Journal of Agricultural Science (2014), **152**, 981–993. © Cambridge University Press 2014
doi:10.1017/S0021859614000239

ANIMAL RESEARCH PAPER

Rumen antimethanogenic effect of *Saponaria officinalis* L. phytochemicals *in vitro*

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(*Received 4 February 2013; revised 17 December 2013; accepted 21 February 2014; first published online 8 April 2014*)

SUMMARY

Although the effect of saponins or saponin-containing plants on rumen microorganisms and rumen fermentation has been intensively investigated, this issue still requires special attention. Many of the phenomena occurring in the rumen related to dietary saponin supplementation are still not fully understood. *Saponaria officinalis* is a triterpenoid saponin-containing plant; thus, the aim of the present study was to evaluate the effect of *S. officinalis* L. powdered root, methanolic extract of the *S. officinalis* root (SOR) and the effect of the separated fractions (polysaccharides, saponins and phenolics) of *S. officinalis* on rumen methanogenesis, microbial population and rumen fermentation characteristics in an *in vitro* batch culture fermentation system. The powdered root (raw plant material) and *S. officinalis* extract (SOE) decreased *in vitro* methane production and consequently reduced the microbial population in a dose-dependent manner. The inhibition of methanogenesis was accompanied by changes in the volatile fatty acids profile. *In vitro* dry matter digestibility was not affected by any of the secondary compounds applied. The highest applied doses of SOE caused a higher reduction in methanogenesis (33·5 *v.* 14·4%) than the highest doses of powdered root form. Such results suggest that the basic components of the SOR could interact with phytochemicals or that the phytochemicals became physically less available for microbiota, resulting in a decreased antimethanogenic activity of the powdered root *v.* the extract. Among all the fractions selected, the saponin fraction exerted the greatest impact on ruminal fermentation. In conclusion, saponins decreased methane production by 29% in comparison with the control. This decrease was related to the reduction in protozoa and methanogen counts. It is proposed that *S. officinalis* has the potential to inhibit rumen methanogenesis without affecting rumen fermentation adversely.

INTRODUCTION

Global warming and consequent climate changes are currently blamed mostly on human activity, including burning fossil fuels and management of livestock (Keller 2009). Ruminants such as cattle produce appreciable amounts of the greenhouse gas methane, so management strategies to reduce this are important. One effective strategy for reducing methane production in the rumen involves application of dietary feed additives such as monensin (Guan *et al.* 2006). However, due to potential toxicity of the antibiotics administered to the animal, the risk of chemical residues in food products of animal origin and the development of bacterial resistance to antibiotics, the EU has banned the use of these additives (Official Journal of the European Union [OJEU] 2003). Feeding strategies for decreasing methane production and emission in ruminants now concentrate on bioactive substances that occur naturally in plants (Patra & Saxena 2010).

Bioactivity of plants and plant extracts is correlated with a number of secondary metabolites, which

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include saponins, tannins, essential oils, organosulphur compounds and flavonoids (Patra & Saxena 2010; Szumacher-Strabel & Cieslak 2010). These compounds selectively modulate the microbial population in the rumen (Wallace 2004; Zmora *et al.* 2012), decrease methane production (Busquet *et al.* 2006; Budan *et al.* 2013), improve nitrogen metabolism (Patra & Saxena 2009*a*) and modify ruminant-derived products (Calsamiglia *et al.* 2007). It is still not known which of the secondary plant compounds demonstrate the most pronounced effect. Thus, the aim of the present study was to (i) determine the effect of powdered root of *Saponaria officinalis* L. (SOR) and the methanol extract from *S. officinalis* (SOE) root on rumen fermentation, including methane production; (ii) identify which fraction of *S. officinalis* is responsible for its potential antimethanogenic properties; and to (iii) verify the possible interactions between the basic dietary compounds and the SO phytochemicals.

MATERIALS AND METHODS

All procedures were performed in accordance with the guidelines of the National Ethical Commission for Animal Research (Ministry of Science and Higher Education, Poland). The study was approved by the Local Ethical Commission (license permit no. 9/2010).

Extract preparation

Dry, ground SORs were purchased from a commercial company (Herbapol Krakow, Poland). A representative sample has been deposited at the Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation, State Research Institute, Pulawy, Poland.

Powdered SORs (500 g) were defatted with $CHCl_3$: hexane (1 : 1 v/v) in a Soxhlet apparatus. Defatted and dried material (497 g) was extracted with 70% methanol (MeOH) at room temperature for 24 h. The extract was filtered and the residue was extracted twice under the same conditions. The fourth extraction was carried out with 100% MeOH under reflux for 1 h. The combined methanolic extract was concentrated under reduced pressure and lyophilized (233 g).

Fractioning of *Saponaria officinalis* extract

The extract was suspended in double-distilled water (ddH_2O), applied on a preparative column of C18 (60 × 100 mm, LiChroprep RP-18, 40–63 μm, Merck) and eluted with H_2O (162·99 g), 30% MeOH (8·51 g) and 90% MeOH (49·90 g). In the next step, the 30% methanolic fraction was suspended in H_2O and extracted with ethyl acetate (EtOAc). The EtOAc extraction after evaporation yielded 681 mg of phenolics. The fraction containing sugars (H_2O fraction) was applied on diethylaminoethyl-cellulose (DEAE-cellulose) chromatography column, which was washed with H_2O. This gave 155·06 g of white powder containing polysaccharides. All groups of compounds (phenolics, polysaccharides and saponins) were analysed for their composition using a Waters ACQUITY UPLCTM system coupled to Waters TQ Detector (Waters Corp.) in a full scan mode (m/z = 200–2000) on a HSS C18 (1·0 × 100 mm, 1·8 μm) column.

