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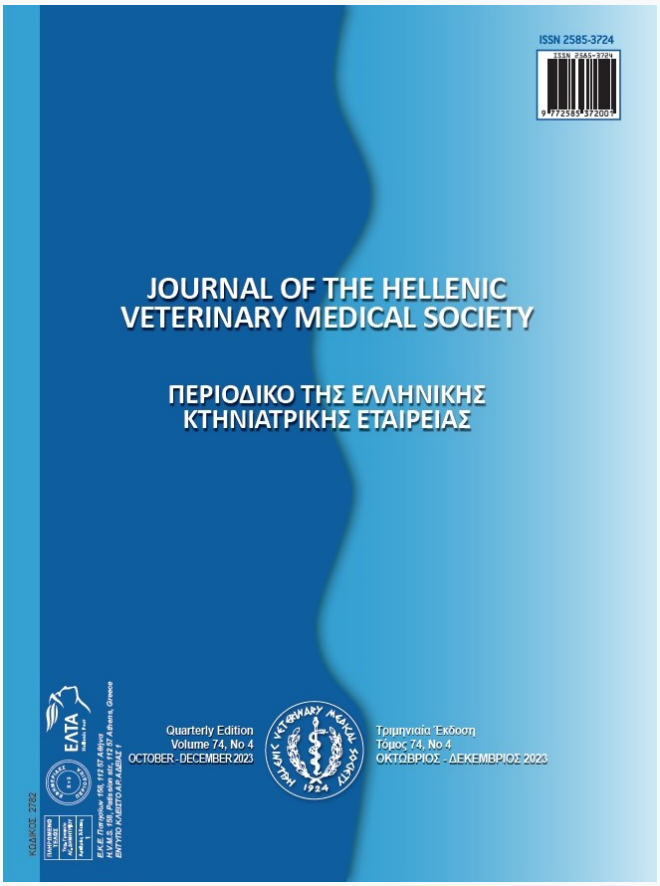

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Investigation of Boron Addition to Dried Alfalfa *In Vitro* Ruminal Profile and Potential for Reducing Enteric Methane Emission

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ABSTRACT: The aim of our study is to investigate the effects of increasing doses of boron on methane gas production amounts, short chain fatty acids level, protozoa number and organic matter digestibility in vitro with HFT (Hohenheim Futterwert Test) technique. In vitro incubation was performed in the Hohenheim Gas test method at 39°C for 24 hours in the study. Dried alfalfa was used as substrate for fermentation. Increasing doses of boron were used on fresh rumen fluid, buffer solution and dried alfalfa. In the study, 54 syringes were used for a total of 6 groups, including 1 control and 5 trial (B1: 25 ppm boric acid, B2: 50 ppm boric acid, B3: 100 ppm boric acid, B4: 200 ppm boric acid, B5: 500 ppm boric acid). Methane gas measurement was performed at 2, 4, 6, 8, 12 and 24 hours of boron addition at increasing doses under in vitro rumen conditions. In terms of time x group interaction, the difference between hours was found to be significant for each group($P<0,001$). However, when looked at for each hour, it was seen that the difference between the groups was significant only for the 24th hour ($P<0,001$). At the 24th hour of fermentation under in vitro rumen condition, acetic acid and total short chain fatty acid values were linearly and cubically affected. With increasing doses of boron, propionic acid, isobutyric acid, butyric acid and valeric acid values were linearly affected The total number of protozoa was not affected by the addition of increasing doses of boron at the 24th hour of fermentation under in vitro rumen conditions. Consequently, the addition of boric acid at increasing doses in in vitro rumen conditions decreased methane production and positively affected the amount of some short-chain fatty acids, organic matter digestibility and total short chain fatty acids. With this study, it can be said that boron has the potential to reduce enteric methane emission. In the light of these findings, it was emphasized that boron has the potential to reduce methane emissions from ruminant animals, considering the greenhouse gas effect.

