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SHORT COMMUNICATION

Rumen microbial fermentation, protozoan abundance and boron availability in yearling rams fed diets with different boron concentrations

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ABSTRACT. The objective of the *in vivo* study was to evaluate whether feeding graded levels of boron affect rumen microbial fermentation including pH, ammonia, volatile fatty acids and protozoa counts. In the experiment 4 Merino rams in a 4 × 4 Latin square design fed forage and concentrate with boric acid (0, 200, 300 and 400 mg · kg^{-1} in control, B1, B2 and B3 group, respectively) were used. Each experimental period lasted 14 days, with 12 first days of diet adaptation. In comparison with control diet, boron supplementation did not increased the total volatile fatty acid concentration before and 3 h after feeding. However in both time points, acetate content was higher in B1 and B2 than in control and B3 groups, whereas propionate content was lower in all boron-supplemented groups. The *iso*-butyrate, *n*-butyrate and *iso*-valerate levels were influenced only 3 h after feeding. The *n*-valerate content was lower in B1 and B2 than in control and B3 groups. Protozoan abundance in the rumen fluid was significantly higher in animals fed B3 and control diets both before and after feeding. The boron content in rumen fluid was increased in boron-supplemented groups to average value 7.32 ppm, but dose effect was not observed. The obtained results showed that dietary boron supplementation had a dose-dependent influence on rumen microbial fermentation and protozoan abundance in yearling rams. However, the boron concentration in rumen fluid did not increased simultaneously with increased dose in a diet. Further studies are needed to estimate the most recommended dose of boron in the ruminant diets and to better understand the boron role in the processes occurring in the rumen.

Introduction

Such trace minerals as selenium, zinc and cobalt, are essential for various physiological functions in animals, and their deficiency may cause many disorders (Rozbicka-Wieczorek et al., 2016). Boron is also an important element which plays a great role in mineral metabolism, enzymes and some hormones actions (WHO, 1998). Multiple studies have been carried out to evaluate the utilization of boron in animal diets. It was shown that boron affects a variety of metabolic functions such as some enzymes activity, energy and lipid metabolism (Devirian and Volpe, 2003; Tanaka and Fujiwara,

2008) and several haematological parameters in poultry (Yildiz et al., 2013; Çinar et al., 2015) and cattle (Kabu and Uyarlar, 2015), however, there is no information about the effects of boron on rumen fermentation and fauna such as bacteria and protozoa. The count of protozoa in rumen milieu is 10^5–$10^6 \cdot ml^{-1}$ rumen fluid, which constitutes a large proportion of the rumen fauna; however protozoa role is still an area of substantial controversy.

Some trace minerals such as molybdenum and copper form complexes with solid rumen digesta including bacteria and protozoa (Allen and Gawthornet, 1987; Spears, 2003). Having this in mind, we hypothesized that the boron as a trace mineral might improve rumen microbial fermentation and protozoa abundance. Therefore, the aim of *in vivo* trial was to evaluate the influence of different boron concentrations on rumen fermentation and to possibly elucidate the underlying mechanism of boron action.

