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EFFECTS OF SELECTED MEDICINAL PLANTS ON RUMEN FERMENTATION IN A HIGH-CONCENTRATE DIET IN VITRO

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ABSTRACT

The objective of this in vitro study was to compare fermentation patterns of seven selected traditional medicinal plants and investigate the effects of medicinal plant mixture (MPM) supplements and a high-concentrate diet (lucerne hay and barley grain, LH+BG, 40:60) on rumen fermentation and fatty acid composition. An MPM of *Taraxacum officinale* L., *Acorus calamus* L., *Calendula officinalis* L., *Hypericum perforatum* L., *Achillea millefolium* L., *Urtica dioica* L. and *Cichorium intybus* L. was used. Qualitative phytochemical screening revealed the presence of medically active compounds (tannins, phenols, steroids, flavonoids, saponins, terpenoids and glycosides). The counts of total protozoan did not differ across medicinal plant fermentations and were positively correlated with the total SCFA concentration (P=0.002), methane production (P=0.001) and *n*-butyrate (P=0.011). Substitution of LH by MPM in proportions of 10%, 50% and 100% resulted in increased in vitro dry matter digestibility (by 6%) and decreased methane production (by 1%) in comparison with a diet without MPM. Only 100% supplementation with MPM increased the content of monounsaturated and polyunsaturated fatty acids, whereas the content of saturated fatty acids decreased in comparison with the diet without MPM. The results point to the promising beneficial effects of MPM in a high concentrate diet, with minimal adverse effect on rumen fermentation.

Key words: Digestibility; batch culture; fermentation; ciliate protozoa; fatty acids; plants.

INTRODUCTION

Medicinal plants have been traditionally used in ethnomedicine practice to treat various digestive disorders not only in human but also in animal health management. Beneficial effects have typically resulted from the content of either a single secondary metabolite or a combination of secondary metabolites from the plants used. However, little information is available on the potential of traditional medicinal plants to modify rumen fermentation in order to enhance nutrient utilization in ruminants (Garcia-Gonzales *et al.,* 2008). Under some feeding regimes, adverse conditions in the rumen may alter the rumen ecosystem and result in various metabolic disorders. Phytogenic additives in the form of whole plants or their extracts can be used to mitigate these negative effects. The major active compounds of medicinal plants (essential oils, saponins, flavonoids, tannins and polyphenols) have been mainly tested as concentrated extracts to examine their anti-microbial activity (Bodas *et al.,* 2012), to decrease rumen methane emissions (Patra *et al.,* 2006) or to modify the lipolysis and biohydrogenation of polyunsaturated fatty acids (Vasta *et al.,* 2009; Jayanegara *et al.,* 2011).

The use of concentrated plant extracts can be toxic and expensive for animal husbandry. On the other hand, it is known that animals grazing in natural areas (grassland) can seek out plants with medicinal effects (Fraisse *et al.,* 2007). However, intensification of animal production prevents such animal self-medication. The maintaining of high productivity in ruminants is associated with the use of high-concentrate diets with possible negative effects on the rumen ecosystem. Therefore, feed rations with a dry mixture of selected medicinal plants could simulate natural grazing conditions, improve the health of animals with high productivity and serve as a cheaper alternative to plant extracts. For this purpose, the evaluation of the nutritive value of selected medicinal plants and the potential of using a mixture of them to manipulate rumen fermentation and lipid metabolism is important. The objectives of the present in vitro study were: (1) to compare fermentation patterns of seven selected traditional medicinal plants and (2) to investigate the effects of a medicinal plant mixture supplement (MPM) and a high-concentrate diet (lucerne hay: barley grain, 40:60), where the lucerne hay in diets was substituted for 10, 50, and 100% of dry matter (DM) by an MPM, on rumen fermentation parameters, ciliated protozoan population and fatty acid concentration.

