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An *in vitro* study on the effect of sage, *Salvia officinalis* L., on rumen fermentation*

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ABSTRACT

Dried leaves of *Salvia officinalis* L., a rich source of phytofactors, were evaluated for their effect on methane production, microbial population, and basic parameters of rumen fermentation in a batch culture system. A 400 mg mixture of meadow hay and barley meal at a ratio of 60:40 was used as the substrate. Dried leaves of *Salvia officinalis* L. were added to the substrate at six levels (4, 10, 20, 40, 100, and 200 mg). A wide range of dried *Salvia officinalis* L. leaves, in quantities even up to 50% of the substrate, was used to test the effect of increasing amounts of phytofactors in the investigated cultures, mostly on methane production. CH_4 production was significantly mitigated by supplementation with 200 mg of sage per incubation vessel. The supplement affected the protozoa and methanogen populations which indicates that methane emission is associated with these populations; *in vitro* dry matter digestibility decreased linearly, however. Although *Salvia officinalis* L. seems to be a plant for which there may be a use in ruminant nutrition and the presented results suggest that the optimal level of *Salvia officinalis* is between 100 and 200 mg, further investigations under long-term *in vivo* conditions are necessary, as are the determination of the optimum dose and the active agent.

KEY WORDS: batch culture, sage, methane, microorganisms, cattle

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INTRODUCTION

Modulation of rumen fermentation in order to improve the digestibility of dietary components, to reduce environmental pollution with methane and nitrogen, as well as to enhance animal performance is one of the most important goals for animal nutritionists. Phytofactors such as saponins, tannins, and essential oils, are considered as good candidates for achieving these goals (Patra et al., 2006; Cieślak et al., 2012). The practical application of these plant components is, however, economically ungrounded and their efficiency is still not satisfying (Benchaar and Greathead, 2011). Moreover, Jouany and Morgavi (2007) proposed supplementation of animal diets with bioactive plant secondary metabolites (PSM) in the form of fresh or dried plants. Hence, our interest was concentrated on sage (*Salvia officinalis* L.), a plant rich in polyphenolics, rosmarinic acid, camphor, and carnasol (Mazzio and Soliman, 2009), which has been used as a food preservative, flavouring and medicinal agent since 1400 A.D. It has been shown that alcohol extracts of sage exert anti-inflammatory, antioxidant, and also antimicrobial effects (Mazzio and Soliman, 2009). The hypothesis of the present study is that sage, as a source of phytofactors, can decrease rumen methane production. Dried leaves of sage were used in this study as a potential modulator of rumen fermentation and the experiment was conducted to evaluate potential antimethanogenic and antimicrobial properties of *Salvia officinalis* L.

MATERIAL AND METHODS

Animals, feeds and feeding

Three cannulated Polish Holstein-Friesian cows weighing 680±23 kg were the donors of rumen fluid for the *in vitro* study. The animals were fed a diet composed of grass silage (7 kg DM), maize silage (5 kg DM), meadow hay (1.8 kg DM), and concentrate (1 kg DM). Drinking water was always available.

Batch culture

The experiment was carried out in a batch culture system according to the modified protocol of Szumacher-Strabel et al. (2004). Briefly, the collected rumen fluids obtained from each cow (before the morning feeding) were pooled, mixed together and squeezed through four layers of cheesecloth into a Scott Duran® bottle (1 l) with an O_2-free headspace and immediately transported to the laboratory at 39°C, where they were used as the source of inoculum. A 400 mg mixture of meadow hay and barley meal at a ratio of 60:40 was used as

the substrate. Dried sage leaves (Herbapol Krakow, Poland) were supplemented to the substrate at six levels (4, 10, 20, 40, 100, and 200 mg). A wide range of quantities of dried sage leaves, even up to 50% of the substrate, was used to test the effect of increasing amounts of phytofactors in the investigated cultures. Each level was tested in four replicates accompanied by blank vessels (no substrate). The complete set comprised four vessels for each tested level and four control vessels without any supplements, as well as 4 vessels as blanks (without substrate). Four hundred milligrams of milled substrate were added to incubation vessels with a 120 ml capacity. Each vessel was filled with 40 ml of the incubation medium consisting of rumen fluid and buffer solution: 292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4$ x $7H_2O$, 64 mg $CaCl_2$ x $2H_2O$, 4 mg Na_2CO_3, and 600 mg cysteine hydrochloride per litre of double-distilled water (ddH2O) dispensed anaerobically at a 1:4 (v/v) ratio. The samples were then incubated at 39°C for 24 h.

