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The potential of the wild dog rose (*Rosa canina*) to mitigate *in vitro* rumen methane production*

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ABSTRACT

This study examined the potential of liquid wild dog rose (*Rosa canina*) seeds oil and solid seeds residue obtained after CO_2 extraction in supercritical conditions to mitigate rumen methane production *in vitro*. Two experiments were carried out. The substrate comprised of a mixture of meadow hay and barley meal (60:40) for the control diets (CON1 in experiment with oil and CON2 in experiment with residue). The control diets were supplemented up to 5% in dry matter of rose seeds oil (RO) and 5% of rose seeds residue (RR). The following parameters were measured: pH, ammonia, volatile fatty acids, ciliate protozoa and bacteria count, methane concentration and methanogens population. In the RO treatment a decrease in methane production and an increase in the *Archea* population were observed. In the RR treatment no change in methane production was reported, whereas some variations in protozoal populations were detected in relation to CON2. The potential to mitigate methane production was reported only in wild dog rose seeds oil treatment. Besides, no negative effect of wild dog rose seeds residue on rumen processes was stated, what may predispose this protein and fibre containing by-product to be utilized as ruminants dietary ingredient.

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INTRODUCTION

During the processing of agricultural raw materials to foods, organic residue defined as waste is produced. Organic waste disposal is now the subject of European discussion because of environmental pollution as well as high utilization costs. Due to its composition fruit waste containing peels, seeds, etc., should be rather recognized as by-product than waste. This is of special interest considering new aspects of utilization of fruit waste as feed additives or supplements with high nutritional value. These are high-value products and their recovery may be economically attractive (Oreopoulou and Tzia, 2007). Fruit waste containing seeds is rich either in basic nutrients or in biologically active compounds and hence may be a valuable component of animal diets. Biologically active compounds may also modulate rumen microbial processes. Wild dog rose (*Rosa canina*) fruits have been already used in Poland as substrate for juice production. During that process seeds oil and seeds organic residue are produced, which are a rich source of unsaturated fatty acids. The seeds residue is also a rich source of phytochemicals, like tannins and flavonoids. The last two factors may act as chemical inhibitors of ruminal methane formation (Broudiscou et al., 2002; Woodward et al., 2002). Rose seeds oil and residues are of special interest because of high content of linolenic acid. Experiments with dietary supplementation of other plant oils that are also the source of this fatty acid showed the potential to reduce methane rumen production (Cieślak et al., 2006a,b).

The United Nations Framework Convention on Climate Change and its Kyoto Protocol have recognized that any effort to address climate change must be directed toward limiting greenhouse gases (GHG) emissions. Once the GHGs have been emitted to the atmosphere, not much can be done to remove these or to mitigate the effects of increasing concentrations on the climate systems (Pulles, 2011). Hence, adequate utilization of organic materials originating from juice production, like wild dog rose seeds oil and seeds residue, as nutrient sources for livestock might be of interest in order to reduce production cost and methane emission to atmosphere. The last became very crucial.

Because of high ether extract content in rose seeds oil and residue, as well as because of phytochemicals concentration in wild dog rose seeds residue, this study was carried out to examine the *in vitro* potential of wild dog rose seeds oil and rose seeds residue to mitigate rumen methane production and also to evaluate the possibility of their utilization as ruminant dietary components.

MATERIAL AND METHODS

Process of extraction

The rose seeds were used as test material separated from dried pomace of fresh fruit processing during concentrated juice production process at Alpex company (Eastern Poland). The rose seeds were crushed using two grinding rolls crusher. Carbon dioxide of food grade was used as a solvent (Nitrogen Plant Production S.A.). The extraction was carried out in The Fertilizers Research Institute's high pressure pilot plant. The extraction tests were carried on a pilot plant (extractor volume 20 l, ID=0.1 m) with a two stage separation. The pilot plant is depicted in Figure 1. The extractor was charged with 8 kg of raw material for each extraction test. CO_2 flow rate of 2.5 kg/m^2 was used.

