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Does humate supplementation affect ciliate population and fermentation parameters in the sheep rumen?

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ABSTRACT. The objective of the study was to examine the effect of supplementing sheep diets with humic substances (HS) on the number of protozoa and short chain fatty acid and methane concentrations in the rumen. The experiment was carried out in three rumen-fistulated sheep. The control (CON) ration was composed of 59.70% hay and 40.30% concentrate. Two experimental diets consisting of the same components were supplemented with two doses of humic substrates, 10 (HS10) or 20 (HS20) g/day/animal. The population of total protozoa and the genera *Entodinium* and *Isotricha* in the rumen was the most abundant before feeding (0 h) and decreased 2 and 4 h after receiving the diets. The populations of total protozoa and the genus *Entodinum* in the rumen were more abundant 2 h after administration of HS10 and HS20 compared to CON. The counts of these groups of protozoa increased 8 h after feeding HS10 compared to CON and HS20. An interaction trend (sampling time × treatment) was detected in terms of the number of *Isotricha* spp. in the rumen. The postprandial (2 and 4 h) pH of the rumen was lower than 0 h and 8 h after feeding CON and HS. Short chain fatty acid concentration was higher 2 and 4 h after feeding compared to 0 h and 8 h postprandial. An interaction trend was as assessed based on acetic acid levels; the highest was observed 4 h after feeding HS20 and the lowest 8 h after feeding HS10. Butyrate concentration was lower 8 h after feeding compared to 2 and 4 h postprandial levels for sheep fed the CON and HS10 diets. Branched chain acid production was the lowest 8 h after feeding compared to 0 h and 2 h after HS10 administration. An interaction trend was observed for methane levels; the highest was showed 4 h after feeding HS20 and the lowest 8 h after feeding HS10 diets. HS supplemented to sheep diets increased the abundance of total protozoa and the genera *Entodinium* and *Isotricha* in the rumen. It seemed that humates could modify the production of acetate and methane in the rumen, as slight increases in these parameters were observed. This suggests that humic substances can intensify methanogenesis in the rumen.

Introduction

Feed additives, e.g. humic substances, fats or herb extracts can modify fermentation, activity and population of microorganisms in the rumen, as well as reduce greenhouse gas emissions and positively affect productivity and health of animals (Majewska et al., 2017a; b; Majewska and Kowalik, 2020).

Recently, there has been a growing interest in the application of humic substances (HS) in the diets of livestock (Trziszka et al., 2011; Marcin et al., 2020; Wang et al., 2020). These substances are safe natural feed supplements with beneficial effects on animal welfare, as well as on the quality of animal products. HS are organic compounds found in soil and formed from natural decomposition of animal

and plant matter (McMurphy et al., 2009). Humic and fulvic acids are the main extractable ingredients of soil humates and most commonly used to improve soil fertility. In soil, HS have been shown to promote microbial growth, as well as absorptive and detoxifying properties (Huck et al., 1991; Islam et al., 2005). Brown coal is a rich source of hummic and fulvic acids. Therefore, HS have been proposed to exert similar effects in the digestive tract, including rumen metabolism, i.e. increasing microbial activity and fermentation in the rumen. Experiments on the application of HS in animal rations have mainly focused on monogastric animals. The positive effects of humates on the growth of broiler chickens, productivity and health of laying poultry, as well as the chemical composition of eggs have been demonstrated in several studies (Trziszka et al., 2011; Mirnawati and Marlida, 2013; Sopoliga et al., 2016). The results of Wang et al. (2020) indicated that humic acids in the rations improved growth performance and health of piglets. Reports regarding the use of humic compounds in ruminant diets are limited and inconsistent. Teter et al. (2021) did not observe any changes in milk yield when dairy cows were fed a diet with HS; however, the coagulation properties were improved, as well as calcium and iron contents. Milk, fat and protein yield was shown to increase after administration of humic substances to goat diets in comparison to control animals (El-Zaiat et al., 2018). The latter authors reported that the total number of protozoa was reduced, while acetate and propionate levels were increased in the goat rumen as a result of HS addition to the diets. On the other hand, Marcin et al. (2020) observed increased counts of protozoa *Entodinium* spp., *Diplodinium* spp. and *Ophryoscolex* spp. and unaltered short chain fatty acid (SCFA) levels in the rumen after HS supplementation to the sheep diet. Farm animals are known to largely contribute to global greenhouse gas emissions, especially methane and carbon dioxide. Sheng et al. (2019) showed that humic substances reduced methane production in an *in vitro* study. In contrast, Terry et al. (2018b) did not observe the effect of humic substances on total gas, methane, and carbon dioxide production in the rumen simulation technique (RUSITEC) study. The results of previous studies were inconclusive in terms of evaluating the effect of humic substances in ruminant feed (Majewska et al., 2017a; El-Zaiat et al., 2018; Terry et al., 2018a; Sheng et al., 2019). This variability may be attributed to variation in the chemical composition and structure of HS, extraction methods, doses and concentration of other compounds, e.g. minerals, vitamins, phenolic acids or bioactive organic groups (Islam et al., 2005).