MATERIALS AND METHODS

Plant materials, diet substrates and chemical analyses: The following seven dry medicinal plants from commercial sources were used: roots of dandelion (*Taraxacum officinale* L.) and calamus (*Acorus calamus* L.), flowers of marigold (*Calendula officinalis* L.), and

whole overground herbs of St. John's-wort (*Hypericum perforatum* L.), yarrow (*Achillea millefolium* L.), nettle (*Urtica dioica* L.) and chicory (*Cichorium intybus* L.). Lucerne hay (LH) was used as forage and barley grain (BG) as concentrate in high-concentrate diets (lucerne hay: barley grain, 40:60). The dry medicinal plant materials were mixed in equal proportions and the mixture of medicinal plants (MPM) was standard throughout the in vitro experiment. The LH in the high-concentrate diets was substituted for 10, 50, and 100% of DM by a MPM, and four diets were examined: LH+BG, MPM100+BG, LH+MPM50+BG and LH+MPM10+BG, respectively. Plant materials and diet substrates were ground, sieved (particle size of 0.15-0.40 mm), bulked and stored in sealed plastic containers until needed. Chemical analysis of the substrates was performed in triplicate, and standard methods (AOAC, 1990) were used to determine the DM (No. 967.03), ash (No. 942.05), nitrogen (No. 968.06), fat (No. 9836.23) and crude protein (No. 990.03). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined through a procedure (Van Soest *et al.,* 1991) using a Fibertec 2010 (Tecator Comp., Höganäs, Sweden). NDF was assayed without (forages) or with (concentrates) heat stabile amylase. NDF and ADF were expressed inclusive of residual ash. The chemical composition of the selected individual medicinal plants is presented in Table 1. The chemical and fatty acid composition of the mixture of medicinal plants, lucerne hay and barley grain is presented in Table 2.

For the qualitative tests of the presence of secondary plant metabolites, samples (20 g) of each of the ground medicinal plants were extracted in 200 ml of ethanol (ethanol/distilled water, 1:1) and stirred for 24 hours at 20°C. This mixture was filtered and 100 ml of ethanol was added. Afterward, the mixture was filtered again and concentrated using a vacuum concentrator (Concentrator Plus 5305, Eppendorf). Chemical tests for the screening and identification of bioactive chemical constituents in the medicinal plants under study were carried out in extracts using standard procedures as described by Yadav and Agarwala (2011).

Experimental design: Two batch culture experiments were performed. The first experiment consisted of 24 h in vitro batch fermentations with the seven medicinal plants used as the sole substrates. Three replicates (3 incubation bottles) were prepared for each plant. The experiment was repeated three times within three consecutive days (n = 3 x 3). The second experiment consisted of 24 h in vitro batch fermentations of high concentrate diets with three proportions of medicinal plant mixture supplement (i.e., LH+BG, MPM100+BG, LH+MPM50+BG, LH+MPM10+BG, respectively). For each diet, nine replicates (9 incubation bottles) were prepared. The experiment was repeated three times within three consecutive days (n = 3 x 9). At the same time, 3 replicate bottles were also used for the blank (rumen inoculum, no substrate).

In vitro incubation and measurements: The rumen fluid used in the present experiment was collected from three rumen-cannulated rams (Lacaune *versus* Suffolk; 2 years of age; 45.0 ± 2.5 kg weight) before the morning feeding for three consecutive days. The rams were housed separately in pens and fed a diet consisting of 800 g/kg DM meadow hay and 300 g/kg DM barley grain divided into two rations per day, with free access to water. The rumen fluid was transferred to the laboratory, squeezed through four layers of gauze and mixed with McDougall's buffer (McDougall, 1948) at a ratio of 1:1. Volumes of 35 ml were dispensed by an automatic pump into preheated 120 ml serum bottles containing 0.25 g of substrate. The fermentation bottles were filled up with CO_2, closed with a butyl rubber stopper, aluminum-sealed and incubated in the incubator for 24 h at 39 ± 0.5°C. The volume of accumulated gas released from the incubated serum bottles was measured after 24 h using the pressure transducer technique (Váradyová *et al.,* 2005). Gas accumulation volume was determined from the recorded pressure or the volume of gas produced. Gases from each fermentation bottle were collected in 2 ml glass gas-tight syringes (Sigma, St. Louis, MO, USA) at the end of incubation (for each bottle separately) and immediately analysed for methane concentration by gas chromatography (Perkin-Elmer Clarus 500 gas chromatograph, Perkin-Elmer, Inc., Shelton, CN, USA). Short-chain fatty acids (SCFA) were determined in the medium at the end of the incubation period by gas chromatography (Cottyn and Boucque, 1968) using a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc., Shelton, CN, USA), with crotonic acid as the internal standard. In vitro dry matter digestibility (IVDMD) was determined from the difference in the substrate weight before and after incubation. Samples of fermentation fluid for counting the ciliated protozoan population were collected in duplicates and were fixed with an equal volume of 8% formaldehyde. The ciliated protozoan cells were counted microscopically, and ciliates were identified according to Williams and Coleman (1992). Fatty acids (FA) in the batch fermentations were determined in lyophilized samples. Lipids were extracted and analyzed from 500 mg of freeze-dried fermentation sample with a mixture of chloroform: methanol (2:1) with purified samples as described by Váradyová *et al*. (2008). The FA methyl ester peaks were identified by authentic standards of C4-C24 FA methyl ester mixture (Supelco, Bellefonte, PA, USA) by gas chromatography using a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc., Shelton, CN, USA),