Experiment 1

Experiment 1 consisted of a short-term *in vitro* batch culture trial with different doses (0·01, 0·25, 0·5, 1·0, 2·5 and 5·0 mg/ml buffered rumen fluid) of the powdered SOR. During one incubation period, rumen fluid was incubated in four replicates for each experimental dose, control and blank. Finally, 32 vessels were used: four vessels for each level of SOR (24 vessels), four vessels served as controls without any supplements and four vessels were blanks (without substrate).

Inoculum preparation

The rumen fluid was collected from three ruminally cannulated, Polish Holstein–Friesian dairy cows (age 3 years, mean body weight 600 ± 25 kg) fed 20 kg/day dry matter (DM) of a 62 : 38 forage to concentrate diet. The diet was composed of maize silage (5·4 kg DM), alfalfa silage (5 kg DM), meadow hay (1·6 kg DM), wet brewers' grains (0·5 kg DM), soybean meal (1·4 kg DM) and concentrate (6·1 kg DM). Drinking water was available *ad libitum*. The rumen fluid was sampled from each cow before the morning feeding. The fluid was squeezed through four layers of cheesecloth, pooled and mixed. Then it was transported within 30 min under anaerobic conditions (at 39 °C) to the laboratory and used as a source of inoculum.

Batch culture

The batch culture method was carried out according to the modified protocol of Szumacher-Strabel *et al.*

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(2004). Briefly, the rumen fluid was mixed with the buffer solution (292 mg dipotassium hydrogen phosphate (K_2HPO_4), 240 mg potassium dihydrogen phosphate (KH_2PO_4), 480 mg ammonium sulphate [$(NH_4)_2SO_4$], 480 mg sodium chloride (NaCl), 100 mg magnesium sulphate heptahydrate ($MgSO_4$) × 7 H_2O, 64 mg calcium chloride dehydrate ($CaCl_2$) × 2 H_2O, 4 mg sodium carbonate anhydrous (Na_2CO_3), and 600 mg/l cysteine hydrochloride in 1 litre of ddH_2O) at a 1 : 4 (vol/vol) ratio. The bottles were constantly purged with O_2-free CO_2. Incubation of each experiment was run at 39 °C under CO_2 in 40 ml buffered rumen fluid added to pre-warmed 125 ml vessels. The basal substrate consisted of 240 mg of meadow hay (164 g crude protein (CP)/kg DM, 494 g neutral detergent fibre (NDF)/kg DM) and 160 mg of barley (138 g CP/kg DM, 180 g NDF/kg DM), both ground to 1 mm. The basal substrate was supplemented by various doses of SOR. The incubation flasks were sealed with rubber stoppers and aluminium caps, placed in an incubator for 24 h and mixed periodically.

Estimation of pH and ammonia concentration

After 24 h of incubation, rumen fluid pH was measured (pH-meter CP-104, ELMETRON, Zabrze, Poland). Ammonia concentration was determined using the Nessler's method as described by Szumacher-Strabel *et al*. (2002).

In vitro dry matter digestibility

For the *in vitro* dry matter digestibility (IVDMD) the same experimental design as for the batch culture was used: 40 ml of buffered rumen fluid was incubated with 400 ± 1 mg of substrate for 24 h at 39 °C. After incubation, the content of the incubation flasks was transferred to previously weighed crucibles. The residues of incubation were washed with 50 ml distilled water and dried at 105 °C for 3 days. The percentage loss in weight of the feed DM was determined and presented as IVDMD.

Estimation of gas and methane production

After 24 h of incubation, gas production was estimated by the displacement of the syringe piston, which was tightly connected to the incubation flasks. Net gas production was calculated by subtracting the gas produced in blank flasks (without a substrate) from the total gas produced in the flasks containing buffered rumen fluid and substrate. For methane determination, 500 μl of gas was sampled from the headspace of the incubation flasks in a gas-tight syringe (GASTIGHT® Syringes, Hamilton Bonaduz AG, Switzerland). The sampled gas was injected into an SRI 310 gas chromatograph (Sri Instruments, Torrance, USA), equipped with a thermal conductivity detector (TCD) and Carboxen – 1000 column (mesh side 60/80, 15 FT × 1·8 INS.S, Supelco, Bellefonte, USA). Nitrogen was used as the carrier gas at a constant flow of 30·0 ml/min. The oven temperature was initially programmed at 180 °C for 1·5 min, and then the temperature was increased to 220 °C at 20 °C/min before injection of gas samples (500 μl). The observed peaks were identified by comparison of the retention times with the appropriate gas standards (mix gases 5·63% CO_2, 5·56% CH_4, 5·10% H_2 and N_2 remains, Multa S.C. Poland) using PeakSimple ver. 3.29.

Protozoa and bacteria counts

The protozoa counts were determined according to Michalowski *et al*. (1986), using a drop of buffered rumen fluid with defined volume (100 μl) under a light microscope (Zeiss, type Primo Star no. 5, Jena, Germany). The protozoa were divided into *Holotrichs* and *Entodiniomorphs* groups. The bacteria were counted under a microscope (400×) in a Thom chamber (0·02 mm depth, Blau Brand, Wertheim, Germany), according to Ericsson *et al*. (2000).

