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Original article

EFFECTS OF HUMIC SUBSTANCES WITH UREA ON PROTOZOAL POPULATION AND FERMENTATION IN THE RUMEN OF SHEEP

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Summary

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The aim was to investigate the effects of humic substances (HS) combined with urea, on pH, ammonia, total volatile fatty acids (∑VFA), protozoal population, amylolytic and cellulolytic activities in rumen fluid (RF) of 12 female crossbred merino sheep (n = 6 in experimental EG and control CG groups). The basic daily ration consisted of 1.25 kg grass hay, 0.25 kg cereal grains – equally wheat and barley (daily intake of crude protein 113.45 g, crude fibre 394.19 g, crude fat 17.27 g and ash 71.85 g) and 10 g urea. HS were applied at 20 $g.day^{-1}$ per animal in EG on day 2–18. Increased protozoal cells ($P<0.05$) of *Entodinium spp.* by 243.19, of *Diplodinium spp.* by 5.11 and of *Ophryoscolex spp.* by 2.06 $10^3.mL^{-1}$ were observed in EG on day 3. The effects on the amylolytic and cellulolytic activities as well as pH values were not significant. Ammonia was higher ($P<0.05$) in EG by 5.78 at 3 h and by 12.75 at 6 h on day 2, by 10.62 at 9 h on day 3, and by 7.84 $mg.100\ mL^{-1}$ at 6 h on day 4 after feeding. ∑VFA were increased ($P<0.05$) by 3.89 $mmol.L^{-1}$ at 3 h on day 18 after feeding. The feed intake of HS combined with urea had significantly positive effects on the course of ammonia and protozoal population in RF.

Key words: ammonia, *Diplodinium, Entodinium,Ophryoscolex,* rumen fluid, sheep

INTRODUCTION

Humates are raw materials utilisable in the plant and animal husbandry as sources of organic and mineral substances with the positive effects on their biological characteristics. These natural products are geological deposits located in the earth superficial layer which originated from the process of decomposition of plant and animal matter via the activities of microorganisms (McMurphy *et al.*, 2011). Humic substances (HS) are mainly bound to inorganic components such as clay and oxides. Some of them is dissolved in the soil suspensions mainly in the alkaline

conditions (Islam *et al.*, 2005). The positive effect of humates on the growth and feed conversion of broiler chickens was demonstrated in several studies (Demeterová *et al.*, 2009; Mirnawati & Marlida, 2013).

Abdel-Mageed (2012) and Sopoliga *et al.* (2016) reported the positive influence of HS on the egg production and their quality of quails and pheasants. The results of Ji *et al.* (2006) indicated the improvement of growth performance of pigs and the reduction of ammonia emission from manure.

The rumen provides a suitable environment for the existence of microorganisms, predominantly bacteria, protozoa, and anaerobic fungi which can be influenced by HS after feed intake. These microorganisms are essential for digestion and fermentation of the large amounts of fibrous feeds which ruminants consume, but otherwise would not be able to utilise (Yokoyama & Johnson, 1988).

The scientific hypothesis was based on the positive effects of urea and ammonia (NH_3) combined with HS on the proteosynthesis of rumen protozoa, which have potential to improve fermentation characteristics in the rumen fluid (RF).

The aim of this study was to investigate the effects of peroral intake of HS preparation combined with urea, as non-protein nitrogen, on the protozoal species, pH values, the concentration of NH_3, the content of total volatile fatty acids (∑VFA) as well as amylolytic and cellulolytic activities in RF of sheep.

MATERIALS AND METHODS

Animals and diets

Twelve female crossbred merino sheep (two years of age, live weight 50.6±1.96 kg) were used in the feeding experiment. The animals were housed in two groups in the identical loose pens with the same hygienic conditions for 18 days. Sheep were fed daily with the basal diet consisting of 1.25 kg grass hay and 0.25 kg of cereal grains mixture divided into two equal parts at 6.00 a.m. and 3.00 p.m. (Table 1). The daily intake of the analysed nutrients from the plant feed was the following: crude protein 113.45 g, crude fibre 394.19 g, crude fat 17.27 g and ash 71.85 g. The crystal urea (CH_4N_2O, molecular weight 60.06 g, Merck Slovakia Ltd.) was added to feed of the experimental (EG, n=6) and the control groups (CG, n=6) at dose 10 g/day/animal mixed into grain mixture between days 2–18.

