31	0.18	0.19	0.19	0.01	0.012	0.242
In vitro volatile fatty acid production										
Acetate (mM)	39.7	40.0	30.6	38.2	30.0	31.7	26.2	1.15	0.229	0.319
Propionate (mM)	12.3	12.2	8.6	12.0	8.8	13.62	7.7	0.46	0.001	0.021
Butyrate (mM)	9.0	8.6	6.4	9.2	6.6	7.4	5.9	0.29	0.922	0.704
A:P ratio[4]	3.2	3.3	3.6	3.2	3.3	2.3	3.4	0.08	0.825	0.359
Total VFA[5] (mM)	66.5	66.5	49.4	65.2	48.8	57.0	43.6	1.98	<0.001	0.068

[1]TGP – total gas production,
[2]IVDMD – *in vitro* dry matter digestibility
[3]The ratio of methanogens in the population of microorganisms dyed with DAPI
[4]A – acetate, P – propionate,
[5]VFA – volatile fatty acids
[6]SEM – standard error of the mean.

DISCUSSION

The present study was focused on investigation of the SOTE potential to modulate *in vitro* rumen microbial fermentation and thus to mitigate enteric methane production. Results from this study confirmed the antimicrobial properties of SOTE and revealed that exhibited effect was achieved by decrease in microbial populations. We have also shown that SOTE contain the concentration of tannins that may have antimethanogenic properties, but only at the higher doses even when expressed per units of total gas production or volatile fatty acids whereas no changes occur when calculated per g of *in vitro* dry matter digestibility.

S. officinalis tannin extract composition: The polymeric resin-based sorbent, Amberlite XAD used in this study is especially useful for selective enrichment and purification of tannins. In our procedure, SOTE was obtained by a modified method based on earlier works where Amberlite XAD16 was used for polyphenol purification (Seeram *et al.,* 2005). However, we achieved a notable increase in gallotanins and gallic acid. Seeram *et al.* (2005) using similar procedure with other plant

material (e.g. pomegranate peel), observed enrichment in ellagitannins. Ellagitannins are a distinct class of hydrolysable tannins structurally related to gallotannins. Hence, the method adapted to *Sanguisorba radix* is very effective in tannin extraction. The carbohydrate content that could influence the microbial proliferation by delivering additional energy source was also significantly lower in the final product than in the crude extract.

Effect of S. officinalis on rumen fermentation parameters: Some studies demonstrated that plant extracts and their constituents might decrease feed digestibility (Agarwal *et al*., 2009). Also the results of presented study suggest that plant extract inhibit the digestibility of feed components but only at the higher level of SOTE supplementation (linear $P<0.01$). Patra *et al.* (2006) highlighted that an inhibitory effects of plant extracts on feed digestibility was caused by the reduction of rumen cellulolytic bacterial populations. It might be due to the inhibition of enzymes activity (e.g. carboxymethylcellulase, xylanase and acetylesterase) by volatile essential oils, tannins and other metabolites present in analyzed plants (Patra *et al.*, 2010). The *in vitro* dry matter digestibility is also dependent on the pH and ammonia concentration. In the present study, dietary supplementation with SOTE increased the pH (linear $P<0.01$) and had also effect on ammonia production (quadratic $P<0.01$), what is partly consistent with some previous studies (Devant *et al.*, 2007; Suharti *et al.*, 2011). In the paper by Suharti *et al.* (2011) the concentration of ammonia slightly increased with the increasing level of lerak extract although, likewise in our experiment the increase was not statistically significant. In the research by Devant *et al.* (2007) average rumen ammonia concentration was not affected by treatment (blend of plant extract). It is well known that the use of tannins in ruminants' nutrition reduces protein breakdown in the rumen (Jouany and Morgavi 2007). Also the data presented in Table 2 clearly showed the stimulating effect of the highest dose of tannins on ammonia release. Probable explanation of above observation is degradation of microbial protein (decreased bacteria and protozoa numbers) and using the degradation products i.e. microbial amino acids as substrates utilized in the mentioned catabolic processes.