Quantification of methanogens

The methanogen population was determined by the fluorescence *in situ* hybridization (FISH) technique, according to Stahl *et al*. (1995) with some modifications as described by Pers-Kamczyc *et al*. (2011). A domain-specific oligonucleotide probe targeting all methanogens (S-S-Arc-0915-a-A-20) was used and the hybridization temperature was 56 °C. Slides were evaluated under a fluorescence microscope (Zeiss Axiovert 2000, Carl Zeiss Light Microscopy, Göttingen, Germany; 945×) with the AxioImage Observer Software and the specific signal was determined with filters specific for 4′-6-diamidino-2-phenylindole (DAPI) and Rhodamine immunofluorescence. The images were taken with an AxioCamMRm Rev. 3 Fire Wire video camera and methanogens were counted manually. The probe was labelled with Cy3 (red signal, Rhodamine) whereas all the bacteria were stained with DAPI (blue signal). All cells expressing the merged red/blue signal were considered

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to be methanogens (FISH positive). The methanogen count was expressed as ratio of methanogens to the number of all DAPI positive cells.

Estimation of volatile fatty acids

At the end of the incubation period 3·6 ml of fermented rumen fluid was stabilized with 0·4 ml of 46 mM mercury chloride ($HgCl_2$) solution and frozen until high performance liquid chromatography (HPLC) analysis (Waters 2690, Santa Clara, CA, USA). After thawing, the mixture was centrifuged at 12 000 rpm for 10 min and filtered (0·22 μm pore). Clear supernatant (10 μl) was injected into the HPLC Waters 2690 equipped with Waters 2487 Dual λ detector and Aminex HPX-87H column (300 × 7·8 mm). The mobile phase of 0·004 M sulphuric acid (H_2SO_4) was used and a 10 μl sample volume was injected into the column. The quantitative and qualitative evaluations of individual peaks were made using the external standard method prepared by mixing individual volatile fatty acid (VFA) (Supelco) and analysed with the Millennium 2001 software (version 2.15, Waters Corporation, Manchester, England).

Experiment 2

The levels of SOE used in the batch culture experiment were half the amount of SOR used in Expt 1 (i.e. 0·005, 0·13, 0·25, 0·50, 1·25 and 2·50 mg/ml of buffered rumen fluid). The content of active substances in the SOE in Expt 2 was the same as in the SOR in Expt 1. A batch culture experiment was performed and all parameters of rumen fluid were analysed as described in Expt 1. During one incubation period, rumen fluid was incubated in four replicates for each experimental dose, control and blank. Finally, 32 vessels were used: four vessels for each level of SOR (24 vessels), four vessels as the control without any supplements, and four vessels as blanks (without the substrate).

Experiment 3

To identify the fraction of *S. officinalis* with potential antimethanogenic activity, a batch culture experiment was undertaken. The amount of supplements was calculated based on the most promising effect on methane mitigation from Expt 2 (2·5 mg SOE/ml buffered rumen fluid). A set of 24 vessels was used which included four control vessels, four vessels for each of the three SOE fractions (polysaccharides, saponins, and phenolics), four vessels of fraction mixture and four blank vessels. The content of each biologically active fraction was the same as for the highest SOE level used in Expt 2. Batch culture was set up and all of the rumen fluid parameters were analysed as described in Expt 1.

Statistical analyses

For all rumen fluid parameters, four samples were analysed for each group within every experiment. The data obtained from Expts 1 and 2 (SOR and SOE, respectively) was subjected to one-way analysis of variance (ANOVA). Multiple comparisons between groups (SOR or SOE levels) were evaluated statistically by the Holm–Sidak pairwise *post hoc* analysis. Moreover, the data was analysed with the polyanova routine within the GenStat 15.0 software (VSN International, Hemel Hempstead, UK). Such data allowed for the assessment of *S. officinalis* by partitioning variance into linear and non-linear (quadratic) contrasts. In Expt 3, multiple comparisons between groups (different fractions) were evaluated statistically with the use of one-way ANOVA with the Holm–Sidak pairwise *post hoc* analysis. Degree of freedom was partitioned among different fractions. P values <0·05 were considered significant.

RESULTS

Impact of *Saponaria officinalis* powdered root on *in vitro* fermentation

Relative to the control, the pH and IVDMD values were similar among groups regardless of the SOR level. However, a significant linear and quadratic response was indicated for ammonia concentration (both $P<0·001$). Compared to the control, increasing dietary supplementation with SOR caused a significant decrease ($P<0·05$) in ammonia production, i.e. 42·8% after the addition of 2·5 mg SOR (Table 1). Moreover, increasing SOR supplementation was associated with decreases in methane (by *c.* 14·4%) as compared with the control. Also, all of the rumen microbes analysed decreased concurrently with increases in SOR supplementation (Table 1). The concentration of total VFA increased linearly in the medium due to inclusion of SOR (Table 1).

Impact of extract on *in vitro* fermentation

The extraction of SORs using aqueous methanol yielded 500 g extract/root kg. The extract

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Table 1. *Effects of Saponaria officinalis L. powdered root (SOR) on the in vitro ruminal fermentation. Data are presented as mean with S.E.M.*