Statistical analysis: All data were analysed using analysis of variance (Graphpad Instat, Graphpad Software Inc., San Diego, CA, USA), with the substrate as the fixed effect and incubation run as a random effect. When the overall treatment effect was significant (P<0.05), individual treatment differences were determined using Tukey's multiple comparison post-test and considered to be significant when P<0.05. All data on individual medicinal plants were compared by determining Pearson's correlation coefficients between the protozoan counts and all of the fermentation parameters.

RESULTS

Rumen fermentation patterns of individual medicinal plants: Qualitative phytochemical screening revealed the active compounds present to be: tannins, phenols, steroids and flavonoids in all medicinal plants, saponins and terpenoids (*T. officinale, A. calamus*, *C. officinalis, A. millefolium*, *U. dioica, C. intybus*) and glycosides (*C. officinalis, A. millefolium*, *U. dioica* and *C. intybus*). The IVDMD of *T. officinale* was the highest of all the medicinal plants tested and differed from the IVDMD of *C. officinalis, H. perforatum*, *A. millefolium, U. dioica* and *C. intybus* (P=0.001) (Table 3). The value of the IVDMD was lowest in *A.millefolium*. The values of gas and methane did not differ across treatments. The values of total SCFA (P=0.001) and the molar proportions of acetate (P=0.002), *n*-butyrate (P=0.001), *i*-butyrate (P=0.002) and *i*-valerate (P=0.001) varied among the medicinal plants tested. Hydrogen recovery was positively correlated with methane production, with *r*=0.571; however, a negative relationship occurred between hydrogen recovery *versus* SCFA and hydrogen recovery *versus* propionate (P=0.001) (Table 4). The counts of total ciliate protozoan did not differ across medicinal plant fermentations and were positively correlated with the total SCFA concentration (P=0.002), methane production (P=0.001) and the molar proportion of *n*-butyrate (P=0.011).

Rumen fermentation parameters of a high concentrate diet with a medicinal plant mixture supplement: Substitution of LH for 100, 50, and 10% with the MPM resulted in an increase (P=0.001) of the IVDMD in the MPM substituted diets compared with the LH+BG diet (Table 5). On the other hand, the values of methane production, total SCFA and molar proportion of propionate for the MPM-substituted diets decreased. The values of 2H-recovery were highest in MPM100+BG (P=0.001). The number of total ciliate protozoan cells and number of *Entodinium* spp. were lowest in the LH+MPM10+BG diet.