A decrease in total gas and methane production by plant extracts could be mediated through: (i) a reduction in protozoa and/or methanogen populations associated with the protozoa surface (Patra and Saxena 2009; Cieslak *et al.*, 2012); (ii) a direct inhibition of methanogen population (Zmora *et al.*, 2012); (iii) changes in volatile fatty acids profile and the acetate to propionate ratio (Busquet *et al.*, 2006) and (iv) inhibition of fibrolytic enzymes activity and thus the feed digestibility (Patra *et al.*, 2010). In the present study, the mitigation of methanogenesis by SOTE contained tannins was accompanied by a several factors like *in vitro* dry matter digestibility (linear $P<0.01$), TGP (linear $P<0.01$) and microbial population (bacteria, *Entodiniomorpha*, *Holotricha* and methanogens; linear $P<0.01$) what is consistent with Patra *et al.* (2010) and Cieslak *et al.*, (2013). Moreover, the differences were also observed in TGP (linear and quadratic, $P<0.01$) when SOTE was added. It should be noted, that groups with lower doses of SOTE had a numerically higher value of TGP. We hypothesized, that it could be partly due to the addition of soluble sugars to the reaction mixture through inclusion of the extracts. The soluble carbohydrates might be immediately transformed to the gases form and increased the total gas production, as well as blocked the action of tannins on methanogenesis. The relatively low sugar content, however, did not allow the statistically important changes to occur. The highest doses of SOTE in our study decreased gas as well as methane production. The effect of bioactive compounds like tannins depends on their chemical and physical structures and their concentration in the diet (Waghorn and McNabb 2003; Cieslak *et al.*, 2013), what was confirmed by the present experiment. Some previous study stated that the concentration of tannins must be less than 50 g/kg dietary dry matter not to cause negative influence on rumen ecosystem (Jouany and Morgavi 2007). In our study only the highest dose of SOTE (100 mg of SOTE/128 g of tannins per kg of dry matter) negatively affected rumen parameters. It must be also mentioned that conclusion by Jouany and Morgavi (2007) concerns the condensed tannins (CTs) whereas SOTE used in the present study was a mixture of the hydrolysable (HTs) and condensed forms. Effect of bioactive compounds depends also on the diet composition. Cieslak *et al*. (2014b) pointed out that basic components (like crude protein, crude fiber) can interact with phytochemicals and consequently the phytochemicals became physically less available for microbiota. This interaction results in a decreased activity of the whole plant as sources of phytochemicals vs. the pure extract.

The mitigation of *in vitro* rumen methanogenesis might change the rumen fermentation to redirect hydrogen from methane to hydrogen utilizing products, *i.e.*, propionate (Suharti *et al.*, 2011). Such responses could be observed in reports, where methanogenesis was decreased by anti-methanogenic compounds, such as secondary plant metabolites (Tatsuoka *et al.*, 2008; Goel *et al.*, 2008). Many researchers have demonstrated the decrease of methane production within the increase of propionate and butyrate and decrease of acetate (Busquet *et al.*, 2006; Devant *et al.*, 2007; Patra *et al.*, 2010; Suharti *et al.*, 2011). Simultaneously, the total volatile fatty acids content should not be affected due to the fact that VFA represent the main energy supply for ruminants (Van Soest 1982) and its reduction would be nutritionally unfavorable for the host animals. The situation, when

methane production is decreased and total VFA is changed has been demonstrated only partly in the present study when the 100 mg of SOTE was supplemented. However, addition of 40 mg of SOTE resulted in reduction only in methane production without altering the total VFA. In the present study other doses (1, 4, 10 or 20 mg of SOTE) did not influence methane production whereas total VFA concentration decreased at 4 and 20 mg of SOTE supplementation. Similarly to these results, garlic oils modulated rumen fermentation without altering the VFA patterns after 24 h of incubations (Kamel *et al.,* 2008). The lack of the effects of SOTE on VFA profile can be caused by the resistance of, *inter alia*, propionate-producing bacteria, such as *Selenomonas ruminantium*, to the analyzed biologically active compounds. Patra *et al.* (2010) in the research with dietary supplementation of plant extract rich in tannins, demonstrated opposite effect, namely the increase of A:P ratio by direct inhibition of *Selenomonas ruminantium* population.

