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EFFECT OF SUPPLEMENTAL HUMIC ACID WITH UREA AS A NON PROTEIN DIETARY NITROGEN SOURCE ON RUMEN VARIABLES IN SHEEP

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Abstract

The aim of this study was to investigate the effects of humic substances (HS) combined with urea as feed additives on the rumen fermentation indicators (pH, concentration of volatile fatty acids VFAs, ammonia concentration, protozoal population) in rumen fluid of 12 female crossbred merino sheep (n = 6 in test and control group). The daily ration for both groups consisted of 1.25 kg grass hay and 0.25 kg cereal grain mixture and 10 g urea. The humic substances were applied at a dose of 20 g/day per animal of the test group orally drenched with a probe directly into rumen (day 1 – 3) at the morning feeding or mixed into feed (day 4 – 18). Rumen samples were taken on day 0, 3 and 18 at 3, 6 and 9 h post morning feeding. The results revealed that HS increased ammonia concentration and protozoal cells of *Entodinium spp.* by 243.2 and of *Diplodinium spp.* by 5.1 ($\times 10^3$ /ml) on day 3, while the total VFAs concentration; acetate and propionate proportions as well as pH values of rumen fluid and the acetate : propionate ratio were not changed. Ammonia was higher ($P \leq 0.05$) in the test group by 7.9 at 6 h and by 5.6 mg/100ml at 9 h on day 3. Results suggest humic substances addition with urea as a non-protein dietary nitrogen source may improve nitrogen retention in the rumen, but there was no impact on VFAs production. The feed intake of HS with urea had significantly positive effects on the protozoal population in rumen fluid.

Key words: humic substances, ammonia, rumen fluid, urea, protozoa

Introduction

Humate substances, or humic acids, are geological deposits in the earth's soil composed mainly of decaying plant and animal matter through the biological activities of microorganisms (McMurphy et al., 2011). Humates, based on solubility in acids and bases and by molecular weight, can be fractionated into three categories: fulvic acid, humic acid and humin (Stevenson, 1982). Humic and fulvic acids are the major extractable components of soil humates and are predominantly used to improve soil fertility and enhance nutrient uptake by plants (Rajendiran et al., 2016). Humic acid is an end product of biodegradation processes of soil organic substances used as growth promoter (Galip et al., 2010). Humic substances are known to exhibit a high affinity for nitrogen (N), a property that has been postulated to improve rumen microbial synthesis and decrease N excretion into the environment (McMurphy et al., 2011). These nitrogen-binding

qualities could prove to be beneficial in the retention of ammonia nitrogen (NH₃-N) in the rumen. Urea has commonly become an accepted non-protein nitrogen ingredient in the diets of ruminants. It is rapidly hydrolyzed by rumen bacterial urease to ammonia and the ammonia is utilized for the synthesis of microbial proteins (Jin et al., 2018).

Humic acid as a product of decomposition of animal and plant tissue has been proposed as a feed supplement to stimulate the growth of animals and as a potential replacement for antibiotic growth stimulators (Kocabagli et al., 2002; Karaoglu et al., 2004; Wang et al., 2008; Demeterová et al., 2009). Humates have been shown to reduce ammonia emissions when utilized as an amendment to soil, feces and urine (Shi et al., 2001) or when used as a feed supplement in swine (Ji et al., 2006).

The aim of this study was to investigate the effects of peroral intake (orally drenched or mixed into feed) of humic substances preparation combined with urea, as non protein nitrogen, on the protozoal species, pH values, the concentration of ammonia, the sum of total volatile fatty acids as well as acetate : propionate ratio in rumen of sheep in the time dependence.