	SOR level (mg/ml buffered rumen fluid)										
Items	Control*	0·01	0·25	0·5	1·0	2·5	5·0	S.E.M.	*P*	Linear	Quadratic
pH	6·6	6·6	6·5	6·6	6·7	6·6	6·6	0·01	0·011	0·352	0·026
Ammonia concentration (mM)	17·2	17·9	17·2	16·6	13·9	10·9	9·9	0·59	<0·001	<0·001	<0·001
IVDMD	0·32	0·34	0·35	0·32	0·36	0·34	0·34	0·078	0·803	0·796	0·587
Methane (mM)	10·0	10·5	9·3	9·3	7·6	7·5	8·5	0·13	<0·001	<0·001	<0·001
TGP (ml)	65·5	69·2	63·2	63·0	61·2	67·5	74·7	0·87	<0·001	<0·001	<0·001
Microbial analysis (cells/ml)											
Total bacteria ($\times 10^8$)	34·3	34·6	32·8	32·3	27·2	20·4	14·4	1·41	<0·001	<0·001	<0·001
Total methanogens†	32·6	33·6	29·7	26·0	20·3	21·3	14·2	1·31	<0·001	<0·001	<0·001
Total protozoa ($\times 10^3$)	58·3	59·5	73·6	60·5	51·9	34·1	18·7	3·31	<0·001	<0·001	<0·001
Entodiniomorphs ($\times 10^3$)	54·8	55·6	69·8	56·3	48·5	31·8	18·5	3·08	<0·001	<0·001	<0·001
Holotrichs ($\times 10^3$)	3·6	3·9	3·8	4·2	3·4	2·3	0·1	0·25	<0·001	<0·001	<0·001
Volatile fatty acids (mM)											
Total VFA	60·6	62·9	62·9	62·8	64·6	67·7	72·0	0·78	<0·001	<0·001	0·469
Acetate (A)‡	62·8	61·2	62·1	61·9	60·0	58·1	56·0	0·47	<0·001	0·043	0·960
Propionate (P)‡	22·5	24·3	23·4	24·0	25·9	28·0	28·3	0·44	<0·001	<0·001	0·013
Butyrate‡	14·7	14·5	14·5	14·1	14·1	13·9	15·7	0·14	<0·001	<0·001	0·019
A/P	2·8	2·5	2·6	2·6	2·3	2·1	2·0	0·06	<0·001	<0·001	<0·001

TGP, total gas production; IVDMD, *in vitro* dry matter digestibility; VFA, volatile fatty acid; A/P, acetate to propionate ratio.

* No addition of *S. officinalis* L. powdered root.

† The ratio of methanogens in the population of microorganisms stained with 4′-6-diamidino-2-phenylindole (DAPI).

‡ VFAs as molar proportions.

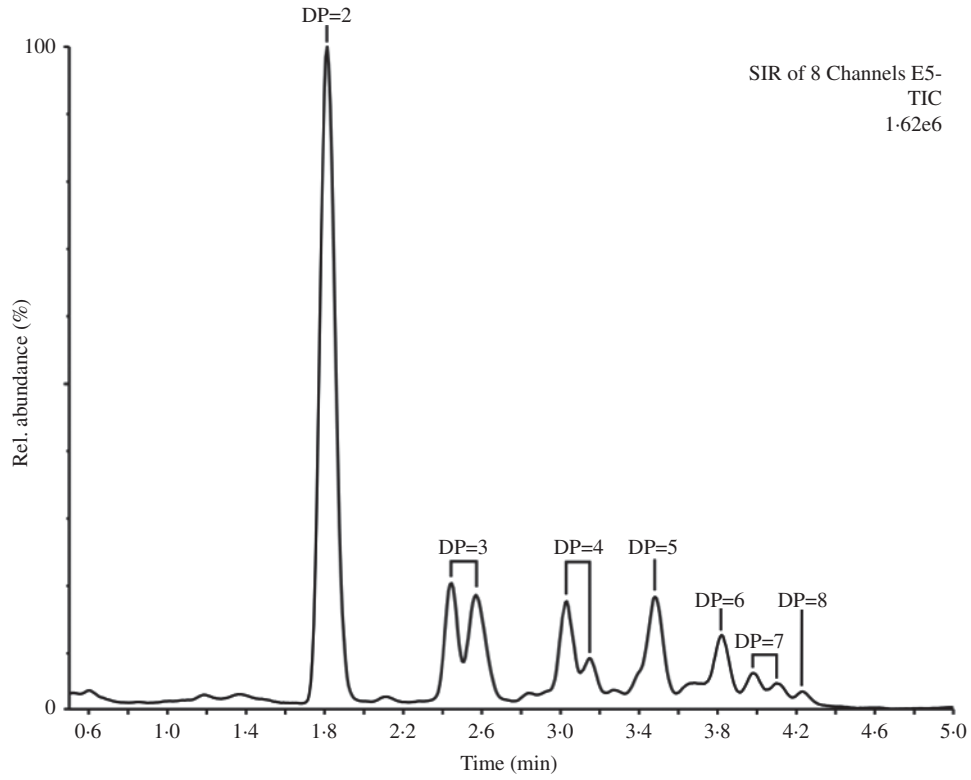

Fig. 1. The ultra-performance liquid chromatography–tandem mass spectrometer (UPLC–MS) profile of *Saponaria officinalis* L. oligosaccharides contained in the water fraction; DP = 2, 3, 4, 5, 6, 7, 8 – compounds having a degree of polymerization respectively two, three, four, five, six, seven or eight.

composition was characterized by gas chromatography and the following compound fractions were identified: polysaccharides (69·5 mg/g), saponins (21·4 mg/g), polyphenols (3·65 mg/g) and others, separated mostly as catechins. Within the separated fractions, polysaccharides were the most polar. The composition of different polymers from two up to eight sugar units (Fig. 1) was observed after the structural ultra-performance liquid chromatography–tandem mass spectrometer (UPLC–MS) analysis. Dimer was the most abundant polymer type, and the amount of higher polymers decreased gradually. The peaks observed for the polyphenol fraction displayed an ultra-violet (UV) spectrum characteristic for phenolic acid (Fig. 2). Chromatography of the saponin fraction revealed a number of compounds with masses ranging from *m/z* 795–1905 (Fig. 3). Based on MS/MS analyses some of the peaks could be identified as saponariosides A, B, C, D, F, G, I, K, L, vaccarioside D, dianchineoside B and other as glycosides of quillaic, gypsogenic and 16α-hydroxygypsogenic acids.