In the present study, we hypothesized that different amounts of humic substances added to the sheep diets could affect protozoan populations and fermentation parameters in the rumen.

Considering the above, the aim of the study was to evaluate the effect of two doses of humic substances added to the sheep diets on the count of total protozoa and the genera ciliates, concentration of short chain fatty acids and methane in the rumen of sheep.

Material and methods

All procedures on animals were accepted by the 2nd Local Animal Care Ethics Committee for Animal Experiments in Warsaw, Poland, permission no. 50/2016.

Animals and feeding

The experiment was carried out using three female Polish Lowland sheep (3 years old; 55.5 kg ± 0.5 average body weight) equipped with a rumen cannula (~8 cm ID; self-made cannulas). The animals were divided into three sub-groups, one sheep each. The sheep were fed a control diet (CON) consisting of (g/day/animal): 600 meadow hay, 300 concentrate, and 20 vitamin-mineral mixture (Polfamix O-K, Trow Nutrition, Grodzisk Mazowiecki, Poland) or two experimental diets based on the CON diet supplemented with humic substances (Košice, Slovakia), which were supplied in dose of 10 (HS10) or 20 (HS20) g/day/animal. The humic substances used in this study contained the following organic acids (%): 65.0 humic and 5.0 fulvic acids and minerals (mg/kg DM): calcium 42.28, magnesium 5.10, iron 19.05, copper 15.00, zinc 37.00, manganese 142.00, cobalt 1.42, selenium 1.67, vanadium 42.10, molybdenum 2.70. The maximum moisture of the preparation was 15% and the particle size was up to 100 μm. The preparation was added and mixed with a concentrate before each feeding. Diets were formulated according to the ruminant nutrition recommendations of IZ PIB-INRA (2009) to cover nutrient requirements of sheep. Dietary composition are given in Table 1. Treatments were administered successively to each group in 3 different sequences (CON-HS10-HS20, HS10-CON-HS20 and HS20-HS10-CON) that formed 3 study periods. Each experimental period lasted 32 days and included 10 days of gradual diet transition, 21 days of adaptation to the new ration and 1 day of sampling. All sheep during the study were housed in individual pens on rubber mats with separate facilities for forage and concentrate with *ad libitum* access to water and salt licks. The sheep were fed twice a day, at

Table 1. Composition of diet

Ingredients	Contents, g/kg DM	Chemical composition	Contents, g/kg DM
Meadow hay	597.03	Dry matter, g/kg	899.94
Crushed barley	283.91	Crude protein	152.12
Soybean oilmeal	98.36	Crude fat	24.81
Vitamin-mineral premix[1]	20.70	Starch	246.81
		Crude ash	58.01
		NDF	511.35
		ADF	259.50
		UFV, kg	0.80

DM – dry matter, NDF – neutral detergent fibre, ADF – acid detergent fibre, UFV – feed unit of maintenance and meat production;[1] Polfamix O-K (Trouw Nutrition, Poland) consisted of: g: Ca 240, Na 60, P 120, Mg 65, Zn 2.5, Mn 3.0, Se 0.003, Co 0.015, vit. E 1.5; IU: vit. A 300 000, vit. D_3 30 000

7:00 and 15:00. Feed intake was checked daily before the morning feeding.

Rumen fluid sampling

The samples of rumen fluid were collected just before the morning feeding (0 h) and 2, 4 and 8 h after the feeding from the middle and ventral sacs. The samples were aspirated by suction using a copper tube connected to a syringe via a rubber tube as described by Majewska et al. (2017b). Rumen fluid samples were precisely mixed and passed through two layers of gauze in order to remove large food particles.

Chemical analysis of animal diets

Meadow hay, barley and soybean oil meal samples were collected during each sampling period. These samples were oven-dried at 55 °C to a constant weight, ground and passed through a 1-mm sieve. Afterwards, feed samples were stored in sealed plastic bags at room temperature until chemical analysis. Dry matter (DM) (934.01), crude fat (930.09), crude ash (942.05), total nitrogen (978.04) and starch (996.11) contents in the feedstuff were assayed according to AOAC International (2005). Natural detergent fibre (NDF) in each feed was analysed using a heat stable amylase according to Mertens (2002) and expressed excluding residual ash. Acid detergent fibre (ADF) in feedstuff samples was expressed excluding residual ash and analysed according to AOAC International (2005).

Protozoa, short chain fatty acid (SCFA) and pH

Ruminal fluid samples for counting protozoa were fixed in 4% formaldehyde (Avantor Performance Materials S. A., Gliwice, Poland) solution and stored in tightly sealed containers at 4 °C. Protozoa in formalin solution were identified and classified based on morphological criteria, according to Dehority (1993), and counted under a light microscope.

