doi: 10.17221/17/2016-CJAS

Effects of diet supplementation with herbal blend and sunflower seeds on fermentation parameters, microbial population, and fatty acid profile in rumen of sheep

M. Wencelová, Z. Váradyová, P. Pristaš, K. Čobanová, I. Plachá, S. Kišidayová

Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic

ABSTRACT: The study evaluated the rumen fermentation pattern, microbial population, fatty acid composition, and antioxidant status in the rumen fluid and blood of sheep in response to a diet supplemented with herbal blend (HB), sunflower seeds (SS), and a combination of both HB and SS (HBSS). The HB consisted of medicinal herbs typical for Central Europe. Four rumen fistulated rams were used in a 4 × 4 Latin square design and were fed a basal diet consisting of meadow hay (720 g dry matter (DM)/day) and barley grain (540 g DM/day) in the ratio of 60 : 40 and supplemented with no additive (Control), HB (10% replacement of meadow hay), SS (180 g DM/day), and HBSS (HB + SS). An increase in the molar proportion of *iso*-butyrate (P = 0.009) and *iso*-valerate (P = 0.034) was observed in the SS and HBSS diets as well as of ammonia N concentration in the HB, SS, and HBSS diets compared to control (P = 0.003). Concentrations of monounsaturated fatty acids of the HB, SS, and HBSS diets were increased (P = 0.041) compared to control, whereas concentrations of polyunsaturated fatty acids in the SS and HBSS diets were decreased (P = 0.023). Phagocytic activity in the blood was higher with all the diets vs control (P = 0.001). Qualitative evaluation of microbial population by denaturing gradient gel electrophoresis (DGGE) and principal component analysis (PCA) revealed no effects of dietary treatments on the composition of both eubacterial and ciliated protozoal populations. However, in spite of the supplementation of forage-concentrate diet by HB in the presence of SS, no negative effects on the fermentation patterns and rumen microbial population have been detected.

Keywords: 16S-PCR-DGGE; medicinal herbs; phagocytic activity; lipid metabolism; rumen fluid; PUFA source

INTRODUCTION

Due to the increased incidence of metabolic disease in human population, food composition is today becoming more important than ever. Ruminant derived products are perceived by many consumers as unhealthy because of the low level of beneficial polyunsaturated fatty acids (PUFA) and the predominance of saturated fatty acids (SFA). These findings have in turn stimulated research on the modification of the fatty acid composition of these products through dietary means. In order to find ways of enhancing the outflow of PUFA and biohydrogenation intermediates from rumen, it is necessary to understand the microbial processes of lipolysis and biohydrogenation.

Medicinal herbs, as a replacement for antibiotics, contain an extensive variety of secondary compounds and have the potential to affect lipolysis and biohydrogenation (Wencelova et al. 2015), rumen fermentation, and methane production (Bhatta et al. 2013). It is known that phytogenic additives enhance a number of important processes in the animal body and positively affect feed quality, health of animals as well as animal products by means of their specifically efficacious substances (Karaskova

Supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences (VEGA 2/0009/14) and by the Slovak Research and Development Agency (APVV 14-0169).

doi: 10.17221/17/2016-CJAS

et al. 2015). On the other hand, the addition of a fat source into the ruminant diet has been recognized as a strategy for raising the energy density of the diet, affecting methanogenesis and augmenting the concentrations of health-promoting PUFA in ruminant-derived food (Glasser et al. 2008). Dietary fat is extensively metabolized and hydrogenated in the rumen, and this process is associated with the formation of SFA and the accumulation of a wide range of biohydrogenation intermediates. Selective inhibition of bacteria capable of converting vaccenic acid to stearic acid would provide more unsaturated fatty acids, including vaccenic acid and conjugated linoleic acid (Durmic et al. 2008).

The objectives of this experiment were to determine the effects of herbal blend (as a component of a diet ration) and sunflower seeds (as a source of PUFA) on rumen fermentation and lipid metabolism of sheep fed forage-concentrate diet (60 : 40), and whether the supplementation of diets with herbal blends affects the rumen microflora in the presence of sunflower seeds.

MATERIAL AND METHODS

Animals, diets, and experimental design. Four rams (Lacaune vs Suffolk; 1.5 years of age; 45.0 ± 2.5 kg weight) fitted with rumen cannulas were used in a 4 × 4 Latin square design. The experimental protocol was approved by the Ethical Committee of the Institute of Animal Physiology SAS and the State Veterinary and Food Office (Ro-2061/13-221). The rams were housed in separated pens with free access to water. The experimental treatments were (1) basal diet (control) composed of 720 g dry matter (DM)/day meadow hay and 540 g DM/day barley grains (600/400, w/w); (2) basal diet enriched with a herbal blend (HB) (10% replacement of meadow hay by HB); (3) basal diet enriched with sunflower seeds (SS) (180 g DM/day); and (4) basal diet enriched with a combination of HB and SS (HBSS). The diets were divided into two equal meals per day that were supplied at 6:00 and 15:00 h. The crushed SS and HB were mixed with barley grain. The HB consisted of seven medicinal herbs typical for Central Europe; additionally, the medicinal herbs were chosen according to their anti-inflammatory and carminative effects (Kresanek and Kresanek 2008). 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.) were obtained from commercial sources in the form of dry plant material. After being ground up (particle size of 0.15–0.40 mm), the individual herbs were mixed in equal amounts; the plant mixture was stable through the whole experiment. The chemical composition of the meadow hay, barley grain, herbal blend, and sunflower seeds is given in Table 1.