Effect of S. officinalis on rumen microbes: A reduction in numbers of rumen microorganisms by plant extracts is usually attributed to the presence of secondary plant metabolites, such as tannins, essential oils and saponins. Moreover, the antibacterial effect is dose-dependent, what was confirmed by our study. Only the highest dose of SOTE decreased bacteria population count. Shan *et al.* (2007) has shown a link between the concentration of phenolic compounds in plant extracts and their antibacterial activity. However, the sensitivity of bacteria to the plant extracts depends on species, strains, cell wall structure and outer membrane (Shan *et al.,* 2007). Gram-positive bacteria are generally more sensitive to the plant extracts than Gram-negative one (Lopez *et al.*, 2005). Nevertheless, plant extracts may contain anti-Gram-negative bacteria substances, which also affected e.g. *Escherichia coli* (Shan *et al*. 2007). Moreover, in this study we demonstrated the antiprotozoal (against *Entodiniomorpha* sp.) effect of plant extract only at the highest dose of supplement, what is consistent with Patra *et al.* (2010) who reported that the inclusion of higher levels of bioactive compounds decreased the protozoa populations. At one of the lower doses of SOTE (4 mg of SOTE) evaluated in the present study the number of *Entodiniomorpha* increased. Initially, we thought that the cause was the presence of the soluble sugars, but there has already been well stated that *Entodinia* prefers starch not soluble sugars as major energy source whereas the numerous species of large *Ophryoscolecids* readily engulf and digest starch as well as plant particles rich in structural carbohydrates. For this reason, other factors like relatively small amount of bioactive compounds should be taken into account. Cardozo *et al.* (2006) confirmed our conjectures, that low concentration of analyzed factors was stimulatory to some species of protozoa. For example, dietary supplementation with cinnamaldehyde (180 mg d^{-1}) and eugenol (90 mg d^{-1}) increased the number of *Holotricha*, but the higher concentrations of cinnamaldehyde (600 mg d^{-1}) and eugenol (300 mg d^{-1}) caused no effect.

Since a reduction in methanogenesis was achieved with doses that showed antiprotozoal activity (statistically confirmed in the case of *Entodiniomorpha* sp and the tendency in the case of *Holotricha* sp) and because of lack of effects on total methanogen populations, interpretation of obtained result is very difficult. Technique used to determine the number of methanogens (FISH) does not allow us to estimate the methanogens associated with protozoa and free-living cells. Thus, in our studies we could not determine the separate effect of SOTE on methanogens attached to the protozoal surface and free-living. Moreover, it is likely that SOTE can only inhibit the metabolism of methanogens, without reducing their number, what was probably observed when 40 of SOTE was added. This hypothesis was previously suggested by Dohme *et al.* (1999) in their research on the role of the rumen ciliate protozoa for methane suppression caused by coconut oil compared diets containing either coconut oil or rumen protected fat in Rusitec fermenters filled with faunated and defaunated rumen fluid. They reported an inhibition of *in vitro* methane production without reduction of methanogens number either in faunated or defaunated rumen fluid.

Conclusion: Our study showed that SOTE has a potential to modulate rumen fermentation and to inhibit methane production. 40 mg of SOTE should be used in further long-term studies. This dose indicated antimethanogenic effect even when expressed per units of total gas production or volatile fatty acids. 40 mg of SOTE had no negative impact on *in vitro* rumen microbial populations, dry matter digestibility and volatile fatty acid content. Moreover the obtained data of the present study clearly showed the stimulating effect of the highest dose of tannins on ammonia release. Hence, further experiments are required to assess the long-term ability of SOTE to reduce methane emission and ammonia metabolism from ruminants.

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