Material and methods

Animals, diets, and experimental design

Four 12-month-old Merino rams weighting approximately 60 kg were fed four different experimental diets (control – basal diet with 0 mg $\cdot$ kg^{-1} boron, B1 – basal diet with 35 mg $\cdot$ kg^{-1} boron, B2 – basal diet with 52.5 mg $\cdot$ kg^{-1} boron, and B3 – basal diet with 70 mg $\cdot$ kg^{-1} boron) in a 4 × 4 Latin square design. Rams were fed lucerne pellets (500 g $\cdot$ d^{-1}), barley straw (400 g $\cdot$ d^{-1}) and concentrates (500 g $\cdot$ d^{-1}). Basal diet contained, %: maize 25, barley 24, soyabean meal 3, sunflower meal 12, rasmol 25, full fat soya 2, molasses 4, $CaCO_3$ 3, salt 1 and mineral-vitamin supplement 1 (Ekol A. S., Istanbul, Turkey) (mg $\cdot$ kg^{-1}: retinol 3 000, cholecalciferol 75 000, tocopherol 30 000, thiamine 980, niacin 99 500, biotin 20, Mn 50 000, Zn 50 000, Fe 50 000, Cu 10 000, I 8000, Co 200, Se 300, Mg 250). Boric acid (H_3BO_3; cat no. A0150965 032, Merck KGaA, Darmstadt, Germany) was used as a source of boron. Each animal was placed in an individual pen (area 24 m^2) with *ad libitum* access to water. The feed was provided twice a day (9:00 and 16:30) and residues were available until the next feed. The nutrient composition and energy content of raw ingredients of feed materials and experimental diets (Table 1) were analysed according to AOAC methods (1995), whereas the metabolic energy levels were calculated according to the Turkish Standards Institute (TSE, 1991). The experimental area and equipment were disinfected prior to the study, and anticoccidial drugs (oxane and sulfametine) were administered twice during the study. The room temperature was 10–24 °C, and ventilation was ensured by open doors and windows. All procedures were performed in accordance with the National Regulations for Animal Use in Research and approved by the local Ethics Committee of Ankara University for Animal Experiments (20071557).

Table 1. Chemical composition and energy contents of forages and experimental diets with or without boron

Indices	Forages		Experimental diet			
	lucerne	straw	control	B1	B2	B3
Dry matter, %	92.8	89.0	90.8	90.7	91.1	92.0
Organic matter, %	84.6	83.7	82.5	81.9	82.1	81.1
Crude protein, %	9.5	2.7	15.0	15.9	15.7	16.5
Crude fat, %	30.8	39.6	6.4	6.8	6.26	6.5
Acid detergent fibre, %	46.0	51.0	ND	ND	ND	ND
Ether extract, %	1.10	2.18	4.20	4.16	4.01	3.96
Metabolizable energy, kcal $\cdot$ kg^{-1}	1495	1168	2647	2616	2637	2595
Boron, mg $\cdot$ kg^{-1}	39.1[1]	23.9[1]	10.2	42.5	61.7	80.1

[1] values were reported by Serbester (2013); ND – not detected

Faeces, rumen and blood sampling

The experiment lasted 14 days and included 12 days of adaptation to the experimental diet and 2 days (days 13 and 14) of data collection. Rumen fluid was collected before (0 h) and 3 h after feeding (3 h) using a rumen probe. It was analysed for pH and ammonia concentration by using electrode. Protozoan abundance was measured as described by Ogimoto and Imai (1981) after fixation and staining with methyl-green formalin-saline solution (0.6 g $\cdot$ l^{-1} methyl green, 100 ml $\cdot$ l^{-1} 37% formaldehyde and 8.0 g $\cdot$ l^{-1} NaCl). A part of the rumen fluid was frozen at −20 °C to determine the concentration of volatile fatty acids (VFA). Rumen fluid was centrifuged at 2218 *g* for 15 min at 4 °C, and the supernatant was dissolved into two Eppendorf tubes with 1 ml met-phosphoric acid solution (100 g $\cdot$ l^{-1}). Subsequently, both tubes were centrifuged at 13150 *g* for 10 min at 4 °C, and the obtained supernatant was used to analyse VFA with use of VFA standard (Supelco Volatile Free Acids Mix 46975-U; Sigma-Aldrich, St. Louis, MO, USA) by a Shimadzu GC-2010 gas chromatograph (GC; Shimadzu Co., Kyoto, Japan). The GC was equipped with a flame ionization detector (FID) and a Teknokroma (TR-151035, TRB-FFAP 30 m x 0.53 mm x 0.50 µm; Teknokroma, Barcelona, Spain) capillary column. Temperatures of injector and detector were 170 and 190 °C, respectively. Helium was used as carrier gas with a flow rate of 1 ml $\cdot$ min^{-1}.

Faeces and blood were collected at day 14 and placed at −20 °C until further analysis. Samples were thawed in a microwave oven, and boron concentration was determined by a Dionex-3000 ion chromatography system (Thermo-Fischer Scientific, Waltham, MA, USA) as described by Vanatta et al. (1999).