Table 1. Chemical composition and composition of fatty acids of diet substrates

	Diet substrates			
	MH	BG	HB	SS
Dry matter (g/kg)	916	868	896	930
N (g/kg DM)	25	19	28	21
CP (g/kg DM)	150	115	168	126
ADF (g/kg DM)	380	60	350	235
NDF (g/kg DM)	470	180	402	310
Ash (g/kg DM)	70	34	91	50
Fat (g/kg DM)	21	25	34	515
C16:0 palmitic (g/kg of FA)	320	240	245	60
C18:0 stearic (g/kg of FA)	50	65	45	35
C18:1n9 *cis* oleic (g/kg of FA)	110	160	130	260
C18:2n6 linoleic (g/kg of FA)	260	420	280	630
C18:3n3 α-linolenic (g/kg of FA)	150	50	180	10
Saturated FA (g/kg of FA)	370	305	290	95
Monounsaturated FA (g/kg of FA)	110	160	130	260
Polyunsaturated FA (g/kg of FA)	410	470	460	640

DM = dry matter, CP = crude protein, ADF = acid detergent fibre, NDF = neutral detergent fibre, FA = fatty acids, MH = meadow hay, BG = barley grain, HB = herbal blend, SS = sunflower seeds

Sample collection. During the one-month adaptation period the animals received the basal diet supplemented with HB (20 g DM/day) and SS (60 g DM/day). The experiment consisted of four experimental periods, each with the duration of 2 weeks followed by a 7-day wash out phase. During this phase the rams had access to the basal diet. Samples of rumen fluid and blood were collected from each animal on days13 and 14 of each experimental period before the morning feeding. Blood samples were collected from the jugular vein into heparinized tubes and immediately centrifuged at 1200 *g* for 15 min at 4°C. For microscopic counting

doi: 10.17221/17/2016-CJAS

of microbes (ciliates and bacteria), a 5 ml portion of rumen fluid per animal was preserved with an equal amount of 8% formaldehyde solution and refrigerated until analysis. For polymerase chain reaction denaturing gradient gel electrophoresis analysis (PCR-DGGE) of the rumen eubacterial population, rumen fluid of approximately 10 g per animal was dispensed to microtubes and stored at –80°C until analysis. For evaluation of the antioxidant status, the rumen fluid was squeezed through four layers of gauze and centrifuged at 15 000 *g* for 15 min (4°C). Plasma samples and supernatant of rumen fluid were stored at –80°C until analysis.

Chemical analysis and measurements. Using standard methods (AOAC 1990), the diet substrates were submitted to chemical analysis for DM (method No. 967.03), ash (method No. 942.05), nitrogen (method No. 978.02), fat (method No. 9836.23), and crude protein (method No. 990.03). A Fibertec System 2021 FiberCap (Foss Analytical AB, Höganäs, Sweden) was used to determine the quantity of acid-detergent fibre (ADF) and neutral-detergent fibre (NDF) in the samples of diet substrates (Van Soest et al. 1991). The values of ADF were expressed inclusive of residual ash. The content of NDF in concentrates was assayed using a heat-stable amylase and expressed inclusive of residual ash, while the content of NDF in forages was determined without a heat-stable amylase and expressed inclusive of residual ash. The total phenol amount (i.e. 25 g/kg DM) of HB was estimated according to Makkar et al. (1993) using the Folin-Ciocalteu method and the result was expressed as the tannic acid equivalent. The pH of the rumen fluid samples was measured using a pH meter (InoLab pH Level 1, Weilheim, Germany).

Analyses of short-chain fatty acids (SCFA) were carried out by gas chromatography; 1 µl of sample was directly injected into a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer, Inc., Waltham, USA) using crotonic acid as the internal standard. Standards of SCFA (acetic, propionic, and *n*-butyric acid) with purities higher than 99% (Sigma Aldrich, St. Louis, USA) were used for constructing the calibration curve. A gas chromatograph equipped with flame ionization detection (FID) system for estimating SCFA was used. Separation of the SCFAs was performed using a stainless steel packed column (2 m × 2 mm i.d.) with a phase composition of 10% Carbowax 20M-TPA + 1% H_3PO_4 on a 100/120 Supelcoport (Supelco, Bellefonte, USA), and the peaks at retention times of 3.3, 4.7, and 6.8 min for acetic, propionic, and *n*-butyric acid, respectively, were quantified. The column oven temperature was programmed at 150°C for SCFA. The injector and detector temperatures were programmed at 230°C for SCFA. The gas flow rates were 40 ml/min for hydrogen and 400 ml/min for air. The average nitrogen carrier gas flow was set at 36 psi for SCFA.