Rumen fluid samples for SCFA analysis were preserved in formic acid (Avantor Performance Materials S. A., Gliwice, Poland) and centrifuged; the resulting supernatant was stored in glass vials at 4 °C until SCFA determination. SCFA levels were analysed by gas chromatography (GC-2010, Shimadzu, Japan) using a capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), flame ionization detector and helium as the carrier gas (Miltko et al., 2016a). The pH was measured immediately after sample collection with a pH meter (type 7011; ChemLand, Stargard, Poland).

Methane concentration was calculated using molar proportions of SCFA (acetate, propionate, and butyrate), as described by Moss et al. (2000).

Statistical analysis

The results are presented as mean and standard error of the mean (SEM). The data were subjected to repeated measure analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test. The model included the effects of treatment type, i.e. dietary supplementation with humic substances (CON, HS10, HS20), sampling time (0, 2, 4, 8 h), and their interaction. Furthermore, the effect of periods (I, II, III), as well as animals (three sheep) were also analysed to verify the correctness of the planned experiments. Sampling time was treated as a repeated measure factor in the same unit (within subject). Data normality was tested using the Shapiro-Wilk test, and homogeneity of variance was tested with Levene's test. The results with abnormal distribution were transformed to natural logarithms. The repeated measure ANOVA sphericity assumption (Muchly's test) was also verified. The level of significance was assumed at $P \leq 0.05$ and trends at $P \leq 0.1$. The obtained results are shown in Tables 2 and 3 in the form of raw values, before logarithmic transformation. All statistical analyses were performed using the Statistica 10.0 software package (StatSoft, Kraków, Poland).

Results

Protozoan counts

Generally, the number of total protozoa and the genera *Entodinium* and *Isotricha* in the rumen of sheep was the highest before feeding (0 h) and decreased 2 and 4 h after applying the rations. However, the protozoan count 8 h after feeding

increased and was similar to that before feeding (Table 2). Sampling time and treatment affected the size of populations of total protozoa and *Entodinium* spp. in the rumen (P = 0.000 and P = 0.004, and P = 0.007 and P = 0.000, respectively) (Table 2). Moreover, an interaction trend (T × Tre, P = 0.061, and P = 0.071, respectively) was also observed. The population of total protozoa and the genus *Entodinum* in the rumen was higher 2 h after the administration of HS10 and HS20 diets compared to the CON ration. Moreover, the abundance of these protozoan groups was increased 8 h after feeding HS10 compared to CON and HS20 diets.

There was no effect of treatment on the density of the genera *Diplodinium, Ophryoscolex, Isotricha* and *Dasytricha* in the rumen (P = 0.657, P = 0.538, P = 0.501 and P = 0.923, respectively) (Table 2). Irrespective of treatments, the number of ciliate genera, except for *Ophryoscolex* spp., was the highest before feeding or 8 h after feeding, while it was the lowest at 2 and 4 h after feeding ($P \leq 0.004$) (Table 2). Additionally, an interaction trend (T × Tre, P = 0.083) was detected with respect to the number of *Isotricha* spp. in the rumen; the highest was observed before feeding HS10 and the lowest was 2 h after feeding CON diets.