All procedures were performed with the animals approved by the Animal ethics committee of the University of Veterinary

Table 1. The chemical composition of the diet and formulation of cereal grain mixture

	Grain mixture	Grass hay
DM ($g.kg^{-1}$)	880.70	868.80
Crude fibre ($g.kg^{-1}$ DM)	34.40	356.0
Crude fat ($g.kg^{-1}$ DM)	23.20	11.20
Crude protein ($g.kg^{-1}$ DM)	135.0	77.10
Ash ($g.kg^{-1}$ DM)	15.10	63.10
Formulation of grain mixture (%)		
Barley	50	
Wheat	50	

DM – dry matter.

Medicine and Pharmacy in Košice according to Directive 2010/63/EU (Anonymous, 2010).

Experimental design

The preparation of HS (20 $g.day^{-1}$) was applied in sheep from the EG. The applications of HS were performed with a probe directly into rumen in a water suspension at the time of morning feeding on days 2–4. Subsequently, HS were mixed into grain mixture from day 5 to day 18.

The characteristics of the applied HS preparation (Humac Natur AFM, Humac Ltd., Slovak Republic) were the following: size of particles up to 100 μm, max. moisture 15%, content of humic acids min. 650, fulvic acids min. 50 $g.kg^{-1}$, minerals Ca 42.28, Mg 5.10, Fe 19.05 $g.kg^{-1}$ and microelements Cu 15, Zn 37, Mn 142, Co 1.24, Se 1.67 as well as Mo 2.7 $mg.kg^{-1}$ dry matter (DM).

Measurements and analyses

The chemical analyses of diets were performed according to Commission Regulation (EC) 152/2009 (Anonymous, 2009).

The samples of 100 mL RF were taken from the rumen of sheep using a tube on days 1, 2, 3, 4, 18. The rumen fermentation was evaluated by analysis of pH, NH_3 and ∑VFA at 3, 6 and 9 h after morning feeding. The population of rumen protozoa as well as amylolytic and cellulolytic activities were analysed 3 h after feed intake.

RF was collected and filtered through two layers of gauze and analysed for pH with pH meter (Consort C830, Belgium).

Samples of RF for the isotachophoretic analysis were preserved with 2–3 drops of thymol to prevent fermentation. They were centrifuged and 50 times diluted in distilled water. The analysis of ∑VFA was carried out in a two-capillary isotachophoretic analyser (EA100, VILLA LABECO, Slovak Republic). The leading electrolyte consisted of 10 $mmol.L^{-1}$ HCl, 20 $mmol.L^{-1}$ Ɛ-aminocapronic acid, 0.1% methylhydroxyethylcellulose and the terminating electrolyte contained 5 $mmol.L^{-1}$ capronic acid and histidine.

The quantification of NH_3 was performed by direct distillation and titration of 10.0 mL RF with an automatic N-analyzer (Foss Tecator 2300) (Cunniff, 1995).

The long-term fixation of rumen protozoa was performed by 10-fold dilution of 1.0 mL RF with 3% formaldehyde solution. The diluted RF was used for the quantification of protozoal cells using the light microscopy (Marcin *et al.*, 1992). The differentiation of protozoal species was performed according to a technique of Williams & Coleman (1992).

The analyses of cellulolytic or amylolytic activities were performed in 1.0 mL sample of fresh RF at pH 6.70 according to the method of Lever (1977) for the determination of reducing carbohydrates. The methylhydroxyethylcellulose (Merck Slovakia Ltd.) or the soluble starch (Fisher Slovakia Ltd.) were used as substrates.

The data are expressed as means±the standard error of the mean (SEM) of single values. The results from the treatments were compared by the one-way analysis of variance and the linear correlation with software CoStat Ver. 6.45 (Cohort Software). Significance was declared at levels below $P<0.05$.