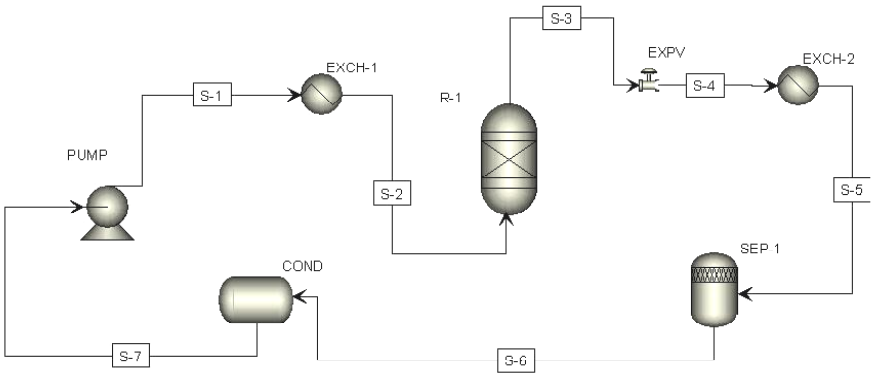

Figure 1. A simplified diagram of the high pressure extraction pilot plant

Liquid carbon dioxide filled in a condenser/storage tank (COND) was used as a solvent. The cooled CO_2 was fed to the piston pump (PUMP) and compressed up to the operating pressure. Then, the compressed carbon dioxide passed through a heat exchanger (EXCH-1) to reach the extraction temperature level. The extraction column (R-1) of 20 l volume with 100 mm inside diameter and 2 m length was packed with grinded rose seeds. The extraction column was heated with a hot water and its temperature was controlled by a PLC computer based system at the desired level. Then, the superheated CO_2 at supercritical condition passed through the extraction column, where extraction process took place. Mixture of extract and CO_2 flew through the expansion valve (EXPV) and downstream the valve the pressure was reduced to the separation level (approx. 60 bars). Simultaneously, the stream was heated up in the heat exchanger (EXCH-2) upstream the separator (SEP-1). The next separation stage, not shown here, was designed to separate water. CO_2 stream free of extract was then redirected to the condenser /storage tank (COND) thus enabling its circulation around the plant.

The extract weight was measured by laboratory balance until no oil was extracted out from the rose seed bed. The extraction process was carried out at pressure 280 bars and temperature 40°C. Extraction resulted in liquid rose seeds oil and solid rose seeds residue used for further studies.

Determination and concentration of phytochemicals in wild dog rose seeds residue after supercritical CO_2 extraction

Powdered residue after CO_2 extraction in supercritical conditions (0.2 g) was extracted with ASE 200 Accelerated Solvent Extractor (Dionex, USA) in two cycles with 40 ml of 90% MeOH at 100ºC for 20 min. Solvent was removed under reduced pressure at 45ºC. The dry residue was dissolved in 5 ml of distilled water, and the solution was loaded on a C18 Sep-Pak cartridges preconditioned with water. The cartridges were washed first with 2 ml of water, and then 5 ml of 50% MeOH. These eluates were evaporated to dryness, dissolved in 1 ml of 50% MeOH, and used for determination.

The high performance liquid chromatographic (HPLC) analyses of flavonoids were performed according to previously described procedure (Stochmal and Oleszek, 2007). Calibration curve was performed and the content of flavonoids determined based on the kaempferol glucoside. For identification and determination of tannins the liquid chromatography-mass spectrometry technique was used (Janda et al., 2007). The amount of tannins was calculated using (+)-catechin as an external standard.

Determination and concentration of fatty acids in wild dog rose seeds oil and seeds residue after supercritical CO_2 extraction

Samples of extracted seeds oil (RO) and seeds residue (RR) after CO_2 extraction in supercritical conditions were analysed according to AOAC (2007) for dry matter (method no. 934.01) and ash (method no. 942.05). Crude protein was determined by Kjel-Foss Automatic 16210 analyser (method no. 976.05), ether extract by Soxtec System HT analyser (method no. 2003.05), and crude fibre by Tecator Fibertec System I (method no. 978.10). The fatty acid composition in oil and residue (Table 1) were analysed according to the protocol described by Cieślak et al. (2009b) (Table 1).