Material and methods

Twelve crossbred merino ewes (two years of age, no pregnant, not lactating) were used in 18 day experiment. The animals were housed in two groups – test and control. Sheep were fed with the daily basal diet consisting of 1.25 kg grass hay and 0.25 kg of cereal grains mixture divided into two equal parts. The experimental dietary ingredients and chemical composition are presented in Table 1. Urea was fed for both groups at dose 10 g / day / sheep mixed into grain mixture at the morning feeding. Water was available *ad libitum*. All procedures were performed with the animals approved by the Animal ethics committee of the University of Veterinary Medicine and Pharmacy in Košice according to Directive 2010/63/EU.

Table 1: Chemical composition of the diet and formulation of cereal grain mixture

	Grain mixture	Grass hay
Dry matter (g/kg)	880.70	868.80
Crude fiber (g/kg DM)	34.40	356.0
Ether extract (g/kg DM)	23.20	11.20
Crude protein (g/kg DM)	135.0	77.10
Ash (g/kg DM)	15.10	63.10
Formulation of grain mixture, % as fed		
Barley	50	
Wheat	50	

DM – dry matter; Urea was fed for both groups at dose 10 g / day / sheep mixed into grain mixture at the morning feeding.

We used two different systems of the application of additives during the test period especially for confirmation of our hypothesis of differences in nitrogen-binding capacity in the rumen after two diverse ways of administration. Urea was used as non-protein nitrogen feed source due to fast transformation into ammonia. We tested our hypothesis in order to assess the main effects of additives after the administration by drenching technique or simultaneously intake in the diet with urea.

Sheep received basal diet either without humic substance (HS) (control group, CG) or were orally drenched with 20 g HS / day per animal (test group, TEG) at the time of morning feeding on day 1 – 3. Each HS dose was diluted in fresh water before each morning feeding and orally drenched to each sheep in order to ensure that each sheep receive the full dose of HS. Subsequently HS in the same dose were mixed into daily grain mixture dose from day 4 – 18.

The characteristics of the applied natural HS preparation (HUMAC®Natur AFM; Humac s.r.o., Slovak Republic) were the following: the size of particles up to 100 µm, max. moisture 15%, the content of humic acids min. 650 g/kg, fulvic acids min. 50 g/kg.

The chemical analyses of diets were performed according to Commission Regulation (EC) No 152/2009. The rumen contents were taken from rumen using a tube at 3, 6 and 9 hrs after morning feeding on day 0, 3, 18. The population of rumen protozoa was analysed 3 h after feed intake. Day 0 was control day before starting of experiment (no addition of urea and HS). The level of rumen fermentation was evaluated by analysing rumen pH, ammonia content (NH₃) (mg /100ml), the sum of volatile fatty acids (ΣVFA) (mmol/l) and the acetate: propionate ratio. Rumen fluid were collected and filtered through two layers of gauze and analysed for pH with pH meter (Consort C830, Belgium). The analysis of VFA was carried out in a two-capillary isotachophoretic analyser (EA100, VILLA LABECO, Slovak Republic). The quantification of NH₃ was performed by direct distillation and titration of 10.0 ml ruminal fluid with an automatic N-analyzer (Foss Tecator 2300). The long-term fixation of rumen protozoa was performed by 10-fold dilution of 1.0 ml ruminal fluid (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 and Coleman (1997).

The data were expressed as a mean ± standard error (SEM) and analysed using an unpaired t-test in Graph-Pad Prism (Graph Prism software, USA). Significance was declared at levels below P<0.05.

Results and discussion

Effects of supplementing sheep with urea and HS on the ruminal fermentation parameters, the protozoa counts, and the ruminal pH are presented in *Table 2*. The supplementation of humic substances with urea combination showed the following in hourly dynamics in the test group: significantly higher level of the ammonia content on day 3 at 6 h (P<0.05) and at 9 h (P<0.05) after administration in comparison with the control group. The slower decrease of ammonia in the rumen in between the third and the sixth hour (5.0 mg/100ml in the TEG and 9.9 mg/100ml in the CG, respectively) after feeding was observed in the test group, especially on day 3 after an oral drench addition of HS. The tendency of slower decrease of ammonia was also detected in between the third and the sixth hour (10.4 mg/100ml in the TEG and 13.4 mg/100ml in the CG, respectively) and the sixth and the ninth hour (11.7 mg/100ml in the TEG and 14.1 mg/100ml in the CG, respectively) on day 18 after peroral intake of HS (mixed into concentrate mixture) in the TEG.