Quantification of rumen fermentation parameters and methane production

After 24 h incubation, the pH of buffered rumen fluid was measured (pH-meter CP-104, ELMETRON). The ammonia concentration was determined by the method of Nessler (Szumacher-Strabel et al., 2002), with some modifications. Briefly, a 3.6 ml sample of buffered rumen fluid was centrifuged at 12 000 rpm for 5 min. A 100 µl sample of the supernatant was then transferred to a tube containing 200 µl of 1% polyvinyl alcohol, 200 µl of 20% potassium sodium tartrate, 200 µl of Nessler reagent, and 19.3 ml of ddH_2O and incubated 10 min at room temperature. The incubation was followed by measurement of the absorbance of the incubated mixture at 400 nm using a HALO SB-10 UV-VIS spectrophotometer. Total gas production was estimated on the basis of the displacement of the piston in a syringe tightly connected to the incubation vessel. The volume of gas produced by fermentation of the substrate was calculated by comparison of gas production in blank vessels with the relevant data of control and experimental cultures, respectively. For determination of methane, a 500 µl sample of gas was taken from the headspace of incubation vessels using a gas-tight syringe (GASTIGHT® Syringes, Hamilton Bonaduz AG, Switzerland) and injected into a SRI310 gas chromatograph equipped with a thermal conductivity detector (TCD) and Carboxen - 1000 column (mesh size 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. The observed peaks were identified by comparison of retention times with appropriate gas standards (gas mix: 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 software.

Quantification of the microbial population

For protozoa determination, the contents of the vessels after incubation were mixed thoroughly and 1 ml samples were mixed with 6 ml of 4% formaldehyde. To determine the total number of bacteria, 20 µl were added to 6980 µl of Hayem solution (2.5 g $HgCl_2$, 25 g Na_2SO_4, and 5.0 g NaCl per litre of ddH_2O). The protozoa were counted microscopically (at 150 x) in a drop of buffered rumen fluid of a defined volume, classifying them into *Holotrichs* and *Entodiomorphs*. The bacteria were enumerated microscopically (400 x) in a Thoma chamber (0.02 mm in depth, Blau Brand, Wertheim, Germany). Fluorescence *in situ* hybridization (FISH), as described by Pers-Kamczyc et al. (2011), was used to quantify the methanogen population. The specific oligonucleotide probe (TibMolBiol, Poznań, Poland) was designed for all methanogens (Lin et al., 1997) and was complementary to 16S rRNA. Hybridization was done at 37°C in a hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl, 38% formamide, and 0.1% sodium dodecyl sulphate (SDS) with 50 ng/well fluorescent dye Cy3 at the 5' end of the oligonucleotide probe. Staining with 4',6'-diamidino-2-phenilindol (DAPI, Vectashield Mounting Medium, Vector) was used to corroborate that the fluorescence observed with the FISH technique corresponded to bacterial 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).

Estimation of volatile fatty acids content

A 3.6 ml sample of buffered rumen fluid was stabilized with 0.4 ml of 46 mM $HgCl_2$ and frozen until analysis by HPLC. After thawing, the mixture was centrifuged at 12 000 rpm for 10 min, filtered through a 0.22 µm filter, and 10 µl of clear supernatant were injected to a Waters 2690 High Performance Liquid Chromatograph equipped with a Waters 2487 Dual λ detector and Aminex HPX-87H column (300 mm x 7.8 mm). The mobile phase was 0.005 M H_2SO_4. Quantitative and qualitative identification of individual peaks was performed using the method based on an external standard prepared by mixing individual VFA purchased from Supelco and using Millenium 2001 software (version 2.15).