Supplementation of the rumen fluid with increasing amounts of SOE did not change the ruminal fermentation parameters (e.g. pH, IVDMD and ammonia). A decrease in methane concentration and microbial populations has been stated and confirmed by significant quadratic and linear responses ($P < 0{\cdot}05$; Table 2). The concentration of total VFA, as well as molar proportions of propionate, elevated with increasing amounts of SOE (Table 2).

Supplementation of ruminal fluid with the different fractions of *S. officinalis* decreased ammonia production. Polysaccharides had the biggest impact (9·61 *v*. 14·22 mM/l, $P < 0{\cdot}05$) (Table 3). The IVDMD did not differ when compared with the control, however there were some significant ($P < 0{\cdot}05$) changes between the fractions (polysaccharides or phenolics *v*. saponins) (Table 3). The total gas production decreased with the addition of all separated *S. officinalis* fractions ($P < 0{\cdot}05$; Table 3). Furthermore, each fraction caused a significant decrease ($P < 0{\cdot}05$) in methane concentration, polysaccharides (by 11·6%), saponins (by 29·0%) and polyphenols (by 15·8%) (Table 3). In addition, each fraction altered total protozoa and methanogen populations ($P < 0{\cdot}05$; Table 3). The effect was fraction-dependent and ranged from a 13·0% reduction of *Entodiniomorph* for the group with polysaccharides to a 79·4% inhibition of methanogen

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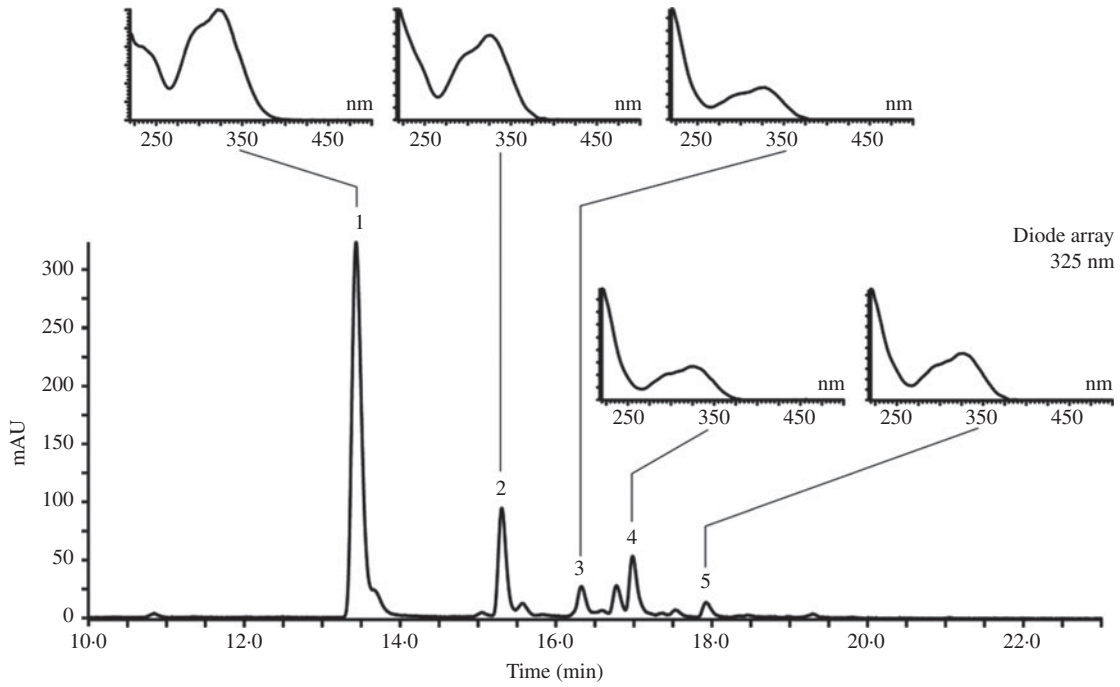

Fig. 2. The ultra-performance liquid chromatography–ultraviolet (UPLC–UV) profile of *Saponaria officinalis* L. phenolic compounds contained in the 30% methanolic fraction. Monitoring at a wavelength 325 nm. Insets: UV spectra of peaks 1–5.