Fatty acids from freeze-dried samples of rumen fluid were extracted and esterified. Volumes of 100 ml of rumen fluid were freeze-dried and weighed. Lipids were extracted from 500 mg of freeze-dried sample with a 2 : 1 mixture of chloroform : methanol, by the method of Bligh and Dyer (1959) and the samples were purified with 20% HCl. The extracted lipids were dissolved in 1 ml of hexane. Tridecanoic acid (Fluka Chemie GmbH, Buchs, Switzerland) was used as the internal standard. The fatty acid methyl ester peaks were identified using authentic standards of a C4–C24 FA methyl ester mixture (Supelco) by gas chromatography on a PerkinElmer Clarus 500 gas chromatograph.

The concentration of ammonia nitrogen was assayed by the phenol-hypochlorite method (Broderick and Kang 1980). Ciliated protozoa were microscopically identified and counted according to Williams and Coleman (1992). To estimate the total bacterial biomass, a direct bacterial count through image analysis of pictures was carried out as described previously (Wencelova et al. 2015).

PCR-DGGE analysis of rumen eubacterial population. Total community DNA was isolated from rumen fluid samples (5 ml) using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The quality of the community DNA was assessed by 0.8% agarose gel electrophoresis. The total DNA (50 ng) was used as a template for PCR amplification of the rRNA genes. For the analysis of eubacterial populations universal primers for 16S rRNA fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3'), rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') and the conditions specified by Weisburg et al. (1991) were used to amplify about 1500 base-pair regions of the 16S rDNA genes in the first round of PCR. The obtained 16S rDNA fragments were subsequently used as a template for the second round of PCR using specific bacterial primers GC-clamp-968f

doi: 10.17221/17/2016-CJAS

(5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and 1401r (5'-CGG TGT GTA CAA GACCC-3') and the conditions specified by Nübel et al. (1996). The PCR reactions were performed in a 50 µl PCR mixture containing 1 µl of DNA, 1 × PCR buffer, 2 mmol/l $MgCl_2$, 1 µl of 200 µmol/l of each dNTP, 1.25 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, USA), and 25 µmol of each primer using a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, USA). The specificity of the PCR reactions was monitored by 1.2% agarose gel electrophoresis. Denaturing gradient gel electrophoresis was performed using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories). Polymerase chain reaction products in a total volume of 45 µl were loaded onto 8% (w/v) polyacrylamide gel (40% Acrylamide/Bis solution 37.5 : 1) in 1 × TAE (40 mmol Tris, 20 mmol acetate, 1 mmol EDTA) containing a linear denaturing gradient ranging from 30 to 60% denaturant (a 100% denaturant solution consists of 7 mol urea and 40% formamide). Electrophoresis was run for 17 h at a constant voltage of 50 V and a temperature of 60°C.

Phagocytic activity, antioxidant status, and lipid peroxidation. Phagocytic activity was analyzed by a direct microscopic counting procedure according to Wencelova et al. (2015). The activity of blood glutathione peroxidase (GPx, EC 1.11.1.9) was determined by the method of Paglia and Valentine (1967) with a Ransel kit (Randox, Crumlin, UK). The haemoglobin (Hb) content of blood was analyzed using a commercial kit from Randox. The malondialdehyde (MDA) concentration in plasma was measured with the modified fluorometric method according to Jo and Ahn (1998) using 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) as a MDA precursor in the calibration curve. The total antioxidant capacity (TAC) of the plasma and rumen fluid samples was evaluated by applying the FRAP assay (ferric reducing/antioxidant power) described by Benzie and Strain (1996). This method is based on the reduction of the ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex to the ferrous (Fe^{2+}) form in the presence of antioxidants, which develops an intense blue colour with absorbance at 593 nm. The calibration curve was prepared using a ferrous sulphate solution, and the results were expressed in mmol Fe^{2+} formed per litre sample.

Statistical analysis. Statistical analysis of a 4 × 4 Latin square using the analysis of variance (GraphPad Prism, GraphPad Software, Inc. San Diego, USA) as a repeated measures mixed model was performed. The model included effects for periods, sheep, and treatment. Treatment effects were determined to be significant at $P < 0.05$, and when this occurred individual treatments were compared for significant differences using an unpaired *t*-test. Ciliates and total bacteria counts were evaluated by a nonparametric Kruskal–Wallis test with Dunn's Multiple Comparison Test. The ethidium bromide-stained DGGE gels were recorded using the Gel Logic Pro documentation system (Carestream Health, Inc., Rochester, USA). The DGGE fingerprints obtained were processed using Phoretix1D software (TotalLab Ltd., Newcastle upon Tyne, UK) without any user interference, and the DGGE fingerprints were transformed into a band-matching table. Data on the absence/presence of a specific DGGE band were combined with data on the presence/absence of a specific protozoa genus (*Entodinium* spp., *Polyplastron* spp., *Isotricha* spp., *Dasytricha* spp.) in the single dataset, and covariance analysis was performed on this dataset using Community Analysis Package version 4 (PISCES Conservation Ltd., Pennington, UK).