Fatty acid concentration in rumen fluid: The content of oleic acid and *trans*-11 vaccenic acid in rumen fluid with LH+MPM10+BG was lower when compared with the other diets (Table 6). The higher concentrations of stearic acid, linoleic acid and -linolenic acid in the MPM100+BG were accompanied by lower concentrations of palmitic acid and myristic acid when compared with LH+MPM50+BG and LH+MPM10+BG. The *cis*9, *trans*11-linoleic acid (CLA) concentrations, ranked by diet, were MPM100+BG > LH+BG > LH+MPM50+BG > LH+MPM10+BG (2.80, 2.20, 2.15, 1.35 g/kg of FA, respectively). Lignoceric acid decreased in the diets supplemented with MPM in comparison with LH+BG. The concentration of saturated FA in the MPM100+BG decreased, whereas the concentrations of monounsaturated FA and polyunsaturated FA increased in comparison with the other diets. The fermentation of MPM100+BG increased the total C18 FA in comparison with LH+MPM10+BG and LH+MPM50+BG.

Table 1. Chemical composition of plants (means ± standard error of the mean)

Common name	Scientific name	Part used	Dry matter (g/kg)	Neutral detergent fibre (g/kg DM)	Acid detergent fibre (g/kg DM)	Crude protein (g/kg DM)	N (g/kg DM)	Ash (g/kg DM)
Dandelion	*Taraxacum officinale* L.	Root	897±20.3	100±7.81	90±8.3	100±5.2	16±1.3	106±3.3
Calamus	*Acorus calamus* L.	Root	867±18.1	273±20.7	146±18.7	97±6.8	15±2.1	63±4.3
Marigold	*Calendula officinalis* L.	Flower	893±28.9	242±17.5	220±10.1	202±18.7	33±1.1	141±13.3
St. John´s-wort	*Hypericum perforatum* L.	Plant overground	916±30.7	410±19.2	366±13.3	119±18.3	20±1.9	64±4.9
Yarrow	*Achillea millefolium* L.	Plant overground	911±19.0	580±22.1	557±21.7	67±3.7	11±0.5	79±5.5
Nettle	*Urtica dioica* L.	Plant overground	904±22.3	354±24.6	287±19.7	269±9.5	43±2.4	178±12.2
Chicory	*Cichorium intybus* L.	Plant overground	914±28.2	524±12.0	427±23.5	115±9.5	19±0.8	103±18.2

Table 2. Chemical and fatty acid (FA) composition of diet substrates (means ± standard error of the mean)

	Mix of medicinal plants	Lucerne hay	Barley grain
Dry matter (DM, g/kg)	910±21.3	912±20.5	897±22.1
Neutral detergent fibre (g/kg DM)	375±14.5	453±18.7	177±10.3
Acid detergent fibre (g/kg DM)	340±10.2	319±16.4	45±4.2
Crude protein (g/kg DM)	100±17.0	243±20.9	109±14.3
N (g/kg DM)	16.1±3.7	38±5.4	18±1.9
Ash (g/kg DM)	90±2.2	97±2.5	31±1.9
Fat (g/kg DM	40±0.9	23±0.9	25±1.2
In vitro dry matter digestibility (g/kg DM)	658±18.5	779±22.4	891±14.3
C14:0 myristic (g/kg FA)	42.0±1.71	13.2±1.70	4.7±1.70
C16:0 palmitic (g/kg FA)	239±12.3	260±10.7	240±14.5
C18:0 stearic (g/kg FA)	27.2±3.56	56.5±4.73	65.3±2.91
C18:1*n*-9 oleic (g/kg FA)	33.4±7.2	102±7.1	158±7.2
C18:2*n*-6 linoleic (g/kg FA)	280±11.2	230±10.3	420±12.8
C18:3*n*-3 -linolenic (g/kg FA)	184±7.5	180±8.1	50±6.5
Saturated fatty acids (g/kg FA)	380±13.0	360±10.3	322±16.1
Monounsaturated fatty acids (g/kg FA)	110±16.2	115±12.0	190±20.4
Polyunsaturated fatty acids (g/kg FA)	506±19.4	440±25.7	483±13.1

Mix of medicinal plants: (*Taraxacum officinale* L., *Acorus calamus* L., *Calendula officinalis* L., *Hypericum perforatum* L., *Achillea millefolium* L., *Urtica dioica* L., *Cichorium intybus* L.).