Statistical analysis

Statistical analysis was performed using SPSS 14.01 (SPSS Inc., Chicago, IL, USA). Measurements from the same ram, collected at different times (0 h and 3 h), were considered as repeated measures in the analysis of variance (ANOVA). The effect of time × diet was not significant, so only diet effects within each sampling time were reported. Multiple comparisons among means were conducted using Tukey's post-hoc test. Differences were considered significant at $P < 0.05$.

Results

No significant difference was observed in dry matter intake (DMI) among the experimental groups, since there were no residuals between meals in any group. Thereby, total feed intake of rams was recorded as 1400 g · d^{-1} in this experiment.

The mean pH in rumen fluid was 6.86 and 6.19 before and 3 h after feeding, respectively, and tended to increase ($P = 0.059$) in B1 and B3 groups 3 h after feeding (Table 2).

The ammonia concentration in rumen fluid tended to be lower ($P = 0.076$) in B1, B2 and B3 in comparison with the control but also only 3 h after feeding (Table 2).

In comparison with control diet, boron supplementation did not increase the total VFA concentration in rumen fluid, however the difference was stated between B1 and B2, B3 groups before and 3 h after feeding ($P = 0.037$ and $P = 0.26$, respectively). The molar proportion of acetate was higher in B1 and B2 groups than in control and B3 groups ($P \leq 0.000$ for both time points), whereas the propionate proportion was the lowest in all three boron-supplemented group ($P \leq 0.000$ for both time points). The acetate:propionate ratio was the lowest in control group and the highest in B1 and B2 groups before feeding ($P \leq 0.000$). Three h after feeding no difference was observed between boron-supplemented groups (B1–B3) which have higher ratio than control group ($P \leq 0.000$). Only after feeding the *iso*-butyrate proportion in rumen fluid was increased in B1 and B3 in comparison with control and B2 groups after feeding ($P = 0.006$), and *n*-butyrate proportion was higher only in B3 group in comparison with control group ($P = 0.021$). After feeding the *iso*-valerate proportion in rumen fluid did not differ between control and boron-supplemented groups, but it was higher in B3 in comparison with B2 group ($P = 0.017$). There were statistical differences in the molar proportion of *n*-valerate between the experimental groups both before and after feeding

Table 2. Effects of diet supplementation with boron on rumen microbial fermentation in yearling rams

Indices	Experimental diet				Standard error	*P*-value[1]
	control	B1	B2	B3		
pH						
0 h	6.95	6.84	6.85	6.78	0.04	0.408
3 h	6.11	6.40	6.06	6.20	0.05	0.059
Ammonia (NH_3-N), mmol · l^{-1}						
0 h	401.25	283.75	376.88	363.75	10.07	0.145
3 h	227.50	170.00	161.88	180.63	19.19	0.076
Total volatile fatty acids (TVFA), mmol · l^{-1}						
0 h	57.94^{ab}	55.92^{a}	63.37^{ab}	74.79^{b}	2.69	0.037
3 h	96.12^{ab}	85.81^{a}	103.58^{b}	103.36^{b}	2.53	0.020
Molar proportions, % of TVFA						
acetate						
0 h	42.38^{a}	47.36^{b}	47.68^{b}	43.62^{a}	0.64	0.000
3 h	41.47^{a}	46.45^{b}	46.13^{b}	43.31^{a}	0.59	0.000
propionate						
0 h	27.82^{b}	22.18^{a}	22.20^{a}	23.06^{a}	0.69	0.000
3 h	34.03^{b}	26.51^{a}	27.52^{a}	26.58^{a}	0.88	0.000
iso-butyrate						
0 h	2.60	2.52	2.49	2.50	0.07	0.961
3 h	1.01^{a}	1.42^{b}	1.01^{a}	1.45^{b}	0.07	0.006
n-butyrate						
0 h	20.25	21.30	21.13	23.86	0.57	0.119
3 h	18.34^{a}	20.40^{ab}	20.72^{ab}	22.41^{b}	0.51	0.021
iso-valerate						
0 h	4.13	4.23	3.98	4.16	0.13	0.940
3 h	1.20^{ab}	1.85^{ab}	1.14^{a}	1.94^{b}	0.13	0.017
n-valerate						
0 h	2.44^{c}	2.05^{ab}	1.99^{a}	2.33^{cb}	0.06	0.002
3 h	3.51^{ab}	3.10^{a}	3.02^{a}	3.87^{b}	0.11	0.004
caproate						
0 h	0.42	0.38	0.54	0.43	0.04	0.499
3 h	0.56	0.30	0.46	0.46	0.08	0.737
Acetate/propionate ratio						
0 h	1.53^{a}	2.14^{c}	2.16^{c}	1.90^{b}	0.28	0.000
3 h	1.22^{a}	1.76^{b}	1.68^{b}	1.63^{b}	0.06	0.000
Protozoan abundance, × 10^3 · l^{-1}						
0 h	46.19^{a}	82.44^{ab}	76.88^{ab}	96.88^{b}	6.66	0.030
3 h	23.13^{a}	50.81^{ab}	40.81^{ab}	54.88^{b}	4.37	0.026

