Effects of supplemental humic acid on ruminal fermentation and blood variables in rams

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Abstract

In this study, we particularly aimed to research the effect of supplemental humic acid on ruminal fermentation and blood variables in rams. A trial was conducted to evaluate the effect of humic acid (HA) on protozoa count, percentages of different protozoa types and blood parameters. Three male Kivircik rams with ruminal cannula were used in a Latin square design, during 22 days periods (15 days for adaptation, 7 days for collection). They received 0 control group (CG), 5 g/day or 10 g/day of HA (HA5, HA10, assay groups). HA were added to the ration with grain diet. Ration was consisted of 5% grain diet and 95% alfalfa hay. Rumen contents collected before and, 3h and 6h after morning feeding on days 1 and 7 in each collection period were analyzed. Blood samples were also collected the same days. No significant difference in biochemical and hematological parameters (except eosinophils levels, P<0.05), variables of ruminal fluid (except sodium levels before feeding) and species of rumen protozoa organism (except the percentage Epidinium spp.) were evidenced with the addition of HA.

Introduction

For many years, ruminant nutritionists and microbiologists have been interested in manipulating the microbial ecosystem of the rumin to improve production efficiency by domestic ruminants. Based on growing concern over the use of antibiotics and other growth promotants in the animal feed industry, interest in the effects of microbial feed additives on animal performance has increased during the past 10 to 20 years (Wang et al., 2008; Avcı et al. 2007; Galip, 2006).

Humates are an accessible raw material that can be used in agriculture and animal husbandry in the form of a humate drink or dry feed as a source of mineral and organic substances for growth stimulation. They have long been used as folk remedies for a wide variety of illnesses (Achard, 1986). Humates are the salts of humic acid in which the cation/anion exchange sites of humic acid is Ca\textsuperscript{2+}, Na\textsuperscript{+}, Al\textsuperscript{3+} and Fe\textsuperscript{2+} rather than hydrogen (Griban et al., 1991). Humates are considered a growth-promoting agent in Europe and formed from decayed plant matter with the aid of living bacteria in the soil. The composition of humates includes humus, humic acid, fulvic acid, ulmic acid, and trace minerals, which are necessary for plant development (Kocabagli et al., 2002).

Humic acids have been used for growth stimulation in the area of plant production for a long time. They were also used as a part of the replacement therapy and enhanced feed conversion ratio in dogs, cats and calves with malnutrition and diarrhea (Islam et al., 2005). Furthermore, humates have been used for their anti-inflammatory, anti-oedematous, antibacterial and antiviral effects on animals (Kuhnert et al., 1991). The concept of using humates as an alternative feed additive in animal nutrition has gained increasing importance, particularly after the ban on antibiotic use in feeds as growth promoters (Karaoglu et al., 2004; Kuhnert et al., 1991). More information is needed on this subject because minimal research has been conducted; humic acid has not shown promise as a performance-promoting supplement in ruminant diets. Therefore, in this study, we particularly aimed to research the effect of supplemental humic acid on ruminal fermentation and blood variables in rams.

Materials and methods

Three Kivircik rams (one year of age and average weight of 30 kg) with ruminal cannula were used in a Latin square design, during a 22 days period (15 days for adaptation, 7 days for collection period). The control group (CG) was fed only the basal diet, consisting of 5% alfalfa hay and 95% grain mix (Table 1), whereas assay groups received 5 g/day of humic acid (HA5) and 10 g/day (HA10) of humic acid (HA) respectively. HA were added to the ration with concentrate mixture. Chemical analyses of diets were carried on according to AOAC (1990). Ration was designed to cover 1.25 times of NRC (1985) maintenance requirements for rams. The basal diet was divided into two equal parts and distributed at 09.00 and 16.00 h. The rams were housed individually in loose pens during this period.

Rumen fluid samples were taken through the rumen cannula at 0, 3 and 6 h after morning feeding, (i.e. 9.00 and 12.00 and 15.00, respectively) on days 1 and 7 in each collection period. Values indicated are average of two samplings at day 1 and 7. The pH of each sample was determined immediately with an electronic pH meter (Nel mod 821, Ankara, Turkey). Ammonia nitrogen (NH\textsubscript{3}-N) and total volatile fatty acids (VFA) concentrations of ruminal fluid were measured according to steam distillation method described by Markham (1942). The Na\textsuperscript{+} and K\textsuperscript{+} in rumen fluid were determined with flame photometer (Eksen et al., 1992). One milliliters of rumen fluid was mixed with 49 mL of rumen protozoa counting solution (2.02% formalin and 15.15% glycerol) to determine the counts of rumen protozoa (Mendoza et al., 1993). Diluted rumen fluid samples were used for counting with the help of Fuchs Rosenthal counting chamber by the method of Boyne et al. (1957). The different microscopic counts of protozoa in the ruminal fluid were obtained with the technique of Coleman and Hall (1978). Cellulose activity into the rumen was measured by loss of weight of cotton thread inoculated in sacco during 24 h in each period (Onifade, 1997).