Table 3. The effects of selected individual herbs on rumen fermentation patterns after 24 h incubation in vitro (means ± standard error of the mean)

	***Taraxacum officinale* L.**	***Acorus calamus* L.**	***Calendula officinalis* L.**	***Hypericum perforatum* L.**	***Achillea millefolium* L.**	***Urtica dioica* L.**	***Cichorium intybus* L.**	**P-value**
IVDMD (g/kg DM)	801^{e}±8.3	771^{e}±12.7	672^{d}±9.3	511^{b}±2.2	348^{a}±10.6	584^{c}±4.5	489^{b}±12.8	0.001
Gas (ml/g DM)	187±1.7	182±1.7	184±1.6	186±1.9	183±1.7	182±1.7	183±2.0	0.052
Methane (%)	9.16±0.88	8.63±1.01	9.41±1.01	7.28±1.24	7.19±1.01	6.73±0.51	7.40±1.58	0.115
SCFA (mmol/l)	60.2^{c}±7.64	58.6^{c}±9.59	54.5^{b}±8.70	49.8^{ab}±8.46	47.1^{a}±7.77	51.2^{ab}±8.56	46.3^{a}±6.65	0.001
Acetate (mol%)	64.3^{a}±0.91	62.6^{a}±1.04	67.1^{b}±0.84	68.1^{b}±1.73	65.3^{ab}±0.97	66.1^{b}±0.70	66.4^{b}±0.84	0.002
Propionate (mol%)	17.4±2.03	18.7±0.78	16.6±0.54	15.3±0.97	15.8±1.01	16.1±0.97	15.4±1.14	0.227
n-Butyrate (mol%)	13.4^{b}±0.33	12.8^{b}±0.64	9.4^{a}±0.50	10.4^{a}±0.71	11.4^{ab}±0.50	10.0^{a}±0.43	11.0^{a}±0.90	0.001
i-Butyrate (mol%)	1.27^{a}±0.09	1.65^{a}±0.04	2.37^{b}±0.21	1.91^{ab}±0.31	2.66^{b}±0.21	2.93^{b}±0.23	2.25^{b}±0.52	0.002
n-Valerate (mol%)	1.79±0.11	1.39±0.17	1.74±0.08	1.56±0.30	1.59±0.16	1.66±0.08	1.75±0.13	0.058
i-Valerate (mol%)	1.68^{a}±0.14	2.15^{b}±0.19	2.48^{bc}±0.24	2.54^{bc}±0.57	2.96^{c}±0.34	3.02^{c}±0.35	2.98^{c}±0.29	0.001
2H-recovery (%)	65.4±3.06	67.1±5.0	69.2±5.71	62.1±6.39	64.7±2.31	62.8±6.01	69.2±4.09	0.242
Protozoa (10^3 n/ml)	383±67.7	308±60.9	313±20.1	313±69.2	358±87.2	383±106.4	388±90.0	0.270

IVDMD: in vitro dry matter digestibility; SCFA: short-chain fatty acids.
[a,b,c,d,e] Values within a row with different superscript letters differ at P<0.05.

Table 4. Correlation matrices (i.e., Pearson's correlation coefficients) between protozoan counts and fermentation patterns.

	IVDMD	Gas	SCFA	Methane	Acetate	Propionate	*n*-Butyrate
Gas	-0.209 (0.101)[*]						
SCFA	0.193 (0.130)	-0.018 (0.886)					
Methane	0.125 (0.328)	-0.009 (0.944)	0.363 (0.003)				
Acetate	-0.255 (0.044)	0.157 (0.220)	-0.607 (0.001)	-0.365(0.003)			
Propionate	0.362 (0.004)	0.010 (0.941)	0.688 (0.001)	0.101 (0.433)	-0.704 (0.001)		
n-Butyrate	0.399 (0.001)	-0.208 (0.102)	0.399 (0.001)	0.409 (0.001)	-0.720 (0.001)	0.283 (0.025)	
2H-recovery	-0.014 (0.912)	-0.016 (0.900)	-0.503 (0.001)	0.571 (0.001)	0.044 (0.734)	-0.411 (0.001)	0.054(0.674)
Protozoa	-0.060 (0.643)	-0.126 (0.325)	0.391 (0.002)	0.461 (0.001)	-0.283 (0.025)	-0.080 (0.533)	0.320(0.011)

IVDMD: in vitro dry matter digestibility; SCFA: short-chain fatty acids. [*]Values are correlation coefficients with P-values.