In vitro batch culture system

Effects of wild dog rose seeds oil and seeds residue were examined using *in vitro* batch culture system with mixed ruminal microorganisms. Rumen fluid was

Table 1. Ingredient and fatty acids (FA) composition of extracted oil and residue of wild dog rose seeds

Item	Rose seeds oil	Rose seeds residue
Dry matter, g/kg	998.4	950.5
Organic matter, g/kg DM[1]	-	969.6
Crude protein, g/kg DM[1]	-	85.3
Crude fibre, g/kg DM[1]	-	536.0
Crude fat, g/kg DM[1]	1000.0	7.25
N-free extractives, g/kg DM[1]	-	341.0
g/100 g, FA[2]		
C14:0	0.03	0.83
C16:0	2.61	13.0
C16:1	0.17	0.69
C18:0	0.88	5.66
C18:1 *cis* 9	11.6	12.9
C18:2 n-6	51.1	42.8
C18:3 n-3	33.0	23.4
SFA[3]	3.60	19.5
UFA[4]	96.4	80.5
MUFA[5]	12.4	14.4
PUFA[6]	84.0	66.1

[1] dry matter; [2] fatty acids;[3] saturated fatty acids (C14:0; C16:0; C18:0);[4] unsaturated fatty acids (C16:1; C18:1 *cis* 9; C18:1 *cis* 11; C18:2 n-6; C18:3 n-3); [5] monounsaturated fatty acids (C16:1; C18:1 *cis* 9; C18:1 *cis* 11); [6] polyunsaturated fatty acids (C18:2 n-6; C18:3 n-3)

collected before morning feeding from 680 kg ruminally fistulated Polish Holstein-Friesian cow maintained on grass silage (7 kg DM), maize silage (5 kg DM), meadow hay (1.8 kg DM) and concentrate (1 kg DM). The cow was fitted with ruminal cannulae by surgical procedure approved by the guidelines of Local Ethical Board for the treatment of animals. Ruminal content was squeezed through four layers of cheesecloth into a Schott Duran® bottle (1 l) with an O_2-free headspace and immediately transported to laboratory. The *in vitro* experiments were carried out according modified protocol by Szumacher-Strabel et al. (2004). Briefly, rumen fluid was diluted with buffer (2:3) solution (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 4 mg Na_2CO_3 and 600 mg cysteine hydrochloride per litre) and 40 ml transferred to incubation vessels containing 0.4 g substrate and incubated in anaerobic conditions at pH 6.5 and 39ºC.

Substrate used for batch culture system was a mixture of meadow hay and barley meal (60:40) for the control groups (CON1 in experiment with wild dog rose seeds oil and CON2 in experiment with wild dog rose seeds residue). The control diets were supplemented up to 5% in dry matter of wild dog rose oil extracted from seeds, received after CO_2 extraction in supercritical conditions (RO; 12 mg extracted oil and 148 mg of barley meal) and 5% of seeds residue

after CO_2 extraction in supercritical conditions (RR; 8 mg residue after extraction and 152 mg of barley meal). The amount of RO (up to 5% in dry matter) used in present *in vitro* experiment were established on the basis of the previous experiment (Cieślak et al., 2006b) as a safety level for rumen fermentation, that is also in agreement with NRC (2001) recommendations. The substrates were incubated under anaerobic conditions for 8, 24 and 48 h.

Chemical analysis

The following parameters in rumen fluid after *in vitro* fermentation were measured: pH, ammonia (Szumacher-Strabel et al., 2002), volatile fatty acids (VFA; Czauderna et al., 2008), ciliate protozoa (*Entodiniomorphs* and *Holotrichs*; Michałowski et al., 1986) and bacteria count (Thoma chamber 0.02 mm depth, Blau Brand, Wertheim, Germany). A gas tight syringe (500 µl) fitted with a needle was used as a punch to a rubber stopper on the fermentation bottle and gas samples for further analyses were collected. Methane concentration was quantified by gas chromatography in a SRI 310 (ALLCHROM ANASERWIS, Poland) 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. The oven temperature was programmed as follows: initially 180°C for 1.5 min, then increasing at 20°C/ min to 220°C. One thousand µl gas samples were injected. Observed peaks were identified by comparison of retention times with appropriate gases standards (mix gases 5.63% CO_2, 5.56 CH_4, 5.10% H_2 and N_2 remains, Multa S.C. Poland) using Peak Simple Workstation (Version 3.29).