Statistical analysis

The effect of different doses of dried sage leaves (0, 4, 10, 20, 40, 100, and 200 mg) on the rumen fluid collected from 3 ruminally fistulated Polish Holstein-Friesian cows was analysed. For all rumen fluid parameters, four samples were analysed in each of the groups (7) using the general linear model of SAS (2006). The data were subjected to one-way analysis of variance with the experimental

group as a factor. The all pairwise multiple comparison procedures were applied with the Holm-Sidak test and differences were considered significant at $P<0.05$. The effect of increasing doses of sage on all parameters was subjected to regression analysis. The following parameters: methane and propionate concentrations, *Entodiniomorph* and *Holotrich* counts, as well as the methane-to-total gas production ratio were analysed as linear regressions with the following model: parameter = intercept + slope x incubation dose. Total gas production, total volatile fatty acids and ammonia, as well as butyrate concentration, bacteria and methanogen counts, and acetate-to-propionate ratio were analysed by non-linear regression using the Proc NLIN of SAS (2006). The data are presented as means.

RESULTS

Increasing the supplementation of sage increased the pH ($R_2=0.53$; $P<0.01$). Similarly, a positive correlation was observed between sage supplementation and ammonia concentration ($R_2=0.53$, $P<0.01$; Table 1). Total gas production increased from 32.00 ml for the control group to 35.50 ml for the group with 100 mg supplementation, however, this parameter was observed to decrease at the highest level of supplementation (200 mg; Figure 1). The changes in TGP caused by sage supplementation are described by quadratic regression equations ($R_2=0.43$; $P<0.01$). The methane concentration as well as the methane-to-TGP ratio decreased with increasing levels of sage ($R_2=0.83$, $P<0.01$; $R_2=0.87$, $P<0.01$, respectively). Moreover, a linear decreasing trend of the IVDMD value with an increasing level of supplementation with sage was observed ($R_2=0.21$, $P=0.05$). The concentration of propionate ($R_2=0.90$, $P<0.01$) increased linearly with increasing supplementation, whereas for total VFA, butyrate concentration,

Table 1. Effect of *Salvia officinalis* on the parameters of ruminal fermentation

Indices	Supplementation levels of *Salvia officinalis* mg/incubation vessel							SEM	R^2	P
	Control	4.0	10.0	20.0	40.0	100.0	200.0			
pH	6.67^{ab}	6.65^{b}	6.66^{ab}	6.65^{b}	6.73^{b}	6.75^{a}	6.75^{a}	0.01	0.53	<0.01
Ammonia, mmol/l	13.2^{c}	13.8^{bc}	15.2^{bc}	15.2^{bc}	16.1^{bc}	17.0^{ab}	17.6^{a}	0.37	0.53	<0.01
TGP, ml[1]	32.0^{b}	34.0^{ab}	33.0^{ab}	34.0^{ab}	33.7^{ab}	35.5^{a}	31.0^{b}	0.36	0.43	<0.01
Methane, mmol/l	9.67^{ab}	10.2^{a}	9.91^{ab}	9.27^{b}	9.44^{ab}	9.17^{b}	5.94^{c}	0.26	0.83	<0.01
IVDMD, %[2]	41.6^{a}	33.6^{ab}	36.7^{ab}	33.6^{ab}	32.9^{ab}	33.0^{ab}	28.5^{b}	1.04	0.21	0.05
Volatile fatty acids profile, mmol/l										
total VFA	49.7^{c}	51.4^{c}	53.1^{bc}	53.8^{b}	52.7^{bc}	55.8^{ab}	58.7^{a}	0.60	0.87	<0.01
acetate	31.4	32.1	32.7	33.0	32.2	33.4	32.7	0.24	0.12	0.23
propionate	9.79^{d}	10.4^{cd}	11.0^{cd}	11.3^{c}	11.1^{d}	12.8^{b}	16.2^{a}	0.40	0.90	<0.01
butyrate	6.95	7.07	7.40	7.56	7.59	7.44	7.03	0.07	0.25	0.03
A:P ratio	3.21^{a}	3.08^{ab}	2.97^{ab}	2.93^{b}	2.92^{ab}	2.60^{b}	2.03^{b}	0.07	0.88	<0.01

^{a,b} values within a row with different superscript letters differ $P<0.05$; [1] total gas production; [2] *in vitro* dry matter digestibility; SEM - standard error of the means