***Keywords*:** Boron, *in vitro* rumen, methane, protozoa, short chain fatty acids

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INTRODUCTION

With the microbial digestion of the feed in the rumen, 2-15% of the net energy is converted into methane gas, which is not underestimated, and this gas is released into the atmosphere during rumination. What happens to the economic losses because this gas released cannot fully benefit from the content of the feeds. It poses an ecological problem as it contributes to global warming, which is a major problem for the continuation of life with the increase in emissions in the atmosphere (Kutlu and Özen, 2009). The direct contribution of animals in greenhouse gas emissions due to animal production is due to enteric fermentation. Various substances have been tried to reduce the formation of methane gas. One of the substances used for this purpose is methane inhibitors. Substances used for this purpose are halogenated methane analogs such as chloroform, chloralhydrate, aminochloral, trichloroacetamide, trichloroethyladipate, bromchloromethane, alpha-cyclodextrin, 2-bromoethane sulfonic acid and 9,10-anthraquinone. Their use is not easy in practice and may have negative effects on animal health. In addition, since methanogenic bacteria gain resistance to these substances, their effects do not last long (Moss et al., 2000; Mohammed et al., 2004).

Most feed additives used in ruminant feeding have been developed to improve nutrient use efficiency by reducing the amount of methane or ammonia nitrogen (NH_3-N) production. Ionophore antibiotics developed for this purpose have been very successful and have found a wide area of use (Cardozo et al., 2004). With the prohibition of the use of antibiotics as feed additives, researchers have turned to search for alternative natural products for the regulation of rumen fermentation. The feed additive to be used should both function as a methane inhibitor and positively affect the animal's performance and physiology. Alternative feed additives; probiotics, prebiotics, aromatic plants and boron mineral, which has recently attracted the attention of the scientific world.

Boron is one of the micronutrients (Sharma et al., 2022). Boron is an element that plays important roles in metabolism and is found in the mechanism of many diseases, including cancer. Boron, which has been found to be an essential trace element for humans and animals, has been reported to be effective in mineral metabolism, lipid metabolism and energy metabolism. In addition, it is thought that it has important functions in the brain together with the immune and endocrine system, affects performance positively, and can be effective in the prevention of osteoporosis, osteoarthritis and arthritis (Nielsen, 1997; Yeşilbağ, 2008). The antibacterial effect of boron is one of its most important properties in the field of health. Thanks to this feature, it is used in antibiotics, antiseptics, sterilization processes and antibacterial creams (Yılmaz 2012; Demirci et al. 2015a; Demirci et al. 2015b; Yakıncı and Kök, 2016).

The aim of our study is to investigate the effects of increasing doses of boric acid on methane gas production amounts, short chain fatty acids level, protozoa number and organic matter digestibility in vitro with HFT (Hohenheim Futterwert Test) technique.

MATERIALS AND METHODS

Experimental rations and chemical analysis

Dried alfalfa was used as substrate in fermenters to stimulate in vitro rumen fermentation and for the HFT method during the experiment (Menke et al., 1979). Alfalfa was supplied by a commercial farm. Dried alfalfa was used as substrate in fermenters to stimulate in vitro rumen fermentation and for the HFT method during the experiment (Menke et al., 1979). Alfalfa was supplied by a commercial farm. Nutrient analyzes of dried alfalfa grass used as substrate in in vitro fermentation were determined according to the procedures of AOAC (1990). Plant cell wall structures; acid detergent fiber (ADF) and neutral detergent fiber (NDF) were made by the method reported by Van Soest et al.(1991). Boron was obtained from a commercial company (Merk). In the study, 54 syringes were used for a total of 6 groups, including 1 control and 5 trial (C: Control basal ratio, B1: 25 ppm boric acid added to basal ratio, B2: 50 ppm boric acid added to basal ratio, B3: 100 ppm boric acid added to basal ratio, B4: 200 ppm boric acid added to basal ratio,B5: 500 ppm boron added to basal ratio) Three portions of sample were taken from each syringe for each analysis. The in vitro organic matter digestibility (IVOMD) value was determined with the technique described by Menke et al. (1979).