Table 2. Concentration of protozoa (×10^4/ml rumen fluid) in the sheep rumen

Specification	Tre	0 h$^{\$}$	2 h$^{\$}$	4 h$^{\$}$	8 h$^{\$}$	$Mean_{Tre}$	SEM_{Tre}	*P*-value		
								Main effect		
								T	Tre	T × Tre interaction
Total protozoa	CON	60.42^{x}	$26.65^{a\,yz}$	34.23^{yz}	$50.97^{a\,x}$	43.07	4.286	0.000	0.004	0.061
	HS10	74.58^{x}	$38.50^{b\,yz}$	47.17^{yz}	$67.08^{b\,x}$	56.83	4.626			
	HS20	73.28^{x}	$45.53^{b\,y}$	41.48^{y}	$51.88^{a\,y}$	53.06	3.892			
	$Mean_T$	69.43^{x}	36.89^{y}	40.96^{y}	56.64					
	SEM_T	3.248	2.953	2.099	3.159					
Entodinium	CON	48.52^{x}	$20.70^{a\,y}$	28.31^{yz}	$42.35^{a\,xz}$	34.97	3.664	0.007	0.000	0.071
	HS10	62.31^{x}	$32.56^{b\,y}$	42.17^{yz}	$57.18^{b\,xz}$	48.55	3.904			
	HS20	61.29^{x}	$39.05^{b\,y}$	36.71^{y}	$40.81^{a\,y}$	44.47	3.270			
	$Mean_T$	57.37^{x}	30.77^{y}	35.73^{y}	46.78					
	SEM_T	3.537	2.965	2.250	3.023					
Diplodinium	CON	1.67	0.88	0.92	0.71	1.04	0.121	0.004	0.657	0.399
	HS10	1.35^{x}	0.65	0.77^{y}	1.30^{x}	1.02	0.119			
	HS20	1.38^{x}	0.80	0.83^{y}	0.63	0.91	0.162			
	$Mean_T$	1.47^{x}	0.76^{y}	0.84^{y}	0.88^{y}					
	SEM_T	0.151	0.111	0.101	0.135					
Ophryoscolex	CON	1.70	0.76	1.16	1.08	1.17	0.140	0.599	0.538	0.882
	HS10	1.76	0.57	0.65	0.62	0.90	0.179			
	HS20	1.46	1.00	0.61	1.42	1.12	0.206			
	$Mean_T$	1.64	0.77	0.80	1.04					
	SEM_T	0.194	0.166	0.162	0.167					
Isotricha	CON	2.57^{x}	0.97^{y}	1.09^{y}	1.81	1.61	0.203	0.000	0.501	0.083
	HS10	3.02^{x}	1.41^{y}	0.96^{y}	2.78^{x}	2.04	0.340			
	HS20	1.97	1.14^{x}	1.07^{x}	2.44^{y}	1.66	0.201			
	$Mean_T$	2.52^{x}	1.17^{y}	1.04^{y}	2.34^{x}					
	SEM_T	0.290	0.131	0.132	0.200					
Dasytricha	CON	5.96	3.35	2.76	5.02	4.27	0.489	0.000	0.923	0.974
	HS10	6.14	3.32	2.62	5.20	4.32	0.621			
	HS20	7.18^{x}	3.54	2.26^{y}	6.59^{x}	4.89	0.887			
	$Mean_T$	6.43^{x}	3.40^{y}	2.54^{y}	5.60^{x}					
	SEM_T	0.925	0.297	0.403	0.524					

Tre – treatment, T – time, SEM – standard error of the mean; CON – control, HS10 – humic substances in dose of 10 g/day/animal, HS20 – humic substances in dose of 20 g/day/animal; $^{\$}$ samples were collected before morning feeding (at 0 h), and at 2, 4 h, 8 h after morning feeding; the samples were collected at 1 following day, n = 3 for each group; different letters in a row (^{xyz} – $P \leq 0.05$) show difference between sampling time (0, 2, 4, 8 h); different letters in a column (^{abc} – $P \leq 0.05$) show differences between treatments (CON, HS10 and HS20)

Fermentation patterns

No effect of treatment was determined for pH and SCFA concentration ($P > 0.05$) (Table 3). Rumen pH ranged from 6.52 to 7.07. Postprandial (2 and 4 h) pH of rumen fluid was lower than before and 8 h after feeding in the control and experimental groups ($P = 0.010$).

The highest concentrations of total SCFA, acetate and propionate were recorded 2 or 4 h after feeding, while the lowest before feeding or 8 h after sheep received the diets ($P < 0.001$) (Table 3). However, a suggestive statistical interaction trend (T × Tre; $P = 0.096$) was found for acetic acid concentrations; the highest was recorded 4 h after feeding the HS20 diet and the lowest 8 h after feeding the HS10 diet. The molar proportion of butyrate was lower 8 h after feeding compared to 2 and 4 h postprandial values for CON and HS10. The molar proportions of the sum of isobutyric, valeric, and isovaleric acids were the lowest 8 h

Table 3. Ruminal pH, short-chain fatty acids (SCFA) concentration and composition (mM/100ml) and methane concentration (mM/100ml) in the sheep rumen