Table 5. Effect of high-concentrate diet (lucerne hay: barley grain, LH+BG, 40:60), where LH was substituted for 100, 50, and 10% by a mixture of medicinal plants (MPM) on fermentation parameters in vitro (means ± standard error of the mean).

	LH+BG	MPM100+BG	LH+MPM50+BG	LH+MPM10+BG	P-value
IVDMD (g/kg DM)	579^{a}±5.4	642^{b}±2.5	610^{b}±9.4	655^{b}±1.1	0.001
Gas (ml/g DM)	135^{a}±3.5	146^{b}±4.7	146^{b}±5.1	121^{a}±4.6	0.003
Methane (%)	8.25^{b}±0.01	7.63^{a}±0.06	7.38^{a}±0.26	7.18^{a}±0.15	0.015
SCFA (mmol/l)	48.7^{b}±2.31	42.6^{a}±0.35	41.6^{a}±1.29	36.2^{a}±0.11	0.001
Acetate (mol%)	67.1±0.33	68.6±0.22	67.6±0.13	68.3±0.81	0.159
Propionate (mol%)	16.6^{b}±0.13	15.9^{a}±0.05	15.5^{a}±0.20	15.6^{a}±0.10	0.002
n-Butyrate (mol%)	10.7±0.08	11.3±0.13	11.3±0.35	11.6±0.49	0.267
2H-recovery (%)	64.8^{a}±0.35	76.3^{c}±0.33	65.6^{a}±0.20	68.7^{b}±0.18	0.001
Total protozoan number (10^3 n/ml)	199^{ab}±3.6	221^{b}±8.3	189^{ab}±7.6	162^{a}±11.6	0.006
Entodinium spp. (10^3 n/ml)	194^{ab}±8.6	216^{b}±11.8	184^{ab}±6.4	156^{a}±12.1	0.005
Dasytricha ruminantium (10^3 n/ml)	3.98±0.435	4.03±0.292	3.71±0.280	4.21±0.251	0.751
Isotricha spp. (n/ml)	390±66.5	260±49.9	180±40.2	330±67.7	0.104
Enoploplastron triloricatum (n/ml)	245±57.4	240±49.6	140±29.0	320±38.7	0.090
Polyplastron multivesiculatum (n/ml)	170±44.9	120±27.1	95±11.6	135±11.6	0.296
Ophryoscolex c. tricoronatus (n/ml)	305^{ab}±76.8	260^{ab}±64.2	220^{a}±32.9	516^{b}±92.6	0.039

IVDMD: in vitro dry matter digestibility; SCFA: short-chain fatty acids.
^{a,b,c}Values within a row with different superscript letters differ at P<0.05.

Table 6. Effect of high-concentrate diet (lucerne hay: barley grain, LH+BG, 40:60), where LH was substituted for 100, 50, and 10% by a mixture of medicinal plants (MPM) on fatty acids in rumen fluid in vitro (means ± standard error of the mean)