Blood samples were collected from the jugular vein of each animal on days 1 and 7 in each collection period (2 h after morning feeding). Values indicated are average of two samplings.
at day 1 and 7. Hematological samples were collected in heparin-treated tubes, while samples for serum biochemical parameters were collected without anticoagulant. Serum biochemical parameters were determined with “Technician Dax 72” auto analyzer (Miles Inc., Tarrytown, NY, USA). Numbers of erythrocyte, leukocytes and the haematocrit value were estimated according to the methods reported by Jain (1986). Also mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), were mathematically calculated according to Jain (1986). The concentrations of haemoglobin were measured spectrophotometrically by the cyanmethemoglobin method of Cannon (1958).

Data were analyzed using a 3×3 design model. The model included dietary treatments, rams, periods, and time periods. Differences between means were identified by Tukey’s Test (SAS, 1985). The differences were considered as significant when P<0.10. Because of the low size of ram population, a greater risk was tolerated.

**Results and discussion**

Chemical compositions of the diets and formulation of grain diet were indicated in Table 1. It was shown the rumen variables in rams fed humic acid supplemented diets in Table 2 and the species of rumen protozoa organism (%) in rams fed humic acid supplemented diets in Table 3. It had no significant effect on rumen variables in rams fed humic acid supplemented diets, except sodium levels (P<0.05) before feeding. It was shown the biochemical and haematological parameters in rams fed humic acid supplemented diets in Table 4. The significant differences were not detected among groups after and before beginning of feeding in the biochemical and hematological parameters, except eosinophils (%) levels (P<0.05).

In the present in vitro study humic acid was used in the form of a commercial H60B feed additive preparation. However, there was little information in the literature concerning the use of humic acid as a feed additive for promoting growth in ruminants. Limited studies on the effects of humate or humic acid on feedlot performance, health and production in ruminants showed the positive effect on the utilisation of carbohydrates and protein (Bell et al., 1997; Covington et al., 1997; Brown et al., 2007; Cusack, 2008). But the literatures researching the effects of humic acid on both biochemical and hematological parameters with regard to the species of rumen protozoa organism and the ruminal fluid parameters are not enough.

It had no significant effect on rumen variables in rams fed humic acid supplemented diets, except sodium levels before feeding (Table 2). Sodium levels of ruminal fluid (P<0.05) were found to be statistically different between CG and HA5 diets before feeding. Compared to the control, higher only values were obtained for sodium levels of rams fed HA5 and HA10 diets at 0h before feeding. However there wasn’t any change in HA10 diets and in other hours after morning feeding of HA5. HA was contained a number of functional groups such as carboxylic, carboxylic, phenolic and methoxy groups. Those function groups in HA may combine with some ions and heavy metals. Therefore, HA play many significant roles in stabilizing sediments, regulating the levels of water, metals and other components (Ghabbour et al., 2006). Both ions, metals and organic contaminants can be absorbed by HA in a number of ways such as complexation, ion exchange and reduction (Wood, 1996), forming some hydrosoluble or water-insoluble complex compounds (Schnitzer and Khan, 1972).

Addition of HA had only a significant effect on the percentage Epidinium spp. of different rumen protozoa types (Table 3). HA decreased

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**Table 1. Chemical compositions of the diets and formulation of grain diet.**

|          | Grain mix | Alfalfa hay |
|----------|-----------|-------------|
| Dry matter, % | 88.62 | 88.59 |
| Crude fibre, % DM | 7.74 | 22.25 |
| Ether extract, % DM | 2.25 | 2.57 |
| Crude protein, % DM | 4.5 | 19.20 |
| Ash, % DM | 6.51 | 9.52 |
| Formulation of grain mix, % as fed | | |
| Barley | | 69.20 |
| Corn | | 10.00 |
| Sunflower meal, 30% CP | | 17.60 |
| Limestone | | 2.50 |
| Salt (NaCl) | | 0.60 |
| Vitamin-mineral premix | | 0.10 |

1Provided per kilogram of vitamin-mineral premix, vitamin A 15,000,000 U, vitamin D 3,000,000 U, vitamin E 30,000 mg, Mn 50,000 mg, Fe 50,000 mg, Zn 50,000 mg, Cu 10,000 mg, I 800 mg, Co 150 mg, Se 150 mg.