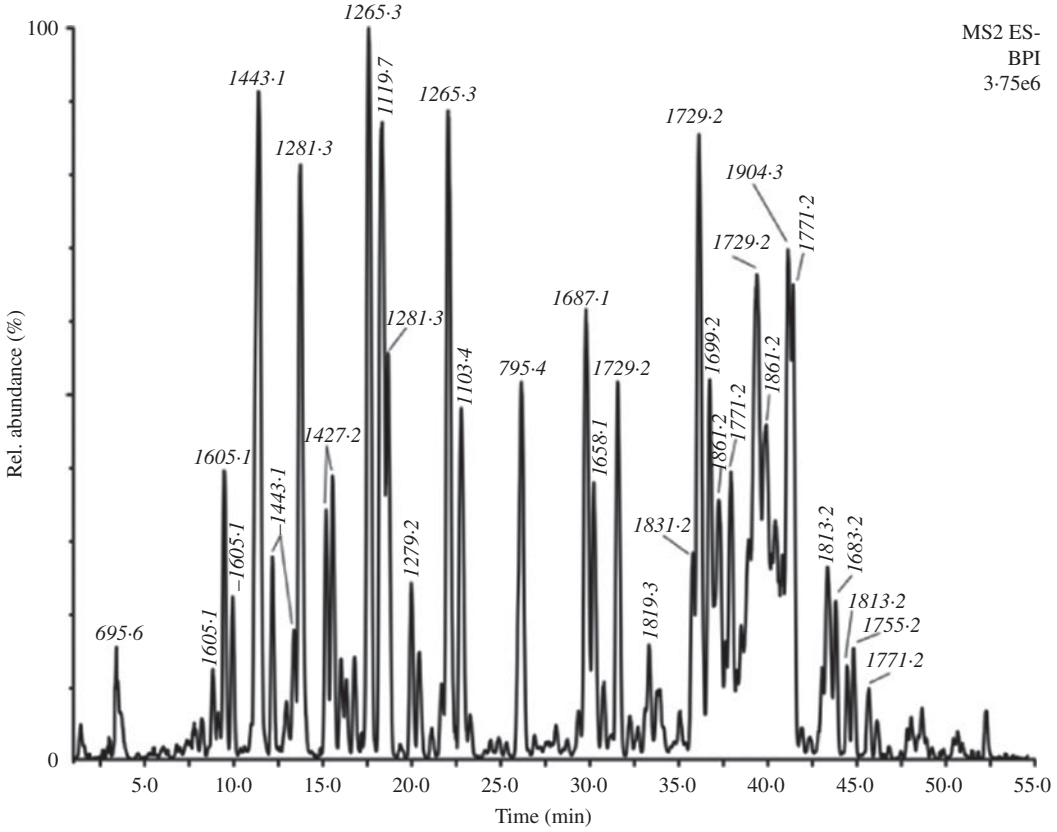

Fig. 3. The ultra-performance liquid chromatography–tandem mass spectrometer (UPLC–MS) (negative mode; full scan) of *Saponaria officinalis* L. saponins contained in the 90% methanolic fraction.

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Table 2. *Effects of Saponaria officinalis L. extract (SOE) on in vitro ruminal fermentation. Data are presented as mean with S.E.M.*

	SOE levels (mg/ml buffered rumen fluid)										
Items	Control*	0·005	0·13	0·25	0·50	1·25	2·50	S.E.M.	*P*	Linear	Quadratic
pH	6·4	6·5	6·5	6·4	6·4	6·3	6·2	0·02	<0·010	0·006	0·445
Ammonia concentration (mM)	17·1	17·6	19·4	16·0	15·7	14·8	13·9	0·71	0·489	0·259	0·511
IVDMD	0·38	0·34	0·33	0·35	0·33	0·31	0·32	0·092	0·439	0·339	0·441
Methane (mM)	10·6	10·3	7·8	7·8	7·9	7·6	7·0	1·14	<0·001	<0·001	<0·001
TGP (ml)	44·0	45·2	45·2	44·0	43·5	43·7	37·2	43·3	0·588	0·904	0·488
Microbial analysis (cells/ml)											
Total bacteria ($\times 10^8$)	32·2	33·2	32·0	31·4	25·2	19·4	12·9	1·41	<0·001	<0·001	<0·001
Total methanogens†	34·0	35·4	29·1	28·1	28·1	26·1	20·4	0·96	<0·001	0·002	0·129
Total protozoa ($\times 10^3$)	46·2	69·4	67·7	64·7	33·3	17·0	11·7	4·37	<0·001	<0·001	0·003
Entodiniomorphs ($\times 10^3$)	43·8	67·0	65·6	62·4	31·5	16·2	11·5	4·23	<0·001	<0·001	0·003
Holotrichs ($\times 10^3$)	2·3	2·4	2·1	2·3	1·8	0·8	0·2	0·16	<0·001	<0·001	0·023
Volatile fatty acids (mM)											
Total VFA	55·9	53·5	44·1	53·6	57·1	59·5	64·4	1·35	<0·001	<0·001	0·629
Acetate (A)‡	64·5	64·6	64·3	63·9	62·2	59·9	58·0	0·47	0·003	0·004	0·984
Propionate (P)‡	20·0	20·6	20·9	21·4	23·7	27·0	27·9	0·58	<0·001	<0·001	0·016
Butyrate‡	15·4	14·9	14·9	14·7	14·1	13·0	14·1	0·17	0·003	0·005	0·098
A/P	3·2	3·1	3·1	3·0	2·6	2·2	2·1	0·08	<0·001	<0·001	<0·001

TGP, total gas production; IVDMD, *in vitro* dry matter digestibility; VFA, volatile fatty acid; A/P, acetate to propionate ratio.

* No addition of *S. officinalis* L. powdered root.

† The ratio of methanogens in the population of microorganisms stained with 4′-6-diamidino-2-phenylindole (DAPI).

‡ VFAs as molar proportions.

Table 3. *Effects of Saponaria officinalis L. fractions on in vitro ruminal fermentation. Data are presented as mean with S.E.M.*

	Adding fraction of *S. officinalis* L.						
Items	Control*	Polysaccharides	Saponins	Phenolic	Mix†	S.E.M.	*P*
pH	6·3	6·1	6·2	6·3	6·0	0·02	<0·01
Ammonia concentration (mM)	14·2	9·6	12·7	13·7	9·5	0·40	<0·001
Methane (mM)	11·7	10·3	8·3	9·8	10·3	0·38	<0·001
IVDMD	0·3	0·4	0·3	0·4	0·3	0·01	0·160
TGP (ml)	53·75	43·5	32·7	33·7	47·0	1·52	<0·001
Microbial analysis(cells/ml)							
Total bacteria ($\times 10^8$)	32·0	41·8	41·9	36·1	34·6	0·87	<0·001
Total methanogens‡	32·5	15·1	6·7	12·6	20·0	2·07	<0·001
Total protozoa ($\times 10^3$)	47·0	40·7	17·1	36·1	21·7	1·82	<0·001
Entodiniomorphs ($\times 10^3$)	45·8	39·8	16·6	35·2	21·0	1·84	<0·001
Holotrichs ($\times 10^3$)	1·3	0·9	0·5	0·9	0·7	0·04	<0·001
Volatile fatty acids (mM)							
Total VFA	82·6	98·3	76·3	81·5	98·1	2·81	<0·001
Acetate (A)§	63·7	62·6	62·2	63·5	61·5	1·19	<0·001
Propionate (P)§	23·8	25·4	26·5	23·4	25·9	0·28	<0·001
Butyrate§	12·5	12·0	11·2	13·1	12·5	0·16	<0·001
A/P	2·7	2·5	2·3	2·7	2·4	0·02	<0·001