RESULTS

The results of the microscopical identification and quantification of rumen pro-

Table 2. Species of rumen protozoa of sheep fed diets with urea (control group, CG) or combined with humic substances (experimental group, EG). Values are presented as mean±SEM, n=6

Species of protozoa	Sampling time (day)	EG (10^3.mL^{-1})	CG (10^3.mL^{-1})	P
Isotricha spp.	1	0.76 ± 0.41	1.24 ± 0.64	0.73
	2	0.78 ± 0.37	0.53 ± 0.18	0.72
	3	2.67 ± 1.90	0.67 ± 0.40	0.38
	4	0.33 ± 0.20	0.25 ± 0.08	0.83
	18	0.28 ± 0.16	0.42 ± 0.24	0.18
Dasytricha spp.	1	2.97 ± 1.36	3.58 ± 2.65	0.87
	2	0.42 ± 0.13	0.69 ± 0.39	0.53
	3	7.22 ± 5.35	2.67 ± 1.57	0.49
	4	3.67 ± 2.67	1.17 ± 0.50	0.95
	18	1.86 ± 0.85	1.62 ± 0.81	0.85
Entodinium spp.	1	96.14 ± 10.53	102.42 ± 15.77	0.76
	2	172.78 ± 13.54	178.67 ± 41.66	0.90
	3	442.83 ± 41.79[a]	199.64 ± 35.43[b]	0.01
	4	371.92 ± 12.42	337.67 ± 46.33	0.55
	18	295.47 ± 44.22	272.62 ± 67.27	0.79
Epidinium spp.	1	1.92 ± 0.62	0.94 ± 0.19	0.21
	2	1.33 ± 0.50	1.36 ± 0.35	0.96
	3	4.44 ± 0.87	1.95 ± 0.49	0.07
	4	0.33 ± 0.17	0.75 ± 0.37	0.63
	18	3.17 ± 1.83	1.01 ± 0.22	0.31
Diplodinium spp.	1	1.21 ± 0.23	0.90 ± 0.45	0.58
	2	4.72 ± 0.62	3.57 ± 0.96	0.37
	3	8.56 ± 0.75[a]	3.44 ± 0.78[c]	0.01
	4	3.17 ± 1.17	4.75 ± 2.08	0.58
	18	1.64 ± 0.20	0.93 ± 0.46	0.23
Ophryoscolex spp.	1	0.22 ± 0.03	0.11 ± 0.07	0.23
	2	0.58 ± 0.31	0.04 ± 0.03	0.16
	3	2.39 ± 0.39[a]	0.33 ± 0.20[b]	0.02
	4	3.17 ± 0.83	3.58 ± 2.08	0.87
	18	0.44 ± 0.15	0.35 ± 0.22	0.74
Polyplastron spp.	1	2.28 ± 1.40	1.63 ± 0.38	0.68
	2	3.93 ± 0.83	4.72 ± 1.91	0.72
	3	10.22 ± 2.09	4.06 ± 1.11	0.06
	4	8.33 ± 0.67	10.08 ± 2.58	0.58
	18	2.81 ± 0.42	3.59 ± 1.11	0.54

P – probability value, means with different superscript letters in the same row differed significantly: [a,b] $P<0.05$, [a,c] $P<0.01$

tozoa species are demonstrated in Table 2. The addition of HS combined with urea had a positive effect on the quantity of some protozoal species in RF. The significant increase of *Entodinium* spp., *Diplodinium* spp. and *Ophryoscolex* spp. was observed in EG compared to CG on day 3. There were not any significant quantitative differences in *Isotricha spp.*, *Dasytricha spp.*, *Epidinium spp.* and *Polyplastron spp.* between groups in the particular sampling times. However, the

counts of protozoal cells of all observed species were continuously increasing from day 1 to 3 except for *Ophryoscolex spp.* which increased from day 1 to 4. The decrease in the number of protozoal cells was observed between day 3 to 18 (*Isotricha spp., Dasytricha spp., Entodinium spp., Epidinium spp., Diplodinium spp., Polyplastron spp.*) or day 4 to 18 (*Ophryoscolex spp.*).