The fluorescence *in situ* hybridization (FISH), according to Stahl et al. (1995) with modification of Soliva et al. (2004), was used to quantify methanogens in the population in the samples where methane production was significantly decreased (RO treatment at 48 h of fermentation). The specific oligonucleotide probe (Tib-MolBiol, Poznan, Poland) was performed for all methanogens (*Archeae*) S-S-GTGCTCCCCCGCCAATTCCT-a-A-20 (Lin et al., 1997), which was complementary to 16S rRNA. Hybridization was performed at 37°C in a hybridization buffer containing 0.9 M NaCl, 20 mM Tris/HCl (pH 7.2), 38% formamide and 0.1% sodium dodecyl sulphate with 50 ng/well-1 fluorescent dye Cy3 at the 5' end. The dyeing 4′,6-diamidino-2-phenilindol (DAPI, Vectashield® Mounting Medium, Vector) was used to corroborate that the observed fluorescence with the FISH technique corresponded to microbial cells. Samples were viewed with a fluorescence microscope (Axiovert 200, Zeiss). Images of the fluorescent signals were taken with a colour video camera (AxioCam MRm, Zeiss) and a software programme (AxioVision 6.0, Zeiss).

Calculations and statistical analysis

In vitro dry matter digestibility (IVDMD) was calculated finally after 8, 24 and 48 h of incubation using the equation as follows:

IVDMD (%) = [(initial DM input - (residue - blank) / initial DM input)*100]

Totally, 90 samples were analysed. Six replications were performed for each time point (3 time point; 8, 24, 48 h) in each group (4 groups; CON1; RO and CON2, RR). Moreover, the blank samples (containing the rumen fluid-buffer only) were also included into experiment design. All together, 3 blank samples in each experiment were incubated in triplicates for each time point (totally 18 samples). Due to culture method used, samples were analysed only at one time point. Obtained data were subjected to t-students test and analysis was done separately for each time point (8, 24, 48 h). Data were described as significantly different at the $P<0.05$. Statistical analyses were conducted with Sigma Plot version 11.0.

RESULTS

The seeds residue is rich in tannins that mean concentration is 0.88 mg/kg DM, and in flavonoids that mean content is 3.17 mg/kg DM. The fatty acids present in analysed organic materials represented mostly unsaturated fatty acids, mainly linoleic acid, either in seeds oil or in seeds residue (Table 1). The highest content of linoleic (C18:2 n-6) and linolenic (C18:3 n-3) acids were detected in oil. Oil and residue of the wild dog rose seeds were also rich in oleic (C18:1 *cis* 9) acid. Moreover the seeds residue comprised 13 g of palmitic acid (C16:0) per 100 g FA.

Wild dog rose seeds oil decreased methane production after 48 h of fermentation by 8.33% (Table 2) whereas the methanogens concentration tended to increase by 14.54%, from 14.9 to 17.1% of methanogens in all microbial cells dyed with DAPI in CON1 and RO, respectively. But, no statistical differences were found between both groups. Ammonia concentration was higher in RO treatments at all tested time points, however statistical differences were observed at 24 and 48 h of fermentation (Table 2). Protozoal and bacterial populations were not affected by wild dog rose seeds oil treatment (Table 2). Molar proportions of acetate and isobutyrate decreased ($P<0.05$) whereas the proportion of propionate was higher ($P<0.05$) with RO diet when compared to control (CON1). *Rosa canina* caused a decrease in propionate to acetate ratio ($P<0.05$). All determined significant effects were observed at 48 h of incubation except for butyrate that firstly significantly increased after 24 h of incubation (Table 3).

Table 2. Effect of wild dog rose seeds oil and rose seeds residue on the rumen microbial populations and basic parameters of rumen fermentation