Specification	Tre	0 h$	2 h$	4 h$	8 h$	$Mean_{Tre}$	SEM_{Tre}	*P*-value		
								Main effect		
								T	Tre	T × Tre interaction
pH	CON	7.06^{x}	6.56^{y}	6.52^{y}	6.86^{x}	6.75	0.097	0.010	0.965	0.615
	HS10	7.02^{x}	6.65^{y}	6.59^{y}	6.95^{x}	6.80	0.107			
	HS20	7.07^{x}	6.66^{y}	6.61^{y}	6.92^{x}	6.81	0.085			
	$Mean_T$	7.05^{x}	6.61^{y}	6.57^{y}	6.91^{x}					
	SEM_T	0.079	0.089	0.096	0.094					
Total SCFA	CON	10.76^{x}	13.30^{y}	14.10^{y}	10.74^{x}	12.23	0.472	0.000	0.872	0.108
	HS10	11.74	13.54^{x}	13.64^{y}	9.91^{y}	12.21	0.546			
	HS20	12.10^{x}	12.81^{x}	14.39^{y}	10.76^{x}	12.52	0.476			
	$Mean_T$	11.54^{x}	13.22^{y}	14.05^{y}	10.47^{x}					
	SEM_T	0.282	0.239	0.407	0.318					
Acetate	CON	7.57^{x}	9.35^{y}	10.03^{y}	7.75^{x}	8.67	0.328	0.000	0.929	0.096
	HS10	8.29^{xz}	9.53^{zy}	9.90^{y}	7.21^{x}	8.73	0.368			
	HS20	8.61^{x}	8.98	10.14^{y}	7.68^{x}	8.85	0.338			
	$Mean_T$	8.16^{x}	9.28^{y}	10.02^{y}	7.55^{x}					
	SEM_T	0.203	0.165	0.283	0.216					
Propionate	CON	1.57^{x}	2.15^{y}	2.23^{y}	1.67^{x}	1.90	0.107	0.001	0.611	0.211
	HS10	1.66	2.05^{x}	1.98^{x}	1.43^{y}	1.78	0.101			
	HS20	1.72^{xz}	2.10^{yx}	2.31^{y}	1.64^{z}	1.94	0.091			
	$Mean_T$	1.65^{x}	2.10^{y}	2.17^{z}	1.58^{x}					
	SEM_T	0.061	0.067	0.095	0.072					
Butyrate	CON	1.14	1.32^{x}	1.35^{x}	1.00^{y}	1.20	0.050	0.004	0.884	0.137
	HS10	1.27^{x}	1.43^{x}	1.33^{x}	0.95^{y}	1.24	0.067			
	HS20	1.28	1.26	1.40	1.06	1.25	0.054			
	$Mean_T$	1.23^{x}	1.34^{x}	1.36^{x}	1.00^{y}					
	SEM_T	0.038	0.046	0.054	0.046					
Branched chain#	CON	0.48	0.48	0.49	0.33	0.44	0.022	0.000	0.505	0.386
	HS10	0.51^{x}	0.53^{x}	0.44	0.32^{y}	0.45	0.031			
	HS20	0.49	0.47	0.54	0.38	0.47	0.022			
	$Mean_T$	0.49^{x}	0.50^{x}	0.49^{x}	0.34^{y}					
	SEM_T	0.016	0.018	0.026	0.019					
Methane	CON	3.43^{x}	4.14^{y}	4.44^{y}	3.43^{x}	3.86	0.137	0.021	0.897	0.075
	HS10	3.79^{xz}	4.29^{yz}	4.44^{y}	3.23^{x}	3.94	0.164			
	HS20	3.91	3.97	4.49^{x}	3.43^{y}	3.95	0.148			
	$Mean_T$	3.71^{x}	4.13^{y}	4.46^{z}	3.36^{xy}					
	SEM_T	0.093	0.073	0.121	0.101					

Tre – treatment, T – time, SEM – standard error of the mean; CON – Control, HS10 – humic substances in dose of 10 g/day/animal, HS20 – humic substances in dose of 20 g/day/animal; $ – samples were collected before morning feeding (at 0 h), and at 2, 4, 8 h after morning feeding; # – isobutyric acid + valeric acid + isovaleric acid; the samples were collected at 1 following day, n = 3 for each group; different letters in a row (^{xyz} – $P \leq 0.05$) show difference between sampling time (0, 2, 4, 8 h)

after feeding compared to 0 h and 2 h after the administration of the HS10 ration.

Methane concentration increased 2 and 4 h after feeding the CON and HS10 diets and 4 h after receiving the HS20 ration ($P = 0.021$) (Table 3). At 8 h after feeding, the concentration of this gas was similar to that before feeding the animals. However, an interaction trend (T × Tre; $P = 0.075$) was observed, as methane concentration in the rumen was the lowest 8 h after the administration of HS10 and the highest 4 h after the HS20 diet.

Discussion

The use of HS as a natural additive for domestic ruminants is a fairly novel approach. The HS used in this study contained the following organic acids (%): 65.0 humic and 5.0 fulvic acids. Other studies investigating the use of HS as a supplement in ruminant rations have reported organic acid concentrations ranging from 55.1 to 89.8% (Váradyová et al., 2009; McMurphy et al., 2011; Degirmencioglu, 2014; Terry et at., 2018b). The current results can be difficult to interpret and discuss, as well as to compare with other studies, because scientists use various HS sources and doses with different content of organic acids and other nutrients.