In vitro fermentation technique

In vitro incubation was performed in the Hohenheim Gas test method at 39°C for 24 hours. The rumen liquid needed in the study was obtained from the Ankara Çubuk Municipality slaughterhouse (the rumen liquid was delivered to the laboratory in a thermos as soon as possible after fresh cutting). The rumen fluid was mixed immediately and filtered into preheated syringes

in an incubator (39 °C) and filled with a buffer solution bubbled with CO_2. For each dose of boron to be added, rumen fluid (10 ml) and buffer solution (20 ml) were added to 200 mg of alfalfa, and 9 syringes which are 100 ml capacity and gas-tight were prepared for each dose. 25, 50, 100, 200 and 500 ppm doses were tried for increased boric acid levels. These doses of boron were added to each syringe and incubated, gas measurement at 24th hours, methane measurement at 2, 4, 6, 8, 12 and 24 hours (Steingass and Menke, 1986). The effect of these doses on methane production was investigated. Its effect on methane production was evaluated using in vitro gas production technique with HGT (Hohenheim Gas Production Test), an incubation medium with boron and rumen fluid added to the ration (alfalfa) (20 ml) was added.

Buffer solution:

Macro Element Solution: Na_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$; Micro Element Solution: CaCl2.2H2O, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $FeCl_3.6H_2O$; Buffer Solution:$NaHCO_3$, NH_4HCO_3; Resazurin Solution:Resazurin; Reductant Solution: $Na_2S.7H_2O$, NaOH

Sampling and analysis

After 24 hours at 39 °C, the syringes were removed from the incubator, placed in ice water to stop fermentation, and sampled for total gas, methane, total protozoa and SCFA (Short Chain Fatty Acids) determination. 2 ml of filtered mixed rumen fluid was immediately acidified with 0.2 ml of formic acid to prevent fermentation (as a 0-hour sample).Then, rumen fluid samples were collected from the syringes of each group and filtered into individual beakers with a sterile cheesecloth. Each rumen fluid sample was transferred to 5 ml tubes and stored at -20 °C for SCFA analysis.

At the end of the experiment, to determine the total number of protozoa in fermented liquids, rumen fluid was taken with MFS solution (1/9 mixed) (0.6 g methyl green, 8 g NaCl, 100 ml 37% formaldehyde) and stored until the reading stage (Tekeli et al., 2010). The protozoan count in the samples taken from the rumen fluid will be made using the Fuchs Rosenthal Lam and light microscope with the method determined by Ogimoto and Imai (1981). Total protozoa were counted under the microscope using counting chambers (0.1 mm and 0.02 mm depth, respectively).

For methan measurement, 10 ml of gas sample was taken from the gas formed in glass syringe type incubators at the end of the incubation. Methane was measured on the gas sample taken using a gas chromatography (GC) device (Shimadzu GC, Shimadzu Co., Kyoto, Japan), Supelco Carboxen-1010 PLOT Capillary GC Column (30 m x 0.32 mm) (Pellikaan et al. , 2011). Fluka methane standard was used for pre-analysis calibration.

SCFA concentration was determined by gas chromatography according to Geissler et al (1976). Frozen rumen samples were centrifuged at 4°C and rumen fluids were centrifuged at 4,000 rpm for 15 minutes at 4°C. One ml of the supernatant was then transferred to an Eppendorf tube and mixed with 0.2 mL of ice-cold 25% metaphosphoric acid solution. The tubes were then placed in an ice bath for 30 minutes. Then, these tubes were centrifuged again at 11,000 rpm for 10 minutes at 4°C and the supernatant was transferred to gas chromatography bottles. acetic acid, propionic acid, butyric acid determination, isobutyric acid, valeric acid and isovaleric acid concentrations. Samples were analyzed using gas chromatography (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with a capillary column (TR151035, TRB-FFAP, 30 m x 0.53 mm). During the analysis, the column temperature was programmed to increase gradually from 120ºC to 160ºC. In addition, the injector port and flame ionization detector (FID) temperatures were fixed at 230ºC and 250ºC, respectively. The injection volume was adjusted to 1 µL and analyzes were performed in two parallels.

Statistical analysis

The effect of group (1,2,3,4,5,6), sampling time (1, 2, 3, 4, 5, and 6) and group x sampling time interactions on methane and total gas measurement, using repeated measurements analyzed. When a significant difference was found, the term significance was compared with Bonferroni correction and simple effects analysis. One-way analysis of variance (ANOVA) method was used for acetic, propionic, isobutyric, butyric, isovaleric, valeric, total short chain fatty acids (SCFA), total protozoa and in vitro organic matter digestibility. Polynomial contrast test was performed to determine the dose effect of using different levels of boron in the groups. Statistical differences and trend analysis were considered significant at $P \leq 0.05$. Statistical analysis was performed with the SPSS software package (2011).