RESULTS

Rumen fermentation parameters and protozoal and eubacterial community. An increase in the molar proportion of *iso*-butyrate ($P = 0.009$) and *iso*-valerate ($P = 0.034$) in the SS and HBSS diets as well as in ammonia N concentration in the HB, SS, and HBSS diets ($P = 0.003$) was observed as compared to the control (Table 2). The rumens of 4 sheep were harboured with the A-type population consisting of 4 genera of ciliates: *Entodinium* (96.3% of the total count), *Polyplastron* (*Polyplastron multivesiculatum,* 1.8% of the total count), *Dasytricha* (*Dasytricha ruminantium,* 1.5% of the total count), and *Isotricha* (*Isotricha prostoma,* 0.3% of the total count). *Entodinium* and *Polyplastron* genera occurred in all the animals; therefore, only these two genera were statistically evaluated. The supplementation of the diet with HB, SS, and HBSS resulted in numerical changes of the protozoal and bacterial counts; however, no significant effects of treatment were observed. Covariance analysis of the combined data of eubacterial and ciliates communities revealed very

doi: 10.17221/17/2016-CJAS

Table 2. Effects of herbal blend (HB), sunflower seeds (SS), and their combination (HBSS) on the fermentation pattern in sheep rumen fluid (n = 8 per each diet, values are means ± SEM)

Items	Dietary treatments				P-value
	Control	HB	SS	HBSS	
SCFA (mmol/l)	53.3 ± 4.7	49.7 ± 3.7	33.1 ± 5.0	47.2 ± 7.7	0.062
Acetate (mol%)	69.6 ± 1.8	69.4 ± 1.8	66.7 ± 1.3	66.4 ± 1.3	0.442
Propionate (mol%)	15.9 ± 1.8	14.9 ± 1.3	15.4 ± 1.1	18.4 ± 0.8	0.299
n-Butyrate (mol%)	10.9 ± 0.8	11.6 ± 0.4	10.8 ± 0.8	10.2 ± 1.9	0.891
iso-Butyrate (mol%)	1.09^{a} ± 0.03	1.10^{a} ± 0.1	1.81^{b} ± 0.03	1.81^{b} ± 0.1	0.009
n-Valerate (mol%)	1.14 ± 0.1	1.27 ± 0.1	1.92 ± 0.2	1.47 ± 0.4	0.114
iso-Valerate (mol%)	1.08^{a} ± 0.2	1.20^{ab} ± 0.2	2.43^{b} ± 0.3	1.50^{ab} ± 0.5	0.034
Ammonia N (mg/l)	81^{a} ± 3.5	114^{b} ± 1.7	148^{bc} ± 10.4	174^{c} ± 20.4	0.003
pH	6.78 ± 0.3	6.88 ± 0.2	6.87 ± 0.2	7.0 ± 0.2	0.945
Total ciliates (10^3/ml)	182 ± 18.7	252 ± 28.5	158 ± 36.9	151 ± 57.3	0.336
Entodinium spp. (10^3/ml)	181 ± 18.6	251 ± 28.4	157 ± 36.8	150 ± 57.0	0.334
Polyplastron multivesiculatum (count/ml)	3040 ± 1876	5460 ± 1305	3700 ± 866	1600 ± 1081	0.148
Total bacteria (10^9/ml)	22.5 ± 2.1	19.8 ± 2.0	15.0 ± 1.9	20.0 ± 2.3	0.258

SCFA = short-chain fatty acids

$^{a–c}$values within a row without a common superscript letter are significantly different ($P < 0.05$)

low effects of the diet treatments on the composition of the communities (Figure 1). However, there is a tendency toward an impact of the absence of the *Dasytricha* and *Isotricha* ciliate genera on the composition of eubacterial community irrespective of the diet treatments (L9, 13, 15, 16).

Fatty acid profile of rumen fluid. Both diets, SS and HBSS, decreased the concentration of

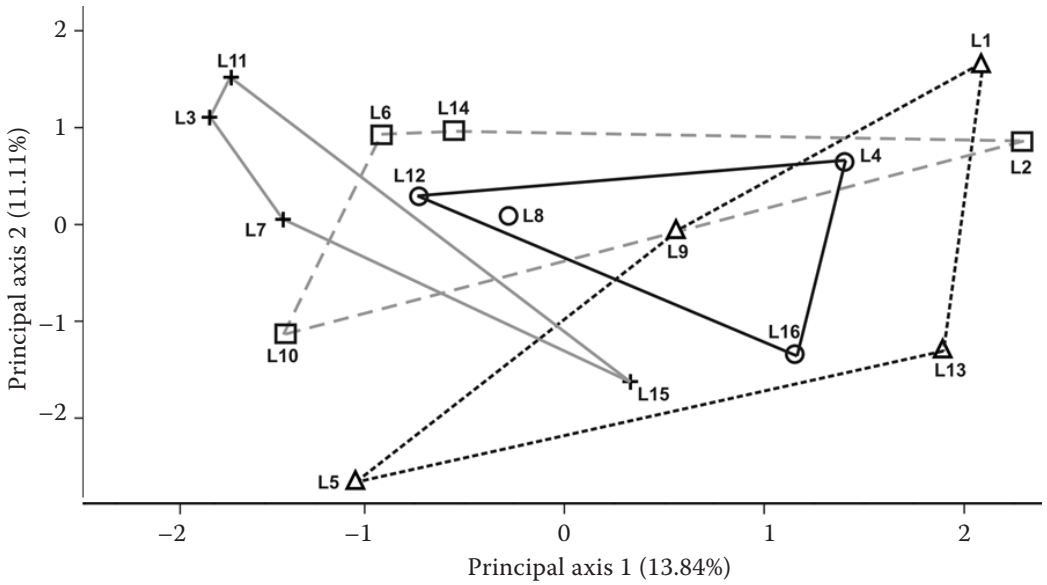

Figure 1. Principal component analysis of the microbial community composition (both eubacteria and ciliates) in rumen fluid (L1–L16) of sheep