[1] *P*-value – according to one-way ANOVA test; ^{abc} – means with different superscripts within each row are significantly different at $P \leq 0.05$ according to Tuckey's post-hoc test

(P = 0.002 and P = 0.004, respectively). Before feeding the *n*-valerate proportion was lower in B1 and B2 groups than in control group, which did not differ from B3 group. There was also observed difference between B2 and B3 groups. After feeding the control group did not differ from other groups, but the *n*-valerate proportion was higher in B3 in comparison with B1 and B2 group. No change in the molar proportion of caproate was observed.

The obtained results showed (Table 2) significantly higher protozoan abundance in B3 group in comparison with control group before and after feeding (P = 0.03 and P = 0.26, respectively).

Table 3. Boron concentration in the blood serum and plasma, rumen fluid and faeces of yearling rams fed basal diet with or without boron, ppm

Indices	Experimental diet				Standard error	*P*-value[1]
	control	B1	B2	B3		
Serum	0.51[a]	6.81[b]	9.73[c]	11.25[d]	1.06	<0.001
Plasma	0.20[a]	6.91[b]	9.92[c]	10.29[d]	1.05	<0.001
Rumen fluid	2.48[a]	7.31[b]	7.36[b]	7.29[b]	0.54	<0.001
Faeces	8.17[a]	16.92[b]	18.17[b]	23.74[c]	1.52	<0.001

[1] *P*-value – according to one-way ANOVA test; [abcd] – means with different superscripts within each row are significantly different at $P \leq 0.05$ according to Tuckey's post-hoc test

The boron concentration in serum and plasma steadily increased with increased dose in the diet ($P \leq 0.000$ for both; Table 3). The rumen fluid concentration of boron reached the concentration of 7.31 ppm after the lowest boric acid dose was added into diet ($P \leq 0.000$), and the further increasing of the boron dose in the diet did not result in its simultaneous increase in rumen fluid. In the faeces the boron concentration was the highest in B3 group and the lowest in control group ($P \leq 0.000$).

Discussion

There is an increasing interest in enhancing the utilization of trace minerals, such as boron, in animal nutrition due to their great economic importance; however, information regarding their effects on rumen fermentation is limited (Yildiz et al., 2013; Sizmaz and Yildiz, 2014). This is the first study to report the effect of boron as a dietary supplement on rumen pH, ammonia and VFA concentrations in ruminants. Data obtained in the presented study clearly demonstrate that boron affects VFA formation, their profile and protozoan abundance in the rumen fluid of rams.