Fatty acids (g/kg of FA)	LH+BG	MPM100+BG	LH+MPM50+BG	LH+MPM10+BG	P-value
C14:0 myristic	17.2^{a}±0.59	18.5^{a}±0.64	27.6^{c}±0.66	23.8^{b}±0.64	0.001
C16:0 palmitic	273^{a}±6.1	270^{a}±3.8	306^{b}±4.6	312^{b}±5.3	0.003
C18:0 stearic	450^{bc}±3.8	462^{c}±5.2	419^{a}±5.8	431^{b}±8.9	0.004
C18:1n-9 oleic	28.2^{b}±0.17	28.2^{b}±1.16	26.2^{b}±1.18	23.9^{a}±1.51	0.039
C18:1trans-11 vaccenic	57.2^{b}±1.45	57.7^{b}±1.83	54.0^{b}±2.03	49.5^{a}±1.44	0.043
C18:2n-6 linoleic	37.9^{a}±1.52	44.8^{b}±1.18	35.6^{a}±1.17	32.3^{a}±0.87	0.007
C18:2cis-9 trans-11	2.20^{ab}±0.17	2.80^{b}±0.33	2.15^{ab}±0.15	1.35^{a}±0.15	0.033
C18:3n-3 -linolenic	18.8^{bc}±0.87	19.8^{c}±0.87	16.0^{ab}±1.01	14.6^{a}±0.93	0.006
C20:0 arachidic	12.7^{b}±0.87	11.1^{ab}±0.88	9.44^{ab}±1.44	7.84^{a}±0.97	0.027
C22:0 behenic	9.45±0.92	8.77±1.45	7.95±0.99	7.45±0.87	0.599
C24:0 lignoceric	26.5^{c}±0.87	17.6^{b}±0.29	17.8^{b}±0.53	9.55^{a}±0.42	0.001
Total C18 FA	597^{ab}±5.6	617^{b}±14.5	556^{a}±17.9	556^{a}±20.2	0.040
Saturated FA	826^{b}±4.4	782^{a}±4.6	847^{b}±5.9	845^{b}±8.4	0.001
Monounsaturated FA	95^{a}±1.25	102^{b}±1.16	93^{a}±1.16	91^{a}±0.88	0.001
Polyunsaturated FA	79^{b}±2.3	112^{c}±2.3	60^{a}±2.9	64^{a}±2.3	0.001

^{a,b,c} Within a row, means without a common superscript letter differ at P<0.05.

DISCUSSION

In the present study, the selection of medicinal plants was based on information available about their beneficial effects in traditional human medicine and their carminative and anti-inflammatory effects (Kresánek and Kresánek, 2008). Our phytochemical tests of the ethanolic extracts of selected medicinal plants revealed the presence of medically active compounds (mainly tannins, saponins, alkaloids, terpenoids and flavonoids) that have curative activity against pathogens (Cowan, 1999).

The IVDMD of individual plants showed a higher trend for the examined medicinal plant roots compared with flowers and overground plants. The higher IVDMD of *T. officinale* and *A. calamus* can probably be ascribed to the content of the more digestible root storage carbohydrates as inulin (Schütz *et al.,* 2006). The value of IVDMD was lowest in *A. millefolium* (348 g/kg of DM); however, in comparison with the *A. millefolium* from high altitude the IVDMD (after 48 h in vitro incubation) was higher and the value reached 610 g/kg of DM (Tufarelli *et al.,* 2010). The lower values of plant DM of *A. millefolium* digestibility could also be

ascribed to the lower nitrogen content in comparison with the other examined herbs. The other IVDMD values varied among the individual medicinal plants; however, they were within the respective values of meadow hay and wheat straw (Váradyová *et al.,* 2005). In our experiment, the substitution of lucerne hay with a dry mixture of selected medicinal plants increased the IVDMD of all diets. These results are consistent with Makkar *et al.* (1995), who suggest that different doses and sources of plant tannins could have different effects on DM digestibility in vitro. However, the impact of root storage polysaccharides cannot be excluded.

The individual medicinal plants tested did not differ in methane production across treatments; however, the measured values of methane were lower compared with the methane values in other in vitro plant studies (Jayanegara *et al.,* 2009; Bhatta *et al.,* 2012). Jayanegara *et al.* (2011) reported a decrease in methane production after 24 h rumen fermentation of *Achillea millefolium*. In addition, Purcell *et al.* (2012) reported lower methane output per unit of feed DM incubated after 24 h rumen fermentation of *Urtica dioica* compared with grass silage. In our experiment, lower methane production was observed in diets with MPM supplement; this is in agreement with the finding that tannins generally decrease ruminal methanogenesis in vitro (Bhatta *et al.,* 2013). The negative correlation between SCFA data for individual medicinal plants *versus* hydrogen recovery and acetate suggests a negative effect on acetogenesis. The positive correlation between methane production *versus* hydrogen recovery data, *n*-butyrate and protozoa count suggests greater effects on methanogenesis than on acetogenesis.