There were no significant difference in pH values in RF between both groups (Table 3).

The addition of HS combined with urea had a positive effect on the amylolytic and cellulolytic activities in RF (Fig. 2) of EG sheep. In the case of the amylolytic activity, there were higher values in EG compared to CG on days 2–4 three hours after morning feeding. The analysed cellulolytic activity increased in EG compared to CG on the same sampling days.

The values of NH_3 in RF were higher in EG at 3 h and at 6 h on day 2, at 9 h on day 3 and at 6 h on day 4 after feed intake (Table 4).

The intake of HS resulted in decreased NH_3 in RF of EG compared to CG at 3, 6 and 9 h on day 3 and on day 18. The decrease of this parameter was observed at 3 and 6 h on day 4 as well.

According to linear correlation analysis, the NH_3 values affected negatively *Isotricha spp.* in rumen of sheep in CG at 3 h after feeding (y=–20.6151x+56.7901, r=–0.8865, P=0.0451) on days 1–18, whereas the positive effect of HS combined

Table 3. Time course of pH value in rumen fluid of sheep from the experimental group (EG) and control group (CG) after the morning feeding. Values are presented as mean±SEM, n=6

Sampling time (day)	EG	CG	P
3 h after feeding			
1	6.65 ± 0.04	6.54 ± 0.08	0.29
2	6.58 ± 0.09	6.64 ± 0.04	0.54
3	6.50 ± 0.06	6.62 ± 0.05	0.21
4	6.74 ± 0.10	6.56 ± 0.16	0.39
18	6.91 ± 0.06	6.68 ± 0.12	0.12
Average	6.68 ± 0.07	6.61 ± 0.09	
6 h after feeding			
1	6.65 ± 0.03	6.70 ± 0.04	0.15
2	6.57 ± 0.07	6.65 ± 0.02	0.32
3	6.31 ± 0.02	6.59 ± 0.09	0.09
4	6.61 ± 0.11	6.47 ± 0.13	0.45
18	6.55 ± 0.10	6.41 ± 0.11	0.37
Average	6.54 ± 0.06	6.56 ± 0.06	
9 h after feeding			
1	6.87 ± 0.02	6.92 ± 0.01	0.11
2	7.05 ± 0.04	6.89 ± 0.06	0.08
3	6.69 ± 0.14	6.77 ± 0.24	0.79
4	6.70 ± 0.06	6.60 ± 0.11	0.55
18	6.74 ± 0.05	6.87 ± 0.11	0.32
Average	6.81 ± 0.06	6.81 ± 0.10	

Table 4. Concentration of NH_3 (mg.100 mL^{-1}) in the rumen of sheep fed diets with urea (control group, CG) or combined with humic substances (experimental group, EG). Values are presented as mean±SEM, n=6

Sampling time (day)	EG	CG	P
3 h after feeding			
1	30.07 ± 1.78	29.12 ± 2.17	0.75
2	49.25 ± 1.31[a]	43.47 ± 1.09[b]	0.015
3	54.35 ± 3.00	50.22 ± 4.75	0.50
4	52.72 ± 1.79	50.03 ± 3.76	0.55
18	44.70 ± 1.88	47.25 ± 1.67	0.34
6 h after feeding			
1	28.36 ± 1.38	27.59 ± 2.17	0.78
2	46.19 ± 0.19[a]	33.44 ± 1.79[c]	0.01
3	46.46 ± 3.49	42.14 ± 2.33	0.36
4	46.85 ± 0.66[a]	39.01 ± 2.03[b]	0.02
18	34.32 ± 0.93	33.85 ± 1.89	0.83
9 h after feeding			
1	18.93 ± 0.37	20.08 ± 0.58	0.17
2	30.50 ± 1.25	29.84 ± 0.73	0.67
3	38.84 ± 1.53[a]	28.23 ± 1.98[b]	0.01
4	nd	nd	nd
18	22.62 ± 0.64	19.69 ± 1.21	0.06

P – probability value, means with different superscript letters in the same row differed significantly: [a,b] - $P < 0.05$, [a,c] - $P < 0.01$; nd=not determined.

with urea was observed on *Isotricha spp.* in EG (y=3.5007x+42.8446, r = 0.3517, P=0.5617). The other observed species of protozoa were not influenced by NH_3 concentration.