diets labels: control (Δ), herbal blend (HB; □); sunflower seeds (SS; ○), combination of herbal blend and sunflower seeds (HBSS; +)

L1, 2, 7, 8, 11, 14 = presence of 4 genera of ciliates (*Entodinium, Dasytricha, Isotricha, Polyplastron*); L3 = absence of *Isotricha*; L4, 12, 13 = absence of *Dasytricha*; L6, 9, 10, 15, 16 = absence of both *Isotricha* and *Dasytricha*; L5 = absence of *Isotricha, Dasytricha, Polyplastron*

doi: 10.17221/17/2016-CJAS

Table 3. Effects of herbal blend (HB), sunflower seeds (SS), and their combination (HBSS) on the fatty acid profile in sheep rumen fluid (n = 8 per each diet, values are means ± SEM)

Fatty acids (g/kg of FA)	Dietary treatments				*P*-value
	Control	HB	SS	HBSS	
C8:0	0.03 ± 0.02	0.28 ± 0.17	0.48 ± 1.22	1.47 ± 0.26	0.326
C10:0	1.42 ± 0.60	0.95 ± 0.21	0.44 ± 0.28	0.54 ± 0.27	0.276
C11:0	0.26 ± 0.12	0.25 ± 0.06	0.27 ± 0.05	0.09 ± 0.12	0.584
C12:0	6.21^{b} ± 0.67	6.19^{b} ± 0.77	3.08^{a} ± 0.30	2.85^{a} ± 0.47	0.001
C14:0	13.6^{b} ± 1.62	16.4^{b} ± 2.01	6.56^{a} ± 0.95	7.25^{a} ± 1.35	0.003
C14:1	0.35^{b} ± 0.10	0.33^{ab} ± 0.12	0.06^{a} ± 0.03	0.10^{ab} ± 0.03	0.031
C15:0	25.5^{c} ± 3.68	22.2^{bc} ± 2.72	10.2^{a} ± 2.40	13.4^{ab} ± 2.19	0.016
C16:0	306^{b} ± 25.8	308^{b} ± 1.90	155^{a} ± 32.6	177^{a} ± 21.8	0.001
C16:1	3.19 ± 0.30	2.67 ± 0.66	1.17 ± 0.31	1.07 ± 0.65	0.052
C17:0	14.1^{b} ± 2.12	14.0^{b} ± 1.26	8.9^{a} ± 0.93	9.4^{a} ± 0.86	0.031
C17:1	0.48 ± 0.25	0.17 ± 0.06	0.23 ± 0.10	0.20 ± 0.13	0.226
C18:0	428^{a} ± 24.5	424^{a} ± 5.51	622^{b} ± 67.2	641^{b} ± 64.4	0.009
C18:1n9 *cis*	38.9^{b} ± 3.89	42.7^{b} ± 6.67	23.6^{a} ± 2.22	23.1^{a} ± 2.41	0.007
C18:1 *trans*-11	31.0^{a} ± 3.04	35.1^{ab} ± 3.74	62.4^{b} ± 3.55	43.9^{b} ± 10.6	0.025
C18:1n9 *trans*	0.90^{a} ± 0.34	1.04^{a} ± 0.37	4.14^{b} ± 0.81	4.62^{b} ± 0.82	0.001
C18:2n6	59.3^{b} ± 4.24	63.2^{b} ± 5.19	24.9^{a} ± 7.44	26.2^{a} ± 7.34	0.004
C18:2 *cis*-9,*trans*-11	0.37 ± 0.17	0.45 ± 0.11	0.74 ± 0.30	0.56 ± 0.16	0.648
C18:3n3	28.6^{b} ± 7.24	23.7^{b} ± 3.15	9.9^{a} ± 2.61	8.9^{a} ± 5.11	0.036
C18:3n6	2.00^{b} ± 0.19	2.19^{b} ± 0.33	0.77^{a} ± 0.39	0.92^{a} ± 0.11	0.010
C20:1	0.55 ± 0.24	0.49 ± 0.13	0.20 ± 0.13	0.31 ± 0.05	0.450
C20:2n6	0.43^{b} ± 0.10	0.17^{ab} ± 0.10	0.09^{a} ± 0.01	0.03^{a} ± 0.02	0.028
C20:4n6	0.47 ± 0.17	0.34 ± 0.07	0.41 ± 0.05	0.12 ± 0.26	0.390
C22:0	6.57^{a} ± 0.76	5.86^{a} ± 0.69	9.33^{b} ± 0.88	7.68^{ab} ± 0.59	0.032
C22:1n9	0.39 ± 0.13	0.44 ± 0.15	0.56 ± 0.07	0.29 ± 0.34	0.695
C22:2n6	0.44 ± 0.16	0.30 ± 0.08	0.89 ± 0.17	0.71 ± 0.16	0.103
C22:6n3	0.44 ± 0.11	0.65 ± 0.19	0.34 ± 0.38	0.64 ± 0.09	0.708
C24:1n9	3.04^{a} ± 0.41	2.65^{a} ± 0.66	8.69^{b} ± 1.51	4.18^{ab} ± 1.46	0.015
SFA	827 ± 9.81	821 ± 5.17	883 ± 20.4	851 ± 39.4	0.352
MUFA	79^{a} ± 4.30	86^{b} ± 3.88	82^{b} ± 5.62	100^{b} ± 4.76	0.041
PUFA	94^{b} ± 9.74	94^{b} ± 4.96	39^{a} ± 10.7	56^{a} ± 15.2	0.023

SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids

$^{a–c}$values within a row without a common superscript letter are significantly different ($P < 0.05$)

lauric acid (C12:0; $P = 0.001$), myristic acid (C14:0; $P = 0.003$), myristoleic acid (C14:1; $P = 0.031$), pentadecylic acid (C15:0; $P = 0.016$), palmitic acid (C16:0; $P = 0.001$), margaric acid (C17:0; $P = 0.031$), oleic acid (C18:1n9 *cis*; $P = 0.007$), linoleic acid (C18:2n6; $P = 0.004$), α-linolenic acid (C18:3n3; $P = 0.036$), γ-linolenic acid (C18:3n6; $P = 0.010$), and eicosadienoic acid (C20:2n6; $P = 0.028$) as compared to control and HB diet (Table 3). The SS and HBSS diets increased the concentration of stearic acid (C18:0; $P = 0.009$), vaccenic acid (C18:1 *trans*-11; $P = 0.025$), elaidic acid (C18:1n9 *trans*; $P = 0.001$), behenic acid (C22:0; $P = 0.032$), and nervonic acid (C24:1n9; $P = 0.015$) as compared to control and HB diet. Concentrations of MUFA of the HB, SS, and HBSS diets increased ($P = 0.041$) compared to control, whereas the concentrations of PUFA of the SS and HBSS diets decreased ($P = 0.023$).

doi: 10.17221/17/2016-CJAS

Table 4. Effects of herbal blend (HB), sunflower seeds (SS), and their combination (HBSS) on blood phagocytic activity, lipid peroxidation, and antioxidant status in blood plasma and rumen fluid (n = 8 per each diet, values are means ± SEM)

Items	Dietary treatments				P-value
	Control	HB	SS	HBSS	
Phagocytic activity (%)	58.4^{a} ± 0.52	61.8^{b} ± 0.43	61.8^{b} ± 0.24	63.4^{c} ± 0.25	0.001
GPx (blood) (U/g Hb)	224 ± 10.5	258 ± 11.9	241 ± 12.4	284 ± 9.3	0.704
MDA (BP) (μmol/l)	0.22 ± 0.004	0.22 ± 0.003	0.21 ± 0.004	0.20 ± 0.004	0.534
TAC (BP) (mmol Fe^{2+}/l)	0.24 ± 0.005	0.26 ± 0.005	0.25 ± 0.006	0.27 ± 0.006	0.348
TAC (RF) (mmol Fe^{2+}/l)	2.01 ± 0.06	2.52 ± 0.08	2.03 ± 0.08	2.54 ± 0.91	0.080

GPx (blood) = activity of glutathione peroxidase in blood, MDA (BP) = activity of malondialdehyde concentration in blood plasma, TAC (BP) = total antioxidant capacity in blood plasma, TAC (RF) = total antioxidant capacity in rumen fluid
$^{a–c}$values within a row without a common superscript letter are significantly different (P < 0.05)

Phagocytic activity, lipid peroxidation status, and antioxidant capacity in the blood and rumen fluid. Phagocytic activity and the activity of GPx in blood, the concentration of MDA, and the total antioxidant capacity in blood plasma and rumen fluid of the sheep are summarized in Table 4. The phagocytic activity was the highest in the HBSS treatment, followed by HB, SS, and control (P = 0.001). The activity of GPx in blood (P = 0.704), the concentration of MDA in plasma (P = 0.534), and the TAC in plasma (P = 0.348) were not significantly affected by the dietary treatments. There was a tendency (P = 0.080) toward higher TAC in the rumen fluid of sheep receiving the HB and HBSS diets compared to those fed the control and SS diets.