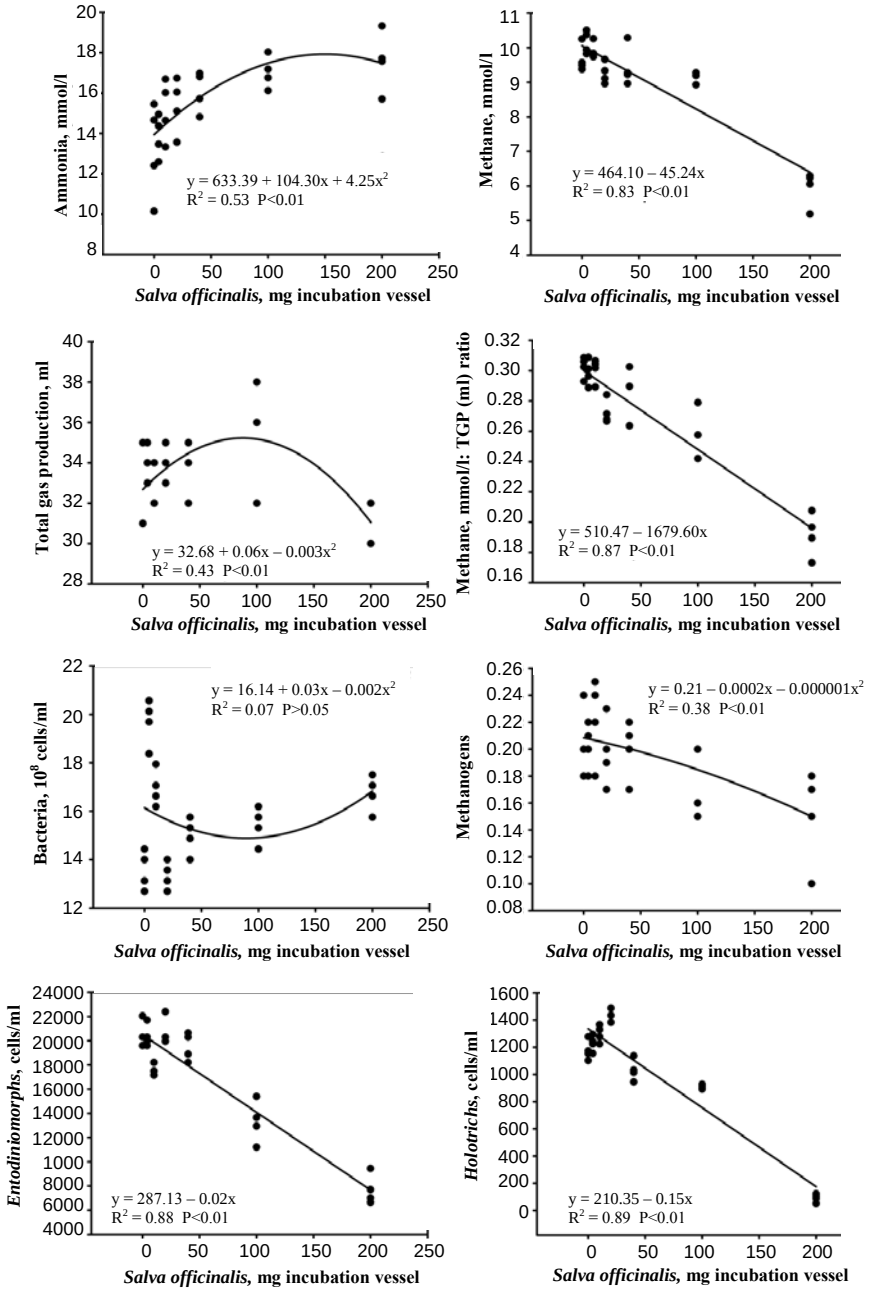

Figure 1. The effect of the *Salvia officinalis* supplementation on the ruminal fluid microbiota and basic fermentation parameters