The role of protozoa in ruminal fermentation and their contribution to the metabolism and nutrition of the host is still the subject of research and controversy. The concentration of protozoa in the rumen is influenced by such factors as diet composition or feeding frequency, and the outflow rate of digestive content to the forestomaches (Michałowski, 1990; Majewska et al., 2017b). The results of our study suggested that HS10 or HS20 added to sheep diet, as well as time after feeding, significantly increased the abundance of total protozoa and the genera *Entodinium* and *Isotricha* in the rumen of sheep. The available data concerning the effect of HS on the abundance of protozoan populations are inconsistent. Váradyová et al. (2009) showed no effect of HS (10 g/kg DM) and diet composition (high forage or concentrate diet) on total protozoa and *Entodinium* spp., and increased abundance of the genus *Isotricha*, but only when HS and a high dose of forage were added to the fermentation bottle. In contrast to our study, Galip et al. (2010) found no significant effect on the number of total ciliates and the genera *Entodinium* and *Isotricha* in the rumen when rams were fed 5 or 10 g/day HS. The increase in ruminal protozoa abundance in the current study could be due to a decrease in rumen fluid volume, lower fluid phase turnover and relationships between protozoa (Miltko et al., 2016b). Moreover, Michałowski (1990) suggested that *Isotricha* spp. could rather occupy a region near the rumen wall and migrate to the ventral sac in the first hours after receiving diets. Our study demonstrated that the population of genera *Isotricha* was increased 8 h after receiving the HS10 and HS20 diets. The identified protozoa produced different hydrolytic enzymes and decomposed various types of carbohydrates. *Entodinium* and *Isotricha* ciliates utilise small starch granules and soluble sugars (Belanche et al., 2014). In turn, our previous study showed that HS supplementation to the sheep diet caused an increased amylolytic activity in the rumen, before and 8 h after feeding (Majewska et al., 2017a). It is likely that this food additive stimulated the activity and growth not only of amylolytic bacteria, but also protozoa, especially *Entodinium* and *Isotricha*.

Physiological rumen digesta pH ranges from 5.5 to 7.5 and depends on the type and form of diet and feeding frequency of ruminants. In the present study, the ruminal pH values decreased 2 and 4 h after feeding and increased 8 h after supplying the diets. Thus, in general, HS did not significantly affect rumen digesta pH values, but they were slightly higher compared to CON. These results were consistent with the findings of Marcin et al. (2020) and Váradyová et al. (2009). Rumen pH fluctuations can reflect the ratio of carbohydrate fermentation to SCFA absorption and the buffering capacity of rumen fluid (Majewska et al., 2021). On the other hand, El-Zaiat et al. (2018) showed that supplementation with 2 g of humic substances per goat significantly increased ruminal pH. According to these authors, HS had a buffering capacity, which in turn possibly led to ruminal pH stabilization. It cannot be ruled out that a decrease in rumen pH after feeding could result from an increased concentration of lactic acid.

SCFAs are the primary end products of ruminal microbial digestion of carbohydrates. The results of SCFA measurements seemed to indicate that rumen fermentation was not affected by the experimental treatments; these results were consistent with the findings of Terry et al. (2018b). However, SCFA levels were increased 2 and 4 h after feeding. Overall, our results appear to be in line with the data reported by Sheng et al. (2019). Ruminal SCFA concentrations were different between the sampling times, which was likely due to changes in the rumen microbial population. According to Newbold et al. (2015), lower SCFA levels observed in defaunated

ruminants appeared to emphasize the role of protozoa in SCFA synthesis and feedstuff degradation in the rumen. The increased abundance of total protozoa before and 2 and 4 h after feeding the HS10 or HS20 diets compared to CON ration administration were accompanied by higher acetate concentrations. Michałowski (1987) showed that protozoa produced mainly acetate and butyrate and only trace amounts of propionate (29–41 and 70–80, and 9–12%, respectively). The ability of ruminal ciliates to absorb exogenous fatty acids may redirect more carbon towards SCFA production in preference to fatty acid synthesis and ultimately increase SCFA content (Newbold et al., 2015). It should be noted that other factors can also affect the concentration of SCFA, such as the liquid passage rate or acid absorption rate (Kasperowicz et al., 2014); however, these factors were not examined in our study.

Methane is produced in the digestive tract of ruminants, particularly in the rumen by a specialized group of microorganisms – methanogenic archaea (Morgavi et al., 2010). In the rumen, methanogens utilise mostly hydrogen and carbon dioxide as substrates for methane production. However, other microorganisms engaged in hydrogen formation also affect methane production. Protozoa belong to microorganisms that produce large quantities of hydrogen. Recently, feed additives that can reduce methane production by ruminants have been of interest. In the present study, the dose of the supplemented diet did not significantly affect methane concentration. However, since a trend of the T × Tre interaction was observed ($P = 0.075$), deviations from the overall pattern were also recorded. Moreover, this interaction was also shown for total protozoa abundance in the sheep rumen ($P = 0.061$). In general, a moderate increase in methane concentrations and protozoan counts was observed in animals fed the HS10 and HS20 diets compared to the CON diet. This suggested that ruminal protozoa could provide hydrogen to methanogens for methane production. Moreover, the increased molar proportion of acetate (observed trend of T × Tre interaction) in the rumen of sheep fed the HS diets in the present study could have also been partially responsible for the increase in methane levels. According to Moss et al. (2000), the formation of acetate from pyruvate in the rumen produces hydrogen, which is the main substrate for methane production. Our results were consistent with the findings of Váradyová et al. (2009) and Terry et al. (2018a). On the other hand, Sheng et al. (2019) reported a decrease in methane levels by 12.8% after 48 h of HS incubation in rumen fluid. However, these authors noted elevated methane concentrations at 6, 9, 12, 24, and 48 h after 0.9 mg/ml HS addition the incubation fluid.