There were no statistically significant differences in the ammonia concentration between groups on day 18.

Table 2: Ruminal fermentation parameters, protozoa counts, and rumen pH in sheep after peroral intake (orally drenched or mixed into feed) of humic substances preparation combined with urea (n = 12; two groups test and control with 6 sheep each). Values are presented as mean±SEM.

Item	ST (h)	Day 0		Day 3		Day 18	
		TEG	CG	TEG	CG	TEG	CG
Ruminal pH	3	6.65±0.04	6.54±0.08	6.50±0.06	6.62±0.05	6.71±0.06	6.68±0.12
	6	6.65±0.03	6.70±0.04	6.31±0.02	6.49±0.09	6.55±0.09	6.41±0.11
	9	6.87±0.02	6.92±0.01	6.69±0.14	6.77±0.24	6.87±0.11	6.74±0.05
Total VFA, mmol/l	3	95.9±1.8	93.2±1.5	93.9±4.9	93.9±1.8	94.4±1.5	95.5±0.5
	6	90.4±2.0	89.5±1.5	101.3±2.5	100.1±5.6	98.3±1.6	98.8±1.1
	9	87.7±0.6	85.5±1.8	92.7±3.3	90.6±1.4	81.1±2.2	85.5±1.7
Acetate (A), mmol/l	3	62.4±1.2	61.1±1.0	60.4±3.2	60.5±1.2	60.1±1.0	61.5±0.3
	6	59.7±1.3	58.2±0.9	63.4±1.7	62.9±3.7	63.8±1.0	63.7±0.7
	9	58.6±0.4	58.1±1.2	61.2±2.2	59.4±0.9	54.9±1.7	57.1±1.4
Propionate (P), mmol/l	3	20.1±0.4	19.4±0.3	20.5±0.9	20.3±0.4	19.2±0.3	19.7±0.1
	6	17.8±0.4	17.6±0.3	22.1±0.6	21.5±1.3	20.0±0.3	20.3±0.2
	9	15.6±0.1	15.2±0.4	17.6±0.7	17.0±0.3	16.1±0.4	16.6±0.3
A : P ratio	3	3.11±0.07	3.15±0.06	2.95±0.19	2.98±0.07	3.12±0.06	3.11±0.02
	6	3.35±0.08	3.29±0.05	2.87±0.09	2.92±0.21	3.18±0.06	3.13±0.04
	9	3.75±0.03	3.81±0.07	3.43±0.13	3.48±0.05	3.41±0.11	3.43±0.08
Ammonia NH ₃ , mg/100ml	3	30.1±1.8	29.1±2.1	52.7±1.8	49.7±3.8	44.7±1.7	47.2±1.9
	6	28.4±1.4	27.6±2.2	47.7±3.5*	39.8±2.3	34.3±0.9	33.8±1.9
	9	18.9±0.4	20.1±0.6	33.8±1.2*	28.2±1.4	22.6±0.6	19.7±1.2
Protozoa, x 10 ³ / ml							
<i>Entodinium spp.</i>	3	96.1±10.5	102.4±15.8	442.8±41.8**	199.6±35.4	295.5±44.2	272.6±67.3
<i>Epidinium spp.</i>	3	1.92±0.62	0.94±0.19	4.44±0.87	1.95±0.94	3.17±1.83	1.01±0.22
<i>Diplodinium spp.</i>	3	1.21±0.23	0.90±0.45	8.56±0.85**	3.44±0.78	1.64±0.20	0.93±0.46

*=P<0.05; **=P<0.01

VFA – volatile fatty acids; ST – sampling time; h – hour; TEG – test group; CG – control group