DISCUSSION

The concentrations of total SCFA, acetate, and propionate and pH were not significantly affected by the HB, SS, HBSS diets compared to the control. According to Jalc and Ceresnakova (2002), if the addition of dietary fat interferes with normal fibre digestion in the rumen, then butyrate and acetate will always be reduced. The general recommendation is that total dietary fat should not exceed 6–7% of dietary DM, otherwise an adverse effect on rumen fermentation could be observed (Jenkins 1993). However, it is not in agreement with present experiment because the fat content of SS and HBSS diets was only 3.3% of DM. The concentrations of *iso*-butyrate and *iso*-valerate were enhanced by SS and HBSS supplementation; these branched-chain volatile fatty acids are formed in the rumen by deamination of branched-chain amino acids, and their concentration is related to ruminal degradation of dietary protein. It is known that rumen fluid is very rich in branched-chain fatty acids, mainly of microbial origin produced from branched-chain SCFA and they are mostly affected by basal diet (Alves et al. 2013). It seems that a higher content of branched-chain SCFA is due to the higher content of protein in SS diet with respect to the control diet. Because of the elevated concentration of ammonia and branched-chain fatty acids in SS and HBSS, we can assume that amino acid catabolism was affected by these diets. Furthermore, a stimulating effect of the SS and HBSS diets on hyper-ammonia-producing bacteria (HAP) could also be considered, because HAP are capable of utilizing certain diet substrates and thereby production of ammonia on varying substrates (Eschenlauer et al. 2002). Hyper-ammonia-producing bacteria are able to utilize amino acids as a source of energy, but they cannot use proteins as a source of nitrogen (Chen and Russell 1989). Therefore, despite the low number of HAP in rumen, they can produce ammonia at a high rate.

It is known that dietary fat inhibits the growth of protozoa and also bacteria, especially fibrolytic species (Patra and Yu 2013). Moreover, Ivan et al. (2004) and Beauchemin et al. (2009) observed an antiprotozoal effect of sunflower seed supplementation to ruminant diets. In the present experiment, neither the protozoal nor bacterial counts were significantly influenced by the experimental diet treatments. Similarly, qualitative evaluation of microbial population by DGGE and principal component analysis (PCA) revealed no effects of dietary treatments on the composition of both eubacterial and ciliated protozoal populations. However, we did observe some tendency

doi: 10.17221/17/2016-CJAS

of isotrichid ciliates (*Dasytricha* and *Isotricha*) to affect eubacterial populations, which was also described in the study of Belanche et al. (2015). In summary, the PCA analysis of DGGE points to the relatively diverse microbial populations in individual animals, which probably reacted in a different manner to diets treatments.

Linoleic and linolenic acids are the main polyunsaturated fatty acids in the diet of ruminants, but due to the complete hydrogenation of these fatty acids the major fatty acid leaving the rumen is stearic acid (Tripathi 2014). Although the concentrations of some saturated fatty acids (palmitic, myristic, lauric acid) were decreased in the SS and HBSS diets, the content of stearic acid was significantly increased in the present experiment. According to Alves et al. (2013), the common findings that a concentrate with PUFA overload inhibits the growth of bacteria able to hydrogenate 18:1 isomers to stearic acid, were not confirmed. In the present experiment, the fatty acid composition is probably due to sunflower seeds effect and this was not modified by herbs supplementation because the level of polyphenols in the ration was low if compared to the study of Vasta et al. (2010). The efficacy of plant additives containing secondary metabolites could be more pronounced for diets containing high amounts of precursors of CLA synthesis (Mandal et al. 2014). In a previous study of Wencelova et al. (2015), a higher concentration of PUFA accompanied by a decrease in stearic acid concentration was observed after the addition of medicinal plant mixture and sunflower oil to the diet *in vitro*. However, these results were not fully confirmed *in vivo* (Wencelova et al. 2015).

After oral administration of herb feed additives the bioactive substances are quickly absorbed and most of them are metabolized. However, the precise mechanisms through which the immune system could be improved by herb feed additives are not clear and further investigations are necessary. Various medicinal herbs can represent natural antioxidants due to the antioxidant activity of herb phenolic compounds (Zheng and Wang 2001). The results of the present experiment suggest that the level of polyphenols in blood was too low to influence the parameters of antioxidative status measured in our experiment. It has been shown that a dose of herb extracts rich in polyphenols may be the limiting parameter to modify total antioxidant status in the plasma of sheep (Gladine et al. 2007).

CONCLUSION

Nutritional manipulation associated with dietary addition of sunflower seeds resulted in increasing concentrations of monounsaturated fatty acids which probably positively affected especially vaccenic acid flow from the rumen. However, results revealed no effects of dietary treatments on the composition of both eubacterial and ciliated protozoal populations. In spite of the supplementation of forage-concentrate diet with herbal blend in the presence of sunflower seeds, negative effects on the fermentation patterns and rumen microbial population were not detected. More studies with higher levels of the herbal polyphenols in the rations are needed.

REFERENCES

Alves S.P., Santos-Silva J., Cabrita A.R.J., Fonesca A.J.M., Bessa R.J.B. (2013): Detailed dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or concentrate supplemented with soybean oil. PLoS One, 8, e58386.

AOAC (1990): Official Methods of Analysis of AOAC International. 15th Ed. Association of Official Analytical Chemists, Arlington, USA.

Beauchemin K.A., McGinn S.M., Benchaar C., Holtshausen L. (2009): Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: effects on methane production, rumen fermentation, and milk production. Journal of Dairy Science, 92, 2118–2127.