From our results it is evident that the substitution of lucerne hay with a dry mixture of medicinal plants in high-concentrate diets decreased the total SCFA by decreasing the propionate concentration and increasing hydrogen recovery. This resulted in a numerical increase of acetate and *n*-butyrate. These differences were probably caused by the inhibitory effect of active plant compounds on ruminal microbes; however, the changes are within the range of the physiological fermentation pattern. However, most active compounds in plants do not affect fermentation parameters, such as SCFA and IVDMD, or the counts of microbial populations in vitro (Zmora *et al.,* 2012). The acetate:propionate ratio (A:P) of the individual medicinal plants tested ranged from 3.35 (*A. calamus*) to 4.45 (*H. perforatum*), and differences in the A:P ratios were probably related to either the plant composition and/or the composition of the microbial population. Previously the A:P ratios produced in rumen fluid fermentation process of barley grain ranged from 1.3 to 2.1, while meadow hay fermented with the same inoculum yielded an A:P of 4.0 and more (Váradyová *et al.,* 2005). These differences were due to the relatively low propionate production of meadow hay. Similar results with low propionate production were also found in our in vitro study. No differences were found in the A:P ratios among the MPM100+BG (4.32), LH+MPM50+BG (4.36) and LH+MPM10+BG (4.38) diets.

In our experiment, a positive correlation between protozoan counts of individual medicinal plants and methane production was observed; in contrast Bhatta *et al.* (2013) observed that methanogenesis is not essentially related to the density of protozoa population in vitro. The effects of MPM supplements on ciliate growth were inconsistent. Only amylolytic *Entodinia* and fibrolytic-amylolytic *Ophryoscolex* were influenced by MPM supplements. Results showed that except for the MPM100+BG and LH+MPM10+BG diets, the other diets did not influence protozoan growth. The substitution of lucerne hay by 10% MPM (i.e., LH+MPM10+BG) positively influenced the growth of *Ophryoscolex c. tricoronatus*. The growth efficiency of the majority of rumen ciliates depends on the amount of concentrate in the animal diet, and rumen ciliate populations usually increase with a concentrate proportion of up to 60% in the diet (Franzolin and Dehority, 1996).

In our study, the MPM100+BG diet contained the highest content of linoleic acid, CLA and -linolenic acid due to the higher proportion of MPM with plant polyphenols added to the diet. Cabiddu *et al.* (2010) found that forage species and the phenological stage, especially tannic polyphenols, affect lipolysis and biohydrogenation in rumen fluid in vitro. In addition, tannins reduce ruminal biohydrogenation through the inhibition of the activity of ruminal microorganisms which hydrogenated *trans*-vaccenic acid to stearic acid (Vasta *et al.*, 2009). The oleic acid and linoleic acid can form *trans*-vaccenic acid for endogenous synthesis of CLA during biohydrogenation to stearic acid. Previously we tested plant oils (sunflower, rapeseed) rich in linoleic acid and oleic acid in combination with organic acids (fumaric, maleic); the increase of the *trans*-vaccenic acid in the batch cultures with both high-fibre and high-concentrate diets was reported (Váradyová *et al*., 2013). In the LH+MPM10+BG diet the lower ciliate count was accompanied by a lower content of *trans*-vaccenic acid, linoleic acid, CLA and -linolenic acid compared with the MPM100+BG diet. However, the forages contained more linoleic and -linolenic acids, and concentrates more oleic and linoleic acids. In our experiment, the content of some fatty acids differed across treatments, probably because the -linolenic acid in dried grass (lucerne hay and mixture of medicinal plants) is in the glycolipid form, which is less susceptible to rumen hydrolysis and biohydrogenation than the free form (Wachira *et al.,* 2000).

It can thus be concluded that for first time a mixture of selected medicinal plants (i.e., *T. officinale, A. calamus, C. officinalis, H. perforatum, A. millefolium, U. dioica* and *C. intybus*) has been used in high-concentrate

diets in vitro, and the majority of the effects of the MPM supplements were beneficial (higher IVDMD and lower methanogenesis). We also observed promising effects on unsaturated fatty acids.

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