The concentrations of ∑VFA in RF are demonstrated in Table 5. The production of ∑VFA was not affected by HS. The only significant difference was observed between groups at hour 3 after feed intake on day 18.

The negative effect of ∑VFA on the counts of *Isotricha spp.* was determined at 3 h after feeding of sheep from CG on days 1–18 (y=–2.7779x+96.3946, r=–0.9463, P=0.0148). This influence of ∑VFA on the mentioned protozoa was diminished by the combination of HS with urea (y = –1.2315x+97.3281, r = –0.4889, P=0.4032) in EG at the same sampling time. On the contrary, the concentration of *Entodinium spp.* in RF of CG was positively influenced by ∑VFA (y= 0.0113x+92.1991, r=0.9244, P=0.0247) at hour 3 after feeding. The effects of HS on the counts of *Entodinium spp.* in EG were negative (y=–0.0085x+98.4978, r=–0.4909, P=0.4011).

DISCUSSION

The novelty of the study was the utilisation of the meaningful potential of the feed additives HS combined with urea (EG) for the positive effect on the protozoal population as well as the fermentative and biochemical characteristics of RF (pH, NH_3, ∑VFA, amylolytic and cellulolytic activities).

Table 5. Total volatile fatty acids (∑VFA) in the rumen of sheep fed diets with urea (control group, CG) or combined with humic substances (experimental group, EG). Values are presented as mean± SEM, n=6

Sampling time (day)	EG (mmol.L^{-1})	CG (mmol.L^{-1})	P
3 h after feeding			
	95.94 ± 1.78	93.20 ± 1.52	0.31
2	97.84 ± 2.98	94.84 ± 0.45	0.36
3	93.90 ± 4.91	93.95 ± 1.78	0.99
4	93.67 ± 1.03	95.91 ± 1.88	0.35
18	99.35 ± 1.54^{a}	95.47 ± 0.46^{b}	0.037
6 h after feeding			
1	90.37 ± 2.02	89.53 ± 1.46	0.75
2	93.23 ± 0.35	88.78 ± 2.00	0.93
3	101.25 ± 2.53	100.07 ± 5.61	0.86
4	92.90 ± 3.71	93.80 ± 3.51	0.87
18	98.84 ± 1.57	98.84 ± 1.10	0.94
9 h after feeding			
1	87.74 ± 0.61	85.52 ± 1.78	0.30
2	87.02 ± 1.35	86.35 ± 2.14	0.81
3	92.74 ± 3.30	90.56 ± 1.35	0.57
4	nd	nd	nd
18	81.07 ± 2.22	85.46 ± 1.70	0.15

P – probability value, means with different superscript letters in the same row differed significantly: ^{a,b} - P < 0.05; nd=not determined.