Group	Time	Biochemical parameters				Rumen fluid microbial counts		
		pH	ammonia, mmol/l	methane mM	IVDMD[1]%	*Entodiniomorph* 10^3 ml^{-1}	*Holotrich* 10^3 ml^{-1}	bacteria 10^8 ml^{-1}
Experiment 1								
CON1	8 h	6.40 ± 0.01	3.02 ± 0.49	3.82 ± 0.11	24.9 ± 0.42	84.5 ± 4.8	2.90 ± 0.14	28.02 ± 1.11
RO		6.40 ± 0.02	4.00 ± 0.61	3.90 ± 0.10	24.8 ± 0.63	78.7 ± 4.86	3.02 ± 0.05	31.79 ± 1.10
CON1	24 h	6.30 ± 0.04	5.63 ± 0.31^b	5.21 ± 0.09	33.8 ± 0.92	104.0 ± 4.71	3.02 ± 0.11	33.20 ± 1.42
RO		6.30 ± 0.02	7.33 ± 0.11^a	5.07 ± 0.09	33.9 ± 0.19	102.3 ± 2.59	3.27 ± 0.22	30.62 ± 0.21
CON1	48 h	6.10 ± 0.01	10.20 ± 0.11^b	6.02 ± 0.03^a	38.5 ± 1.39	127.7 ± 5.45	3.36 ± 0.43	29.01 ± 2.69
RO		6.10 ± 0.01	13.10 ± 0.20^a	5.48 ± 0.11^b	38.6 ± 1.09	129.4 ± 5.19	2.91± 0.16	38.79 ± 2.28
Experiment 2								
CON2	8 h	6.40 ± 0.09	4.06 ± 0.21	4.59 ± 0.20	21.9 ± 3.69	95.6 ± 3.45^a	5.60 ± 0.20^a	19.10 ± 1.04
RR		6.40 ± 0.10	5.09 ± 1.19	4.69 ± 0.11	25.9 ± 4.21	85.7 ± 4.09^b	4.67 ± 0.07^b	21.69 ± 1.89
CON2	24 h	6.20 ± 0.01	9.89 ± 0.11^b	5.39 ± 0.20	37.9 ± 0.21	108.7 ± 5.38^b	4.95 ± 0.30	30.50 ± 1.88
RR		6.20 ± 0.02	12.13 ± 0.21^a	5.50 ± 0.10	39.4 ± 2.49	124.0 ± 2.53^a	4.93 ± 0.09	40.10 ± 3.31
CON2	48 h	6.20 ± 0.10	13.62 ± 0.21^b	6.81 ± 0.20	46.0 ± 1.41	195.4 ± 7.88	4.28 ± 0.04	25.69 ± 0.72
RR		6.20 ± 0.10	15.58 ± 0.12^a	6.81 ± 0.01	46.2 ± 2.93	201.1 ± 8.57	4.28 ± 0.04	27.79 ± 3.01

CON1 - control; RO - wild dog rose seeds oil; CON2 - control; RR - wild dog rose seeds residue; [1] *in vitro* dry matter digestibility; values are shown as least square means (± standard error mean); values with different superscripts within the row of the experiment are significantly different ($^{a.b}$ - $P<0.05$)

Table 3. Effect of wild dog rose seeds oil and rose seeds residue on concentration of individual VFA in the *in vitro* batch culture of rumen sample, mmol/l

Group	Time	Acetic (A)	Propionic (P)	Butyric	Isobutyric	Valeric	Isovaleric	Total VFA	A/P
Experiment 1									
CON1	8 h	21.7 ± 0.18	8.30 ± 0.08	3.41 ± 0.03	1.23 ± 0.03	0.15 ± 0.01	0.36 ± 0.01	35.2 ± 0.26	2.61 ± 0.02
RO		22.3 ± 0.13	8.28 ± 0.07	3.76 ± 0.02	1.04 ± 0.02	0.26 ± 0.01	0.37 ± 0.01	35.9 ± 0.19	2.69 ± 0.09
CON1	24 h	34.0 ± 0.21	12.1 ± 0.08	6.01 ± 0.05	3.00 ± 0.02^{b}	0.48 ± 0.02	0.46 ± 0.02	56.1 ± 0.32	2.80 ± 0.11
RO		32.8 ± 0.23	12.0 ± 0.02	6.59 ± 0.03	3.95 ± 0.09^{a}	0.42 ± 0.03	0.52 ± 0.01	56.3 ± 0.22	2.73 ± 0.08
CON1	48 h	42.4 ± 0.31^{a}	13.8 ± 0.11^{b}	8.77 ± 0.12	0.52 ± 0.01^{a}	1.12 ± 0.02	0.81 ± 0.04	67.4 ± 0.73	3.08 ± 0.05^{a}
RO		40.7 ± 0.33^{b}	14.5 ± 0.08^{a}	8.57 ± 0.11	0.46 ± 0.01^{b}	1.19 ± 0.06	0.84 ± 0.03	66.3 ± 0.56	2.79 ± 0.06^{b}
Experiment 2									
CON2	8 h	25.8 ± 0.21	6.82 ± 0.10	4.88 ± 0.08	0.34 ± 0.01	0.35 ± 0.01	0.44 ± 0.01	38.7 ± 0.51	3.79 ± 0.05
RR		26.0 ± 0.22	6.69 ± 0.05	4.92 ± 0.07	0.33 ± 0.01	0.34 ± 0.01	0.44 ± 0.01	38.7 ± 0.32	3.89 ± 0.10
CON2	24 h	37.4 ± 0.48	8.38 ± 0.14^{b}	7.64 ± 0.10^{b}	0.47 ± 0.01^{b}	1.02 ± 0.02	0.63 ± 0.02^{b}	55.56 ± 0.72^{b}	4.46 ± 0.13
RR		38.0 ± 0.60	9.45 ± 0.15^{a}	8.07 ± 0.17^{a}	0.57 ± 0.01^{a}	1.01 ± 0.01	0.75 ± 0.02^{a}	57.89 ± 0.61^{a}	4.02 ± 0.08
CON2	48 h	44.0 ± 0.73	11.3 ± 0.15	10.3 ± 0.10	0.63 ± 0.02	1.25 ± 0.02	1.27 ± 0.05	68.8 ± 1.01	3.88 ± 0.06
RR		46.8 ± 0.56	11.9 ± 0.10	10.6 ± 0.16	0.67 ± 0.02	1.29 ± 0.02	1.37 ± 0.07	72.7 ± 0.93	3.93 ± 0.06