TGP, total gas production; IVDMD, *in vitro* dry matter digestibility; VFA, volatile fatty acid; A/P, acetate to propionate ratio.
* No addition of *S. officinalis* L. powdered root.
† Mixture of polysaccharides, saponins, and phenolics fractions.
‡ The ratio of methanogens in the population of microorganisms stained with 4′-6-diamidino-2-phenylindole (DAPI).
§ VFAs as molar proportions.

growth for the group supplemented with saponins. In contrast, the number of bacteria increased in the group with additional polysaccharides and saponins ($P<0·05$). The concentrations of the total VFA did not differ among any of the fractions and the control (Table 3). In comparison to the control, molar proportions of acetate decreased in all groups, except for the group with phenolic acid only. The molar proportions of propionate increased in all experimental groups whereas the molar proportions of butyrate increased in the phenolic group only. Furthermore, increasing the amount of diet supplementation with SOR and SOE decreased the acetate to propionate ratio.

DISCUSSION

The present study has found that different fractions of *S. officinalis* may affect ruminal fermentation and microbial community structure. The potential effect of SOR and SOE were evaluated in a batch culture system. To our knowledge, this is the first report on the simultaneous testing of particular fractions extracted from one plant, to observe their potential *in vitro* effect on ruminal fermentation and microbial populations.

A direct effect of phytochemicals (bioactive components of plants) on ruminal microbes has been suggested (Tavendale *et al.* 2005). Roots and extract of *S. officinalis* are rich sources of phytochemicals and the results of the present study demonstrated that the addition of SOR or SOE decreased methane production. Significant linear and quadratic responses were correlated with these parameters. Similarly, *in vitro* studies done on sheep show that supplementing their diet with hydro-ethanolic extract of *S. officinalis* roots decreased methane production numerically (Budan *et al.* 2013).

Moreover, it may be concluded that both *S. officinalis* and its fractions have the ability to decrease methanogenesis by direct changes in protozoa and methanogen communities. These conclusions correspond to the results presented by Zhou *et al.* (2011) but only in the case of protozoa. The changes in microbial communities observed in the present study were not accompanied by a decrease in DM digestibility. The total VFA concentration was higher after the application of SOR or SOE as confirmed by significant linear and quadratic responses.

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These results also confirm the findings of Cieslak *et al*. (2012) on phytochemicals from *Vaccinium vitis idaea*, where methane production decreased without causing a negative effect on DM digestibility or total VFA composition. Hristov *et al*. (1999) reported that the extent of ruminal degradability of DM was not affected by saponins, although the degradation rate for insoluble DM increased. The rate of degradation of insoluble DM, as well as of other DM fractions (not evaluated in the present study) could explain the observed situation. Moreover, the results of the present study show that a decrease in methane production could bring about a decrease in acetate to propionate ratio, resulting in linear and quadratic responses. According to Demeyer & Van Nevel (1975), a lack of negative effects on total VFA values as well as decreased acetate to propionate ratio reflected depressed production of CH_4. This reduction resulted from changed hydrogen utilization in the propionate pathway, without adversely affecting the fermentation process. These results are confirmed by the present study, although, with regard to the *S. officinalis* fractions used in Expt 3, this hypothesis is not always confirmed.

However, the present paper demonstrated that the effects of particular secondary *S. officinalis* compounds differ depending on their chemical structure and dosage. Patra & Saxena (2009*a*) and Cieslak *et al*. (2013) also confirmed these findings, since they showed that the effect of phytochemicals depends on diet composition, microbial structure and microbiota adaptation to the rumen environment. However, Patra & Saxena (2009*a*) and Cieslak *et al*. (2013) thought that the different types of phytochemicals might use different mechanisms of action on ruminal fermentation. Saponins, for example, might decrease methanogenesis by inhibiting the protozoa population (defaunation) and/or via a direct decrease in protozoa activities (i.e. rate of methanogenesis or expression of methane producing genes) and the number of methanogens (Patra & Saxena 2009*b*; Cieslak *et al*. 2013). It was also assumed by Cieslak *et al*. (2013) that saponins may affect specific rumen microorganisms selectively and thus change the rumen metabolism (i.e. VFA profile), which is only partially consistent with the present results.

Saponins exert greater negative effects on protozoa populations than on bacteria. Such an effect was confirmed in Expt 3, where the fraction of saponins was analysed: the 43% reduction in the protozoa population was not associated with reductions in the bacteria population when this *S. officinalis* fraction was used. The negative effect of saponins on protozoa cells may also be related to the presence of sterols in protozoa membranes, but not in bacterial membranes (Williams & Coleman 1992). Thus, the permeability of the protozoa cell membrane is affected, resulting in lysis of the protozoa (Hart *et al*. 2008).