RESULTS

The nutrient component analyzes of dried alfalfa

used as a substrate in fermentation under in vitro rumen conditions are given in Table 1.

Methane gas measurement was performed at 2, 4, 6, 8, 12 and 24 hours of boron addition at increasing doses under in vitro rumen conditions. In terms of time x group interaction, the difference between hours was found to be significant for each group. However, when looked at for each hour, it was seen that the difference between the groups was significant only for the 24th hour. For the most part, the amount of methane produced increased as the measured time interval increased. When we look at the effect of the dose; It is possible to say that the effective dose for the 24th hour is 500 ppm, which is the highest dose (Table 2).

Table 3 shows the values of short chain fatty acids, total protosa numbers and in vitro organic matter digestibility at the 24th hour of boron addition at doses of 0, 25, 50, 100, 200 and 500 ppm under in

Table 1. Chemical composition of dried alfalfa

Chemical composition	
Dry matter, %	93,30
Crude protein, %	9,50
Crude ash, %	8,20
Ether extract, %	1,10
Acid detergent fiber, %	46,10
Neutral detergent fiber, %	63,03
ME/ kcal/kg	1515

Table 2. Influence of boron on methan production in vitro rumen environment (mmol/l).

Groups	Control	B1	B2	B3	B4	B5	SEM	Significance		
Time (Hours)	x̄	x̄	x̄	x̄	x̄	x̄		G	T	GxT
2	$23,27^{A}$	$21,33^{A}$	$23,33^{A}$	$23,11^{A}$	$24,0^{A}$	$20,00^{A}$	1,24	<0,001	0,121	<0,001
4	$37,45^{B}$	$43,11^{B}$	$37,11^{B}$	$35,33^{B}$	$37,77^{B}$	$32,88^{B}$	1,66			
6	$50,18^{C}$	$57,33^{C}$	$48,44^{C}$	$46,88^{C}$	$48,44^{C}$	$42,88^{C}$	2,07			
8	$56,72^{D}$	$68,22^{D}$	$55,55^{D}$	$53,55^{D}$	$55,77^{D}$	$50,00^{D}$	2,26			
12	$62,90^{E}$	$81,11^{E}$	$62,66^{E}$	$60,00^{E}$	$63,11^{E}$	$53,11^{E}$	2,78			
24	$76,36^{b,F}$	$121,11^{a,F}$	$75,77^{b,F}$	$74,00^{b,F}$	$75,55^{b,F}$	$63,11^{b,F}$	3,73			

C: Control basal ratio, B1: 25 ppm boric asid added to basal ratio, B2: 50 ppm boric asid added to basal ratio, B3: 100 ppm boric asid added to basal ratio,
B4: 200 ppm boric asid added to basal ratio, B5: 500 ppm boric asid added to basal ratio. The mean (x̄) and standard error mean (SEM) values of each group.
a-b or A-F: Means in the same row or column followed by different superscripts differ significantly (P <0.05).
Lowercase letters represent the difference between groups in each hour, and uppercase letters indicate the difference between hours in each group.
G= Effect of the trial groups; T= Effect of the relative to incubation time; G x T= The experimental groups by incubation time interaction.

Table 3. Influence of adding increasing doses of boron on some short chain fatty acid concentrations (mM/l), total protozoa (log_{10} cfu/ml) and organic matter digestibility (%, DM) at the 24th hour of the experimentunder in vitro rumen conditions.

Groups	Control	B1	B2	B3	B4	B5	SEM	Significance		
SCFAParameters	x̄	x̄	x̄	x̄	x̄	x̄		L	Q	C
Acetic acid	35,95	33,43	33,56	45,49	55,03	44,17	2,05	**0,007**	0,930	**0,016**
Propionic acid	9,15	8,44	8,51	11,19	12,66	11,79	0,50	**0,009**	0,678	0,098
Isobutyric acid	0,85	0,74	0,70	1,00	1,09	1,39	0,07	**0,011**	0,134	0,694
Butyric acid	10,16	9,85	8,38	12,82	14,22	15,50	0,76	**0,007**	0,269	0,379
Isovaleric acid	1,50	1,30	1,16	1,54	1,70	1,83	0,08	0,103	0,173	0,367
Valeric asid	1,05	0,98	0,93	1,18	1,27	1,31	0,05	**0,029**	0,358	0,290
Caproic acid	0,30	0,30	0,35	0,31	0,32	0,33	0,01	0,503	0,686	,0672
Total SCFA	58,99	55,07	53,62	73,54	86,33	79,34	3,31	**0,005**	0,723	0,046
Total protozoa	6,16	7,5	6,72	8,00	5,61	5,5	0,36	0,298	0,094	0,687
IVOMD	56,71	58,31	57,42	54,76	54,59	58,66	0,64	0,753	0,288	**0,032**