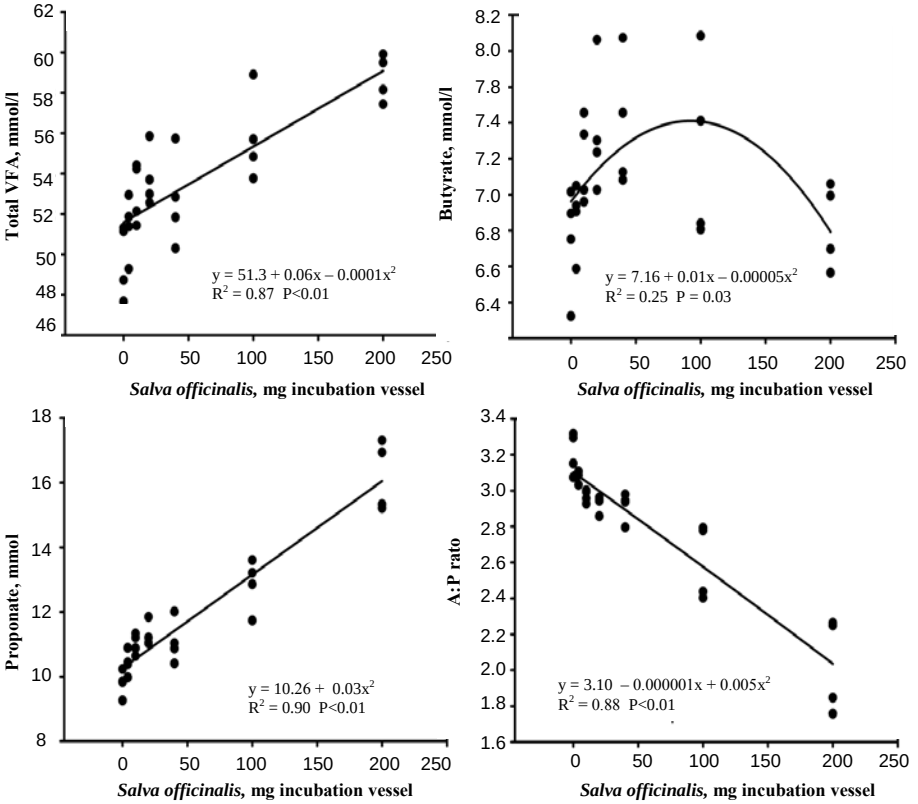

Figure 2. The effect of the *Salvia officinalis* supplementation on ruminal fatty acids (total VFA, butyrate, propionate and A:P ratio)

Table 2. Effect of *Salvia officinalis* supplementation on the microbial population in culture medium of microorganisms incubated *in vitro*

	Supplementation levels of *Salvia officinalis,* mg/incubation vessel							SEM	R^2	P
	Control	4.0	10.0	20.0	40.0	100.0	200.0			
Bacteria 10^8 cells/ml	13.6^{d}	19.7^{a}	16.9^{b}	13.3^{d}	15.0^{cd}	15.4^{cd}	16.7^{bc}	0.41	0.07	0.39
Entodiniomorphs 10^3 cells/ml	20.4^{a}	20.4^{a}	17.5^{b}	20.7^{a}	19.5^{ab}	13.3^{c}	7.70^{d}	0.89	0.88	<0.01
Holotrichs 10^3 cells/ml	1.18^{b}	1.23^{b}	1.30^{b}	1.45^{a}	1.03^{c}	0.91^{c}	0.09^{d}	0.08	0.89	<0.01
Methanogens[1]	0.21^{a}	0.20^{a}	0.22^{a}	0.20^{a}	0.20^{a}	0.19^{ab}	0.15^{b}	0.01	0.38	<0.01

^{a,b} values within a row with different superscript letters differ P<0.05; SEM - standard error of the means; [1] the ratio of methanogens in the population of microorganisms dyed with DAPI

and acetate-to-propionate ratio, effect of sage supplementation is described by quadratic regression equations (R_2=0.87, P<0.01; R_2=0.25, P<0.03; R_2=0.94, P<0.01, respectively). Furthermore, increasing levels of sage caused significant changes in the microbial population (Figure 2; Table 2). Number of both *Entodiniomorphs* and *Holotrichs* decreased as sage levels increased (R_2=0.88, P<0.01; R_2=0.89, P<0.01, respectively). Sage supplementation had no effect on the number of bacteria (R_2=0.07, P=0.39). Supplementation of rumen fluid with the lower (4 and 100 mg) and higher (100 and 200 mg) doses of sage, however, caused a 20% increase in the bacteria count compared with the control. Despite the increasing tendency of bacteria counts presented on the first graph, sage supplementation had no distinct influence on the obtained results.

DISCUSSION

The present study was focused on investigating the potential of *Salvia officinalis* dried leaves to mitigate methane production in an *in vitro* system simulating rumen conditions. *Salvia officinalis*, a known source of phytofactors, showed antimicrobial and antimethanogenic properties. What is noteworthy, this study is the first published trial to utilize not plant extracts or other processed sources of phytofactors, but dried leaves of *Salvia officinalis* in ruminant nutrition.