CON1 - control; RO - wild dog rose seeds oil; CON2 - control; RR - wild dog rose seeds residue; values are shown as least square means (± standard error mean); values with different superscripts within the row of an experiment are significantly different (^{a,b} - $P<0.05$)

Methane production was not inhibited by wild dog rose seeds residue supplementation (Table 2). Changes in protozoal counts showed a relationship with the length of fermentation time (Table 2). *Entodiniomorpha* and *Holotricha* count decreased ($P<0.05$) at 8 h of fermentation. After 24 h of fermentation number of *Holotricha* become stabilized whereas *Entodiniomorpha* increased ($P<0.05$) as the effect of RR. The protozoal population at 48 h was at the same level in both groups and no effect of supplement was detected. No other changes in the rumen parameters value were noticed, except for volatile fatty acids after 24 h of fermentation (Table 3). At this time point propionate, butyrate, isobutyrate, isovalerate and, as the consequence, total volatile fatty acids concentration was increased ($P<0.05$). No such effect was observed after 48 h of incubation.

DISCUSSION

The present study was focused on investigation of the potential of oil and residue from the wild dog rose (*Rosa canina*) to mitigate methane production in *in vitro* system simulating rumen conditions. As a known source of fatty acids, those two components may interact with rumen microorganisms and influence their counts and metabolism. The present study have also shown that wild dog rose residue contain high concentration of phytochemicals, such as tannins and flavonoids, that may have antimethanogenic properties. As far as we know, this study is the first published trial to utilize seeds oil and seeds residue from the wild dog rose in ruminants nutrition.

According to Woodward et al. (2001), phenolic compounds (e.g., tannins), may reduce rumen methanogenesis in sheep and cattle. The mechanism of action is based on both direct effects on methanogen activity and indirect on fibre digestion (Bodas et al., 2009; Szumacher-Strabel and Cieślak, 2010). Flavonoids have been demonstrated to modify the microbial metabolism in the rumen (Broudiscou and Lassalas, 2000). In the experiment of Bodas et al. (2008) *Rheum noblile*, plant rich in flavonoids, was particularly promising since it reduced methane production by 16% in relation to the control group *in vitro*. Patra (2010) reported a decrease in methane emission as a result of diet supplemented with tannins, what was not related to changes in acetate and propionate concentrations. In present study however an effect of wild dog rose seeds oil on methane inhibition was accompanied by the decreased acetate to propionate ratio. Also Patra (2010) concluding the study on effects of phytochemicals on digestibility and rumen fermentation characteristics associated with methanogenesis stated that the decrease in methane production by tannins may be due to inhibition of protozoa, methanogens and, to a lesser extent, hydrogen-producing microbial population. The literature data confirm

the ability to limit rumen methane production by different tannin sources either *in vitro* (Min et al., 2005) or *in vivo* (Waghorn et al., 2002; Woodward et al., 2002). In the present study no such relationship was observed. Effect of tannins on methanogens population depends on tannins chemical structure (Tavendale et al., 2005). Condensed tannins have higher potential to mitigate rumen methanogenesis than hydrolysable once (Bhatta et al., 2009). We can not conclude on hydrogen-producing microbial population because this was out of scope of the present study. In the RO treatment where methane was decreased, *Archeae* population increased and no changes in protozoal population were found in comparison to control (CON1). In the RR treatment no change in methane production was reported, whereas some variations in protozoal populations were detected in relation to CON2.