In the present study, rumen pH (6.06–6.40), which was optimal for microbial growth and activity and fibre digestion (Ørskov and Ryle, 1990), tended to increase in boron-supplemented groups 3 h after feeding. Lu et al. (2008) have shown that the optimum fibre content influences the cellulolytic activity in the rumen and enhances saliva production. Furthermore, the highest dose of boric acid supplementation (400 mg · kg^{-1} diet) increased *iso*-butyrate and *iso*-valerate content originated from dietary proteins or microbial proteins recycling by deamination and decarboxylation of some amino acids (Miltko et al., 2016) and tended to decrease ammonia concentration in comparison with control group 3 h after feeding. Theoretically, a higher production of ammonia due to the degradation and fermentation of proteins or peptides and also amino acids such as valine, leucine and isoleucine leads to higher concentrations of *iso*-butyrate and *iso*-valerate in the rumen (Mathieu et al., 1996). Additionally, *iso*-VFAs might increase digestibility of nutrients (especially dry matter) and microbial population in ruminal fluid (Miltko et al., 2016). This difference could be explained by amino acid degradation of dietary crude protein, since amino acids are protected by bacteria associated with rumen protozoa (Deckardt et al., 2016). In the present study, the increased protozoan abundance was observed after the highest dose of boric acid was added into diet, which could result in the reduction of ammonia utilizing bacteria. Since trace minerals form the complexes with protozoa and bacteria (Allen and Gawthornet, 1987), this form could be effective on bioactivity and metabolism by evaluating the degradation. However, further studies are needed to evaluate the effect of boron on the degradation of nutrients, such as fibre and crude protein, in rumen fluid both *in vitro* and *in vivo*.

In ruminants, forage-rich diets lead to acetate formation, whereas concentrate-rich diets – to propionate formation (Mc Donald et al., 2010). The basal diet used in the present study was a high forage ration, and any changes in VFA profile could be attributed to the interaction of lactate utilizing bacteria with acetate-propionate utilizing bacteria. Lactate is rapidly converted into propionate, *n*-butyrate, *n*-valerate and caproate in the rumen by lactate utilizing bacteria that use acetate as a co-factor, leading to diverse concentrations of acetate and propionate (Mao et al., 2008; Deckardt et al., 2016). Our data showed that the increasing concentration of boron altered the VFA profile in a dose-dependent manner. In comparison with control group the proportion of acetate increased in B1 and B2 groups, *n*-butyrate –

in B3 group, *iso*-butyrate – in B1 and B3 groups and *iso*-valerate – in B3 group, whereas the proportion of propionate was lower in all boron-supplemented groups and of *n*-valerate – in B1 and B2 groups. Our results also documented that the supplementation of boric acid to ram diets increased acetate-to-propionate ratio in comparison with the control diet. Because increasing of proportion of selected VFA, our investigation might have indicated that the boron from dietary boric acid supplementation can increase the fibre digestion and microbial growth, thereby could have increased the production of acetate coming from bacteria. Further studies are necessary to indicate the effects of boron on lipid metabolism such as fatty acid profile, cholesterol concentration in tissues, since acetate plays a major role in energy production as component of acetyl-coenzyme A (acetyl-CoA).

Our study showed that boron concentrations in the blood and faeces were increasing with the increasing level of boron in the diet. These results are in agreement with previous study of Çinar et al. (2015), who reported a significant linear increase in boron concentration in the blood serum of poultry fed diets supplemented with boron. Additionally, the amount of boron in the faeces increased with the increasing concentration of dietary boron in laying hens (Küçükyilmaz et al., 2014). Higher boron content in the faeces could be explained by its availability and the fact that it is not accumulated in any internal organ. The boron concentration in the rumen fluid was lower than that in the faeces, showing that the retention time of boron in the rumen is probably short, and that the mineral is rapidly absorbed by the gastrointestinal epithelia (Hunt et al., 1997). It should be stressed that in the present study boron concentration in rumen fluid reached the concentration of 7.31 ppm when the boric acid was added at a dose of 200 mg · kg^{-1} and the further dose increase did not resulted in simultaneous increase of boron content in rumen fluid.

Conclusions

Dietary boron supplementation had a dose-dependent influence on rumen microbial fermentation and protozoan abundance in yearling rams. However, the boron concentration in rumen fluid did not increased simultaneously with increased dose in a diet. Further studies are needed to estimate the most recommended dose of boron in the ruminant diet and to better understand the boron role in processes occurring in the rumen.

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