According to Woodward et al. (2001), the potential to modulate rumen fermentation has been exhibited by many plants and their extracts due to high concentrations of secondary metabolites, e.g., saponins, tannins, essential oils, flavonoids, organosulphur compounds (Ferme et al., 2004; Szumacher-Strabel and Cieslak, 2010; Zmora et al., 2012). Their mechanisms of action are still unclear, however. Saponins affect methane production by reducing the protozoa count and suppressing the number and activity of methanogens (Hess et al., 2003). Also tannins may modulate ruminal fermentation *via* a direct effect on the protozoa population (Kamra et al., 2006; Cieslak et al., 2012). Furthermore, essential oils, organosulphur compounds and flavonoids can affect CH_4 production by direct inhibition of methanogens, with simultaneously no effect on the protozoa population (Patra and Saxena, 2010). Broudiscou et al. (2000) found that an extract of *Salvia officinalis* L. rich in flavonoids, decreased methanogenesis in continuous culture of ruminal microorganisms without altering the protozoa population. In our study, 200 mg of dried sage leaves mitigated CH_4 production correlated to both methanogens and the protozoa population. The discrepancies among the studies may be caused by different sources of bioactive compounds, hence different mechanisms of action. Broudiscou et al. (2000) used purified plant secondary metabolites or plant extracts. The dried sage leaves used in the present

study contained phytofactors and some other compounds that can interact with PSM and rumen microorganisms (Szumacher-Strabel and Cieslak, 2010).

The mitigation of methane production observed in this experiment might also be caused by modification of the volatile fatty acid profile. Changes in CH_4 production usually occur with an alteration in the VFA profile because of alternative electron sinks to dispose of reducing power. Moreover, the transformation of the feed to propionate usually limits the rate of methanogenesis due to the impact of hydrogen transfer (Ungerfeld et al., 2003). The supplementation with sage increased propionate and total VFA concentrations and also altered the A:P ratio, simultaneously inhibiting digestibility *in vitro*. This last result could be explained by the chemical composition of *Salvia officinalis*. The used additive contained not only bioactive secondary metabolites, but also basic components, such as protein and carbohydrates. The increased VFA production may have resulted in the metabolizing of this component concurrently with dietary components. Garcia-Gonzales et al. (2010) demonstrated that rhubarb decreased CH_4 formation by limited changes in the VFA profile with no effect on dietary dry matter digestibility. In contrast, in our study the *in vitro* dry matter digestibility was reduced by increasing sage supplementation. Similarly, Agarwal et al. (2009) demonstrated that plant extracts may decrease the digestibility of feeds because of the inhibition of cellulolytic bacteria metabolism. Moreover, Patra et al. (2006) highlighted that the inhibitory effects of plant extracts on rumen cellulolytic bacteria may cause a decrease in feed digestibility. On the other hand, the supplement increased the total bacteria population even by 23.4%, which can be explained by ecological succession. Inhibition of the protozoa population probably induced a new ecological niche for bacteria. An increase in the population of one microorganism group without a decrease of another one has been observed by Kumar et al. (2011) in buffalo rumen fluid. These authors applied either a mixture of plants containing secondary metabolites or peppermint oil. The plant mixture stimulated the *Fibrobacter succinogenes* population and inhibited *Ruminococcus flavefaciens* with no changes in the fungal population. In addition, the increase in the bacteria population and decrease in protozoa number can be explained by their interaction. Namely, protozoa engulf bacteria and thus control their population (Diederichs et al., 2003). In an environment with a smaller ciliate number, bacteria do not have so many natural predators and their numbers can grow rapidly. Moreover, alteration in the microbial population in the rumen caused changes in rumen fermentation patterns.

Addition of higher doses of *Salvia officinalis* L. dried leaves to the ruminant diet altered the microbial population and, as a result, decreased methane production and changed the profile of volatile fatty acids. This suggests that the use of *Salvia officinalis* in the diets of ruminants may modulate ruminal fermentation

by reduction of methane production, thus potentially involving productive and environmental benefits, however, *in vitro* dry matter digestibility was decreased linearly. *Salvia officinalis* L. seems to be a plant that can found the application in ruminants nutrition. The presented results suggest that the optimal level of *Salvia officinalis* is between 100 and 200 mg, but further investigations under long-term *in vivo* conditions are necessary and the optimum dose, as well as the active agent, should be determined

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