The potential to mitigate methane production was reported only in wild dog rose seeds oil treatment that is the source solely of fatty acids, not phytochemicals. We suggest that it seems to be related to unsaturated fatty acids content (96.4 g/100 g FA). Fat inclusion in ruminant diets causes a marked decrease in methane production by rumen fluid (Moss et al., 2000; Jalč et al., 2006). Some of the previous studies showed that C18 fatty acids exerted the most pronounced toxic activity on population of the rumen protozoa (Baah et al., 2007). However, fat effects on methane production are not only limited to rumen protozoa because lipids have been shown to inhibit methanogenesis even in the absence of these microorganisms (Dohme et al., 1999). In carried study, dietary supplementation with this oil did not negatively influence the analysed populations of *Entodiniomorpha* and *Holotricha*. With these results we confirmed observations of earlier studies demonstrating none or partial effect of dietary supplementation with C18 rich oils on protozoa population in the rumen ecosystem (Cieślak et al., 2006a; Szumacher-Strabel et al., 2009). Kišidayová et al. (2006a) pointed that the rumen protozoa may respond in different ways to oil supplementation in *in vitro* conditions. Moreover, we have previously observed different metabolic responses of rumen ciliates and bacteria to various sources and concentrations of PUFA (Cieślak et al., 2009a,c). That was earlier pointed by Machmüller et al. (1998). Possible reasons for the different responses may include differences in basal metabolic rate of cellulolytic or amylolytic protozoa. Many authors also postulated that decreased protozoa number in the rumen ecosystem may be due to fibre coated by fat what limited its exposure to microbial digestion. However in the present experiment the diet was supplemented with up to 5% of oil in dry matter as recommended by NRC (2001). This may explain why the protozoa count was not influenced by wild dog rose seeds oil and seeds residue after 48 h of fermentation. According to Moss et al. (2000) the effect of fat inclusion in ruminants diet can not be viewed in isolation. In present study we did not observe

the digestibility breakdown in the rumen and, what's more, the favourable effect on ammonia production was stated. The reason for the beneficial effect of wild rose dog seeds oil and seeds residue on ammonia concentration in the rumen fluid after 24 or 48 h incubations may be their effect on the specific growth rate of the bacteria or some general effect on the rumen environment.

Because the mitigation of methane production was observed only when wild dog rose oil was supplemented to the diet, the further expectations to wild dog rose residue as ruminant diets component seems to come from the content of basic nutrient (protein and fibre).

The crude protein and fibre contents that amounts as much as half of the basic nutrients in the residue after CO_2 extraction in supercritical conditions seems to be a valuable ingredient for ruminants and based upon its protein percentage, might be considered similar to cereals. It is very important that the residue, rich also in fibre, did not negatively affect rumen fermentation processes, because it has been widely accepted that of all nutrients, carbohydrates have the highest methane-producing potential. In general fibre fermentation increases methanogenesis when compared to fermentation of soluble carbohydrates (Hindrichsen et al., 2004). In present study we did not observe such a positive relation and methane concentration was stable at all tested time points in comparison to control group. It should be also emphasized that, except the nutritive value, the analysed residue are biologically stable because of their dry solid form after extraction. In our opinion this is another advantage, besides the environmental aspect, in using that material as feed ingredient. This allows also to avoid cost of drying and difficulties of storage.

CONCLUSIONS

In conclusion, the results of the present *in vitro* experiment confirm the potential of the oil from the wild dog rose (*Rosa canina*) seeds to mitigate rumen methane production without negative influence on rumen fermentation. Besides, no negative effect of wild dog rose seeds residue on rumen processes was stated, what may predispose this protein and fibre containing by-product to be utilized as ruminants dietary ingredient. Based on the obtained results we can suggest that up to 5% of wild dog rose seeds residue and oil may be used in ruminants ration. However, further research are needed to evaluate their effect in animal production.

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