**Table 2. Rumen variables in rams fed humic acid supplemented diets.**

| Variables of ruminal fluid | Sampling times | Treatments | SEM |
|---------------------------|----------------|------------|-----|
| Ruminal pH | 0° | 6.95 | 6.67 | 6.84 | 0.13 |
| | 3° | 5.75 | 5.36 | 5.39 | 0.14 |
| | 6° | 5.72 | 5.54 | 5.53 | 0.57 |
| Protozoa count | 0 | 242.33 | 277.13 | 379.33 | 91.68 |
| | 3 | 181.66 | 218.66 | 186.00 | 36.77 |
| | 6 | 241.33 | 245.00 | 207.66 | 9.27 |
| NH₃-N, mg/L | 0 | 183.33 | 203.33 | 282.33 | 35.2 |
| | 3 | 340.00 | 328.33 | 316.67 | 44.9 |
| | 6 | 326.66 | 378.33 | 284.33 | 34.8 |
| Total VFA, mmol/L | 0 | 34.00 | 32.33 | 48.00 | 15.0 |
| | 3 | 92.33 | 104.00 | 109.33 | 11.7 |
| | 6 | 104.00 | 97.00 | 90.00 | 25.1 |
| Sodium, mmol/L | 0 | 93.50 | 113.50 | 105.00 | 2.24 |
| | 3 | 78.50 | 78.16 | 86.16 | 15.01 |
| | 6 | 78.33 | 79.66 | 86.00 | 12.69 |
| Potassium, mmol/L | 0 | 30.13 | 31.13 | 32.96 | 7.85 |
| | 3 | 51.50 | 53.26 | 55.43 | 1.72 |
| | 6 | 52.96 | 62.90 | 47.26 | 4.31 |
| Cellulose activity, % | 26.66 | 13.68 | 14.73 | 9.76 |

°Before feeding; °3 h after feeding; °6 h after feeding; °Control basal diet; °5 g/day of humic acid with basal diet; °°10 g/day of humic acid with basal diet; “number × 10³ ml⁻¹; ††different superscripts show differences (P<0.05) among groups.
the percentage of *Epidinium spp*. but only the difference between the control group and HA5 group in 3 h after feeding was statistically significant (P<0.05). Protozoa count seems too variable within group for a population that changes very slowly (Table 2). Cellulolytic activity was decreased by HA5 and HA10 in rams, but this difference was not statistically significant (Table 2, P>0.05). This variation could have been due to decrease microbial activity by HA. Also Zora et al. (2009) reported that there was a decrease in VFA production by HA and such decrease in ruminal VFA has been suggested to be due to a lower ruminal cellulolytic activity.

The usefulness of HA as a feed additive for ruminants may be questionable due to their antimicrobial properties and possible effects on rumen bacteria, with a consequent decrease in digestibility, would be deleterious (Ivan et al., 2004). However, supplementation as a feed additive in ruminant diets has not been well reported.

As far as biochemical parameters were concerned, no significant variations in assay groups could be noticed, although some serum concentrations (albumin, A/G, total cholesterol, glucose, sodium, phosphorus, calcium, chloride, ALT, ALP, total bilirubin, direct bilirubin, indirect bilirubin, CK and HDL-cholesterol) tended to be lowered and other serum concentrations (urea, total protein, globulin, potassium and creatinine) seemed to be increased in rams supplemented by HA; although, also serum triglyceride concentrations, AST and LDH activities showed a fluctuation (Table 4). There was only a significant difference in eosinophilia (%) levels (P<0.05) of hematological parameters among HA5 and HA10 with CG. The numbers of eosinophils in blood often rise above the normal range with allergic reactions and parasitic infections as with worms. We could think that humic acid is increasing the value of the eosinophil. However, the mechanisms explaining how humic acid supplementation could induce such modifications on eosinophil metabolism remain unknown and the occurrence of an eventual interest on nutritional status of ram requires further investigations.

### Conclusions

In conclusion, addition of humic acid had no significant effect on blood composition, ruminal fermentation and rumen protozoal populations except for ruminal sodium and rumen protozoa *Epidinium spp*. Particularly, after the addition of humic acid, the levels of sodium increased significantly (P<0.05).