Belanche A., de la Fuente G., Newbold C.J. (2015): Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. FEMS Microbiology Ecology, 91, fiu026.

Benzie I.F.F., Strain J.J. (1996): The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry, 239, 70–76.

Bhatta R., Baruah L., Saravanan M., Suresh K.P., Sampath K.T. (2013): Effect of medicinal and aromatic plants on rumen fermentation, protozoa population and methanogenesis in vitro. Journal of Animal Physiology and Animal Nutrition, 97, 446–456.

Bligh E.G., Dyer W.J. (1959): A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 911–917.

Broderick G.A., Kang J.H. (1980): Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. Journal of Dairy Science, 63, 64–75.

doi: 10.17221/17/2016-CJAS

Chen G., Russell J.B. (1989): More monensin-sensitive, ammonia-producing bacteria from the rumen. Applied and Environmental Microbiology, 55, 1052–1057.

Durmic Z., McSweeney C.S., Kemp G.W., Hutton P., Wallace R.J., Vercoe P.E. (2008): Australian plants with potential to inhibit bacteria and processes involved in ruminal biohydrogenation of fatty acids. Animal Feed Science and Technology, 145, 271–284.

Eschenlauer S.C.P., McKain N., Walker N.D., McEwan N.R., Newbold C.J., Wallace R.J. (2002): Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from sheep rumen. Applied and Environmental Microbiology, 68, 4925–4931.

Gladine C., Rock E., Morand C., Bauchart D., Durand D. (2007): Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. British Journal of Nutrition, 98, 691–701.

Glasser F., Ferlay A., Chilliard Y. (2008): Oilseed lipid supplements and fatty acid composition of cow milk: a meta-analysis. Journal of Dairy Science, 91, 4687–4703.

Ivan M., Mir P.S., Mir Z., Entz T., He M.L., McAllister T.A. (2004): Effects of dietary sunflower seeds on rumen protozoa and growth of lambs. British Journal of Nutrition, 92, 303–310.

Jalc D., Ceresnakova Z. (2002): Effect of plant oils and malate on rumen fermentation in vitro. Czech Journal of Animal Science, 47, 106–111.

Jenkins T.C. (1993): Lipid metabolism in the rumen. Journal of Dairy Science, 76, 3851–3863.

Jo C., Ahn D.U. (1998): Fluorometric analysis of 2-thiobarbituric acid reactive substances in turkey. Poultry Science, 77, 475–480.

Karaskova K., Suchy P., Strakova E. (2015): Current use of phytogenic feed additives in animal nutrition: a review. Czech Journal of Animal Science, 60, 521–530.

Kresanek Jr. J., Kresanek J. (2008): Atlas of Medicinal Plants and Berries. Osveta, Bratislava, Slovak Republic.

Makkar H.P.S., Blummel M., Borowy N.K., Becker K. (1993): Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. Journal of the Science of Food and Agriculture, 61, 161–165.

Mandal G.P., Roy A., Patra A.K. (2014): Effects of feeding plant additives rich in saponins and essential oils on the performance, carcass traits and conjugated linoleic acid concentrations in muscle and adipose tissues of Black Bengal goats. Animal Feed Science and Technology, 197, 76–84.

Nubel U., Engelen B., Felske A., Snaidr J., Wieshuber A., Amann R.I., Ludwig W., Backhaus H. (1996): Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. Journal of Bacteriology, 178, 5636–5643.

Paglia D.E., Valentine W.N. (1967): Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine, 70, 158–169.

Patra A.K., Yu Z. (2013): Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations in vitro. Journal of Dairy Science, 96, 1782–1792.

Tripathi M.K. (2014): Effect of nutrition on production, composition, fatty acids and nutraceutical properties of milk. Journal of Advances in Dairy Research, 2, 115.

Van Soest P.J., Robertson J.B., Lewis B.A. (1991): Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science, 74, 3583–3597.

Vasta V., Yanez-Ruiz D.R., Mele M., Serra A., Luciano G., Lanza M., Biondi L., Priolo A. (2010): Bacterial and protozoal communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins. Applied and Environmental Microbiology, 76, 2549–2555.

Weisburg W.G., Barns S.M., Pelletier D.A., Lane D.J. (1991): 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology, 173, 697–703.

Wencelova M., Varadyova Z., Mihalikova K., Cobanova K., Placha I., Pristas P., Jalc D., Kisidayova S. (2015): Rumen fermentation pattern, lipid metabolism and the microbial community of sheep fed a high-concentrate diet supplemented with a mix of medicinal plants. Small Ruminant Research, 125, 64–72.

Williams A.G., Coleman G.S. (1992): The Rumen Protozoa. Springer-Verlag Inc., New York, USA.

Zheng W., Wang S.Y. (2001): Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry, 49, 5165–5170.

Received: 2016–01–20
Accepted after corrections: 2016–09–14

Corresponding Author

MVDr. Zora Váradyová, Ph.D., Slovak Academy of Sciences, Institute of Animal Physiology, Šoltésovej 4-6, 040 01 Košice, Slovak Republic

Phone: +421 557 922 972, e-mail: varadyz@saske.sk