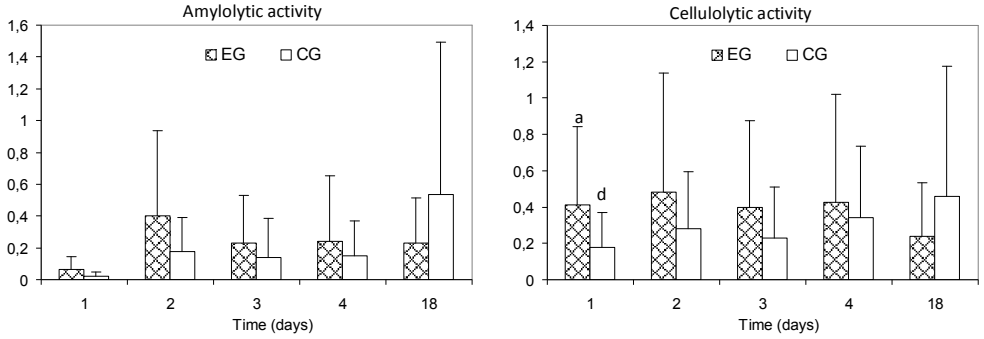

Fig. 1. Amylolytic and cellulolytic activities (glucose mmol/L/min) in rumen fluid of sheep fed diets with humic substances + urea (EG) or only with urea (CG) 3 h after the morning feeding, EG – experimental group, CG – control group. Data are presented as mean ± SEM (n=6), means with different superscript letters differ significantly: ^{a,d} P<0.001.

A positive increase of *Entodinium* spp., *Diplodinium* spp. and *Ophryoscolex* spp. was observed in EG. Similarly, Váradyová *et al.* (2009) demonstrated the

increase in the population of *Ophryoscolex c. tricoronatus* in the artificial rumen after intake of preparation Humacid (SK Organic Farm, India). Otherwise, the total ciliate population of *Entodinium* spp. was not affected and *Isotricha spp.* were increased. On the contrary, Galip *et al.* (2010) observed significant decrease of *Diplodinium spp.* and the increase of *Epidinium spp.* in RF of rams after feed intake of humic substances. The differences between the mentioned experiments can be affected by the differences in physical and chemical characteristics of HS and the absence of additional urea. According to Islam *et al.* (2005), the effects of HS preparations depend on their chemical specifications which are influenced by sources, nature as well as climatic conditions.

The intake of HS had no significant effect on pH values in the rumen.

The amylolytic and cellulolytic activities in RF were enhanced in EG in the period between days 2 and 4. However, the dose of HS applied directly into rumen feed had a positive effect on both enzymatic activities.

As for the enzymatic activities, the different pH levels regulate the fermentation process in the rumen. In the case of lower ruminal pH, the higher amount of amylolytic bacteria are present in RF which compete for soluble carbohydrates as well as for products created in the hydrolysis of starch and hemicellulose (McMurphy *et al.,* 2009). However, Galip *et al.* (2010) observed decreased cellulolytic activity in RF of rams by 13.8 or 12.13% after dietary intake of humic substances 5 or 10 $g.day^{-1}$. They attributed this effect to the antimicrobial properties of HS. On the contrary, it was not confirmed in the presented study.

The continuous decrease of NH_3 in RF was observed in EG compared to CG after feeding.

In addition, Galip *et al.* (2010) observed that the intake of HS without urea caused a decrease of NH_3 in RF at 3 and 6 h after morning feeding. Comparable results were achieved by McMurphy *et al.* (2011) when the dietary intake of humates at the level 5 $g.kg^{-1}$ combined with urea 12.5 $g.kg^{-1}$ reduced NH_3 in the rumen and slightly altered DM intake.

The production of ∑VFA was not affected by HS. Equivalently, McMurphy *et al.* (2011) determined that humate product rich in humic acids do not alter pH value and VFA concentration in RF of Holstein steers after intake of commercial humate product.

The effects of ∑VFA on the counts of *Isotricha spp.* were negative and on *Entodinium spp.* positive in CG. The effect of ∑VFA on *Isotricha spp.* was favourably influenced by the addition of HS with urea, which was not observed in the case of *Entodinium spp*.

The reasons for the different effect of ∑VFA on the observed protozoa were the metabolic differences between *Isotricha spp.* and *Entodinium spp.* As for the effect of ∑VFA on the other species of protozoa, there were no significant differences between groups. On one hand, *Entodinium* metabolises lactic acids and the major products of lactate metabolism are propionic and butyric acids and on the other, VFA are formed during carbohydrate utilisation of *Isotricha* (Williams & Coleman, 1992).

In conclusion, the dietary intake of HS combined with urea significantly increased NH_3 content in RF without significant effects on pH and negative effects on ∑VFA values. The positive effects of additives were observed on the ruminal populations of *Entodinium* spp., *Diplod-*

inium spp. and *Ophryoscolex* spp., and on RF amylolytic and cellulolytic activities.

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