#### Table 3. Species of rumen protozoa organism in rams fed humic acid supplemented diets.

| Species of rumen | Protozoa organism, % | Treatments | SEM |
|------------------|----------------------|------------|-----|
|                  | CG^®                  | HA5^©      | HA10^© |
| *Isotricha intestinalis* | 0° 3.33 | 0.00 | 0.00 | 0.27 |
| *Isotricha prostoma* | 3° 2.00 | 1.16 | 0.56 | 0.49 |
| *Daystricha ruminantium* | 68 | 0.00 | 0.00 | 0.00 |
| *Entodinium spp* | 3° 1.33 | 0.16 | 2.16 | 0.59 |
| *Epidinium spp* | 0 | 0.00 | 0.00 | 0.00 |
| *Diplodinium spp* | 6 | 0.00 | 0.33 | 0.50 | 0.36 |
| *Ophyroscolex caudatum* | 6 | 0.00 | 0.33 | 0.50 | 0.36 |

### Table 4. The biochemical and haematological parameters in rams fed humic acid supplemented diets.

| Biochemical variables | Treatments | SEM |
|-----------------------|------------|-----|
| **CG^®**              | HA5^©      | HA10^© |
| **Haematological variables** |          |      |
| Haematocrit, %        | 31.00      | 31.66 | 30.00 | 0.72 |
| Haemoglobin, g/dL     | 11.20      | 11.90 | 12.06 | 0.54 |
| Leukocytes, 10^9/L    | 4.53       | 6.03  | 4.56  | 0.66 |
| Erythrocytes, 10^13/L | 13.41      | 13.01 | 13.03 | 0.65 |
| MCV, fl               | 23.21      | 24.29 | 22.94 | 0.74 |
| MCH, pg               | 8.35       | 9.07  | 9.28  | 0.48 |
| MCHC, g/dL            | 35.94      | 37.15 | 40.69 | 1.89 |
| Neutrophils, %        | 30.33      | 43.66 | 45.00 | 11.95 |
| Lymphocytes, %        | 65.66      | 50.66 | 51.66 | 11.84 |
| Monocytes, %          | 2.66       | 2.00  | 1.00  | 0.98 |
| Eosinophils, %        | 1.00^b     | 2.66^b | 3.00a | 0.27 |
| Basophils, %          | 0.33       | 1.00  | 0.33  | 0.72 |

| Urea, mg/dL           | 59.33      | 58.66 | 61.33 | 2.17 |
| Total protein, g/dL   | 7.16       | 7.40  | 7.26  | 0.42 |
| Albumin, g/dL         | 1.86       | 1.80  | 1.73  | 0.14 |
| Globulin, g/dL        | 5.30       | 5.60  | 5.53  | 0.28 |
| A/G                   | 0.35       | 0.32  | 0.31  | 0.098 |
| Creatinin, mg/dL      | 0.66       | 0.66  | 0.70  | 0.27 |
| Total cholesterol, mg/dL | 61.00 | 59.33 | 57.00 | 3.86 |
| HDL cholesterol, mg/dL | 34.00 | 34.00 | 33.00 | 2.84 |
| Glucose, mg/dL        | 49.33      | 38.00 | 24.00 | 6.13 |
| Tryglycerides, mg/dL  | 11.66      | 17.33 | 11.00 | 4.10 |
| Sodium, mmol/L        | 144.66     | 143.66 | 141.66 | 2.05 |
| Potassium, mmol/L     | 5.40       | 6.10  | 5.53  | 0.52 |
| Phosphorus, mg/dL     | 8.23       | 8.13  | 7.50  | 1.25 |
| Calcium, mg/dL        | 10.03      | 9.73  | 9.60  | 0.52 |
| Chloride, mmol/L      | 106.33     | 105.33 | 104.66 | 0.54 |
| ALT U/L               | 14.00      | 14.00 | 13.33 | 0.72 |
| AST, U/L              | 157.33     | 112.00 | 265.33 | 104.23 |
| ALP U/L               | 210.66     | 169.33 | 169.33 | 7.09 |

^®Control basal diet; ^©5 g/day of humic acid with basal diet; ^©10 g/day of humic acid with basal diet; ^b,different superscripts show differences (P<0.05) among groups.
ban on antibiotic use in feeds as growth promoters and, because the cost of the humates is usually cheaper than just the other application costs and the concept of using humates as an alternative feed additive in animal nutrition has gained increasing importance, however, further work is needed to define more precisely the relationship among HA and blood composition and ruminal fermentation.

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