Conclusions

According to the results of the present study, humic substances supplemented to the sheep diets increased the abundance of total protozoa and the genera *Entodinium* and *Isotricha* in the rumen. It appeared that humates could modify the production of acetate and methane in the rumen, because modest increases in these parameters were observed. This suggests, that humic substances can intensify methanogenesis in the rumen. However, our results are not unequivocal and future studies on a larger number of animals, using different doses of humic substances, are needed to further elucidate their effect.

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Conflict of interest

The Authors declare that there is no conflict of interest.

References

AOAC Interntional, 2005. Official Methods of Analysis of AOAC International. 18th Edition. Arlington, VA (USA)

Belanche A., de la Fuente G., Newbold C.J., 2014. Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbial. Ecol. 90, 663–677, https://doi.org/10.1111/1574-6941.12423

El-Zaiat H.M., Morsy A.S., El-Wakeel E.A., Anwer M.M., Sallam S.M., 2018. Impact of humic acid as an organic additive on ruminal fermentation constituents, blood parameters and milk production in goats and their kids growth rate. J. Anim. Feed Sci. 27, 105–113, https://doi.org/10.22358/jafs/92074/2018

Degirmencioglu T., 2014. Using humic acid in diets for dairy goats. Anim. Sci. Pap. Rep. 32, 25-32

Dehority B.A., 1993. Laboratory manual for classification and morphology of rumen ciliate protozoa. CRC Press Inc. London (UK)

Galip N., Polat U., Biricik H., 2010. Effect of supplemental humic acid on ruminal fermentation and blood variables in rams. Ital. J. Anim. Sci. 9, e74, https://doi.org/10.4081/ijas.2010.e74

Huck T.A., Porter N., Bushell M.E., 1991. Effect of humates on microbial activity. Gen. Microbiol. 137, 2321–2329 https://doi.org/10.1099/00221287-137-10-2321

Islam K.M., Schumacher S.A., Gropp J.M., 2005. Humic acid substances in animal agriculture. Pak. J. Nutr. 4, 126–134, https://doi.org/10.3923/pjn.2005.126.134

IZ-PIB INRA, 2009. Sheep. In: E. Jarrige (Editor). Ruminant nutrition. Recommended allowances and feed tables (in Polish). National Research Institute of Animal Production. Balice (Poland), pp. 163–166

Kasperowicz A., Stan-Głasek K., Kowalik B., Vandzurova A., Pristas P., Pająk J., Kwiatkowska E., Michałowski T., 2014. Effect of dietary fructose polymers or sucrose on microbial fermentation, enzyme activity, ciliate concentration and diversity of bacterial flora in the rumen of rams. Anim. Feed Sci. Technol. 195, 38–46, https://doi.org/10.1016/j.anifeedsci.2014.06.014

Majewska M.P., Kowalik B., 2020. Growth performance, carcass characteristics, fatty acid composition and blood biochemical parameters of lamb fed diet with the addition of lingonberry leaves and oak bark. Eur. J. Lipid Sci. Technol. 122, 1900273, https://doi.org/10.1002/ejlt.201900273

Majewska M.P., Miltko R., Bełżecki G., Kowalik B., 2021. Population of protozoa and carbohydrate-digesting enzymes in the rumen of sheep fed a diet supplementing with yeast *Saccharomyces cerevisiae*. Small Rumin. Res. 205, 106544, https://doi.org/10.1016/j.smallrumres.2021.106544

Majewska M.P., Miltko R., Skomiał J., Kowalik B., 2017a. Influence of humic acid supplemented to sheep diets on the rumen enzymatic activity. Med. Weter. 73, 770–773, https://doi.org/10.21521/mw.5822

Majewska M.P., Miltko R., Bełżecki G., Skomiał J., Kowalik B., 2017b. Supplementation of rapeseed and linseed oils to sheep rations: effects on ruminal fermentation characteristics and protozoal populations. Czech J. Anim. Sci. 62, 525–538, https://doi.org/10.17221/9/2017-CJAS

Marcin A., Bujňák L., Mihok T., Nad' P., 2020. Effects of humic substances with urea on protozoal population and fermentation in the rumen of sheep. Bulg. J. Vet. Med. 23, 60–69, https://doi.org/10.15547/bjvm.2199