It should also be noted that rumen protozoa showed a different metabolic response to the tested form and to the concentration of the experimental factors, as previously reported by Kisidayova *et al*. (2006) and Cieslak *et al*. (2009). This was confirmed in the current Expts 1 and 2, where the lowest doses of SOR or SOE did not always affect protozoa populations negatively. However, García-Gonzalez *et al*. (2008) showed that phenolics decreased methanogenesis by reducing digestibility and changes in the VFA profile. In the present study, the phenolic fraction of *S. officinalis* changed only the protozoa and methanogen populations, with a numerical increase in numbers of bacteria and IVDMD.

It should be stressed that the term *phenolics* may cover a very broad spectrum of chemicals with diverse structures. According to the present results, the UV spectrum showed that *S. officinalis* phenolics belong to the phenolic acid class. This may explain the discrepancies between the different studies. The explanation indicates the importance of information on the structural features of compounds used in biological tests to present the final conclusions.

The present study also demonstrated probable antagonistic interactions between dietary basic compounds (i.e. CP) and phytochemicals. The effect of SOE was more pronounced than that of SOR in the case of methane production. The highest doses of SOE caused a higher reduction in methanogenesis than the highest doses of powdered root form. Such a result suggests that the basic components of SOR could interact with the phytochemicals or that the phytochemicals were physically less available for microbiota and thus decreased the antimethanogenic activity of the root.

Most of the responses in the present study showed highly significant linear and quadratic responses to increased SOR and SOE doses. No differences in the numbers (even increased protozoa counts) of lower SOR (0·01–0·5) or SOE (0·005–0·25) doses were noticed. The current authors believe that the above data were obtained due to the sugar moiety of saponins used as a nutrient for microorganisms. It is important to note that the potential interactions between dietary basic compounds and phytochemicals should be taken into consideration when interpreting the data.

https://doi.org/10.1017/S0021859614000239 Published online by Cambridge University Press

As indicated in Expt 3, supplementation of the polysaccharides fraction caused a reduction in methanogen and protozoa populations by 53 and 13%, respectively. Total bacteria number increased by 30%.

The data obtained from the present study does not allow for an unambiguous explanation of the observed phenomena, however ammonia utilization in the rumen is intrinsically related to carbohydrate availability (Russell *et al*. 1983). When carbohydrate availability increases, ammonia concentration in the rumen decreases due to the direct incorporation of amino N into microbial protein (Russell *et al*. 1983). Also, Agle *et al*. (2010) showed that increased concentrate proportion in the diet of dairy cows resulted in reduced ruminal ammonia concentration. Only a few studies have investigated the direct influence of polysaccharides on ammonia concentration in the rumen. Leedle *et al*. (1986) showed that ruminal ammonia concentration increased slightly, from 21·3 to 24·9 mM, 2 h after feeding on the high forage diet (rich in sugars) and decreased by 21% after 16 h, to 16·8 mM. Headon *et al*. (1991) suggested that when using glycofractions of the Yucca plant, ammonia concentration decreased due to the ammonia-binding properties of glycofractions. As reported by Headon *et al*. (1991) the decrease in ammonia concentration was also associated with an 8% increase in the bacterial population, from $23{\cdot}1 \times 10^{-9}$/ml before feeding to $24{\cdot}9 \times 10^{-9}$/ml 16 h after feeding.

The present study used a separated fraction of polysaccharides. This fraction, as a main source of rapidly available energy, significantly increased the bacterial population and decreased ammonia concentration by 32% compared to the control group. Hence, ammonia did not limit bacterial growth in experimental diets when polysaccharides were used.

According to some previous studies, plants as a source of saponins can reduce the level of ammonia in the rumen fluid (Wallace 2004; Mao *et al*. 2010). The results from the present study demonstrated highly significant linear and quadratic responses to a decreased ammonia concentration after supplementation of SOR. However, there were no differences and a lack of linear and quadratic responses were noticed when SOE doses were used. The difference described above is difficult to explain. It could be hypothesized that, as mentioned by Szumacher-Strabel & Cieslak (2010), the supplementation of bioactive phytochemicals (e.g. saponins) to the ruminant diet can result in reduced ammonia concentration as a consequence of the decrease in deamination and peptidolysis. These processes were not analysed, but it could be suggested that, unlike in the study by Wallace *et al*. (1994), supplementation of saponins inhibited the rumen proteolytic activity.

Moreover, the decrease in the rumen ammonia concentration may also depend on the indirect result of the decreased protozoal number caused by the supplemented phytochemicals, such as saponins. This was observed by Wallace *et al*. (1994) as well as in the present study. These observations also demonstrate the impact of protozoa on ammonia concentration. The production of excess ammonia in the rumen may therefore be limited. However, based on the results of Expt 3, it can be concluded that the saponin fraction is not the strongest factor limiting ammonia concentrations. A higher decrease in the ammonia concentration was caused either by the polysaccharide (from any of the applied fractions) or by the mixture of all fractions.

CONCLUSIONS

Saponaria officinalis L. has the potential to mitigate methane production by decreasing microbial (methanogens, protozoa) populations without unfavourable alterations in VFA and IVDMD profiles. The SOE caused a higher reduction in methanogenesis than the powdered root (33·49% *v*. 14·4%). This finding suggests that basic components of *S. officinalis* could interact with the phytochemicals or that the phytochemicals were simply physically less available for microbiota and thus decreased the antimethanogenic activity of the root. It was observed that the mechanism of SOE action was similar to that of SOR and it has been proven that the saponins are mostly responsible for the reduction of the *in vitro* methane production.

The research grant (N N311 476339) provided by the Polish Ministry of Science and Higher Education is gratefully acknowledged. The authors would like to thank Marta Cieslik and Marcin Szymczak for their assistance during the experiments, as well as to Zofia Madeja for improvement of the English.

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