Humates have nitrogen-binding qualities that could prove to be beneficial in the retention of ammonia nitrogen in the rumen (McMurphy et al., 2011). The N-binding ability could improve the retention time and slow down releasing of ammonia nitrogen in the rumen. In the rumen, the ammonia can be assimilated by many rumen bacteria for the synthesis of microbial proteins (Owens et al., 1980; Milton et al., 1997). The supplementation of humic substances with urea combination in our study showed the slower decrease of ammonia in the rumen in the time dependence after feeding (in hourly dynamics) in the test group after two different ways for HS administration.

No differences between groups were observed for the total and particular (acetate and propionate) VFA concentrations (mmol/l) in all hourly collections during day 3 and 18 as well as in the pH parameter and the acetate : propionate ratio ($P > 0.05$).

The addition of HS combined with urea had a positive effect on the quantity of some protozoal species in ruminal fluid. The significant increase of *Entodinium* spp. and *Diplodinium* spp. was observed in the TEG compared to the CG on day 3. There was not any significant quantitative differences in *Epidinium* spp. between groups, although the tendency of increase in the number of these protozoal cells was also observed on day 3. The decrease in the number of protozoal cells (*Entodinium* spp., *Epidinium* spp. and *Diplodinium* spp.) was observed on day 18 in the CG. However, there were not any significant quantitative differences on day 18.

A number of studies have examined the value of humic substances as a feed additive for ruminants (Váradyová et al., 2009; Galip et al., 2010; McMurphy et al., 2011; Degirmencioglu, 2012; El-Zaiat et al., 2018; Terry et al., 2018).

McMurphy et al. (2011) determined that humate product high in humic acid content did not dramatically impact some aspects of rumen fermentation (pH value and VFA concentration). Galip et al (2010) observed no significant effect on rumen variables and ruminal protozoal populations except for rumen protozoa *Epidinium* spp. in rams fed humic acid supplemented diets.

A positive increase of *Entodinium* spp. and *Diplodinium* spp. was observed in the EG in our experiment. On the contrary, Galip et al. (2010) observed significant decrease of *Diplodinium* spp. and the increase of *Epidinium* spp. in ruminal fluid of rams after feed intake of humic substances.

The results from El-Zaiat et al. (2018) revealed that humic acid increased ruminal pH, acetate and propionate proportions, while ammonia concentration was decreased. Váradyová et al. (2009) found that when humic substances were added at 10 g/kg DM to a rumen simulation (Rusitec) using sheep inoculum, $\text{NH}_3\text{-N}$ was reduced by 24.4% in a high-forage diet.

Those results contrast with the results from Terry et al. (2018), in which, at a similar concentration, humic substances increased $\text{NH}_3\text{-N}$ concentration. This study indicated that total VFA and their individual concentrations were not affected by the addition of humic substances. The concentration of $\text{NH}_3\text{-N}$ and total protozoa count responded quadratically ($P = 0.03$) to increasing concentrations of HS. The quadratic response of protozoa numbers and $\text{NH}_3\text{-N}$ concentration to HS dose is consistent with the concept that rumen protozoa engulf rumen bacteria that use $\text{NH}_3\text{-N}$ to help meet their N requirements (Koenig et al., 2000; Bach et al., 2005). Thus, increased protozoa numbers may have led to less ruminal NH_3 incorporated into microbial protein and increased NH_3 concentrations (Terry et al., 2018).

Conclusion

The addition of humic substances with urea has confirmed nitrogen-binding capacity in the rumen with longer retention time for potential releasing of ammonia for future synthesis of microbial proteins with significantly increased NH_3 concentration in ruminal fluid. It had no significant effect on the ruminal pH, the VFA content as well as the acetate : propionate ratio. The addition of these additives resulted in increasing of the some ruminal protozoa populations (*Entodinium* spp. and *Diplodinium* spp.).

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