McMurphy C.P., Duff G.C., Harris M.A., Sanders S.R., Chirase N.K., Bailey C.R., Ibrahim R.M., 2009. Effect of humic/fulvic acid in beef cattle finishing diets on animal performance, ruminal ammonia and serum urea nitrogen concentration. J. Appl. Anim. Res. 35, 97–100, https://doi.org/10.1080/09712119.2009.9706995

McMurphy C.P., Duff G.C., Sanders S.R., Cuneo S.P., Chirase N.K., 2011. Effects of supplementing humates on rumen fermentation in Holstein steers. S. Afr. J. Anim. Sci. 41, 134–140, https://doi.org/10.4314/sajas.v41i2.71017

Mertens D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fibre in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85, 1217–1240

Michałowski T., 1987. The volatile fatty acids production by ciliate protozoa in the rumen of sheep. Acta Protozzol. 26, 335–345

Michałowski T., 1990. The distribution of ciliates through the reticulorumen of sheep. Acta Protozool. 3, 213–220

Miltko R., Bełżecki G., Kowalik B., Skomiał J., 2016b. Presence of carbohydrate-digesting enzymes throughout the digestive tract of sheep. Turk. J. Vet. Anim. Sci. 40, 271–277, https://doi.org/10.3906/vet-1507-70

Miltko R., Rozbicka-Wieczorek A.J., Wiesyk E., Czauderna M., 2016a. The influence of different chemical forms of selenium added to the diet including carnosic acid, fish oil and rapeseed oil on the formation of volatile fatty acids and methane in rumen and fatty acid profiles in the rumen content and muscles of lambs. Acta Vet.-Beograd 66, 373–391, https://doi.org/10.1515/acve-2016-0032

Mirnawati Y.R., Marlida Y., 2013. Effects of humic acid addition via drinking water on the performance of broilers fed diets containing fermented and non-fermented palm kernel cake. Arch. Zootech. 16, 41–53

Morgavi D.P., Forano E., Martin C., Newbold C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4, 1024–1036, https://doi.org/10.1017/S1751731110000546

Moss A.R., Jouany J.-P., Newbold J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49, 231–253, https://doi.org/10.1051/animres:2000119

Newbold C.J., de la Fuente G., Belanche A., Ramos-Morales E., McEwan N.R., 2015. The role ciliate protozoa in the rumen. Front. Microbiol. 6, 1313, https://doi.org/10.3389/fmicb.2015.01313

Sheng P., Ribeiro G.O., Wang Y., McAllister T.A., 2019. Humic substances reduce ruminal methane production and increase the efficiency of microbial protein synthesis *in vitro*. J. Sci. Food Agric. 99, 2152–2157, https://doi.org/10.1002/jsfa.9407

Sopoliga I., Hreško-Šamudovská A., Demetrová M., Nad' P., Marcin A., Skalická M., 2016. Effect of humic substances on the production parameters of pheasant hens. Acta Fytotech. Zootech. 19, 11–14, https://doi.org/10.15414/afz.2016.19.01.11-14

Terry S.A., Ramos A.F.O., Holman D.B., McAllister T.A., Breves G., Chaves A.V., 2018b. Humic substances alter ammonia production and the microbial populations within a Rusitec fed a mixed hay - concentrate diet. Front. Microbiol. 9, 1410, https://doi.org/10.3389/fmicb.2018.01410

Terry S.A., Ribeiro G.O., Gruninger R.J., Hunererg M., Ping S., Chaves A.V., Burlet J., Beauchemin K.A., McAllister T.A.J., 2018a. Effect of humic substances on rumen fermentation, nutrient digestibility, methane emission, and rumen microbiota in beef heifers. J. Anim. Sci. 96, 3863–3877, https://doi.org/10.1093/jas/sky265

Teter A., Kędzierska-Matysek M., Barłowska J., Król J., Brodziak A., Florek M., 2021. The effect of humic mineral substances from oxyhumolite on the coagulation properties and mineral content of the milk of Holstein-Friesian cows. Animals 11, 1970, https://doi.org/10.3390/ani11071970

Trziszka T., Dobrzański, Z., Kaźmierska, M., Tronina Ł., Skiba M., 2011. Effect of dietary humic-fatty preparations on egg quality Lohmann Brown hens. Arch. Geflugelk. 75, 84–90

Váradyová Z., Kišidayová S., Jalč D., 2009. Effect of humic acid on fermentation and ciliate protozoan population in rumen fluid of sheep *in vitro*. J. Sci. Agric. 89, 1936–1941, https://doi.org/10.1002/jsfa.3675

Wang Q., Ying J., Zou P., Zhou Y., Wang B., Yu D., Li W., Zhan X., 2020. Effects of dietary supplementation of humic acid sodium and zinc oxide on growth performance, immune status and antioxidant capacity of weaned piglets. Animals 10, 2104, https://doi.org/10.3390/ani10112104