C: Control basal ratio, B1: 25 ppm boric asid added to basal ratio, B2: 50 ppm boric asid addedto basal ratio,
B3: 100 ppm boric asid added to basal ratio, B4: 200 ppm boric asid added to basal ratio, B5: 500 ppm boric asid added to basal ratio.
Polynomial contrasts: L = linear, Q = quadratic, and C = cubic effect of supplemental probiotic and enzyme.
SVFA:Short Chain Fatty Acids.
IVOMD: In vitro organic matter digestibility
DM: Dry matter
The mean (x̄) and standard error mean (SEM) values of each group.

vitro rumen conditions. Acetic acid and total volatile fatty acid values were linearly and cubically affected (P=0.007, P=0.016; P=0.005, P=0.046, respectively). The effective dose for acetic acid and total short chain fatty acid is B4 (200 ppm). With increasing doses of boron, propionic acid, isobutyric acid, butyric acid and valeric acid values were linearly affected (P=0.009, P=0.011, P=0.007, P=0.029, respectively). The total number of protozoa was not affected by the addition of increasing doses of boron. At the end of the experiment, in vitro organic matter digestibility value exhibited a cubic response (P=0,032). In vitro organic matter digestibility value was found to be higher at the lowest and highest dose.

DISCUSSION

Today, reducing methane production comes to the forefront on the basis of research on rumen metabolism. Ruminants are one of the most important factors in global climate changes (Steinfeld et al. 2006). Production of methane gas in ruminates reduction makes significant contributions to the environment and economy. For this reason, intensive studies have been carried out in recent years to reduce enteric methane production in ruminants. In vitro gas production technique is one of the most intensive methods of these studies. Feed additives have different mechanisms of action to reduce enteric CH_4 production (eg inhibition of protozoa, stimulation of propionate, reduction of hydrogen production, direct inhibition of methanogens). The efficacy of these feed additives may be strongly linked to ruminant fermentation conditions caused by pH modification components (Castro-Montoya et al., 2012).However, interactions between feed ingredients and feed additives used as substrates in the basal diet have often been poorly described in previous studies. Saponins not only inhibit cellulolytic activity, but also increase amylolytic activity, resulting in decreased H_2-CH_4 formation(Castro-Montoya et al., 2012). For this reason, we preferred alfalfa as a substrate in the *in vitro* rumen environment due to its saponin content in our study. Thus, we observed the synergistic effect of boron's potential to reduce CH_4 formation together with the saponin contained in alfalfa. In our study, boron, which has the potential to reduce CH_4, was investigated in vitro together with the feedstuff used. Methane gas measurement was performed at 2, 4, 6, 8, 12 and 24 hours of boron addition at increasing doses under in vitro rumen conditions. In terms of time x group interaction, the difference between hours was found to be significant for each group. However, when looked at for each hour, it was seen that the difference between the groups was significant only for the 24th hour. The effective dose for the 24th hour was500 ppm. Boron (B) is defined as a beneficial *micronutrient* for some farm animals (Sharma et al., 2022).The role of micronutrients in rumen fermentation and digestion cannot be underestimated, as they can form complexes with solid rumen digestion, including trace minerals, bacteria and protozoa, affecting feed utilization in ruminant animals (Spears, 2003). However, the potential for boron to reduce enteric methane formation in ruminants is unclear. The number of articles in vitro and in vivo, which is studied on this effect of boron, are limited. In our article, we discussed firstly according to alfalfa used as substrate in ruminal fermentation and secondly, boron feed additive used to reduce methane formation. Firstly, in this context, the composition of the feed given to ruminants significantly affects methane production. With the adjustments made in the feed composition, methane production can be reduced by up to 90%. When ruminants are fed with poor quality roughage that is poor in vitamins, minerals, protein and energy, fermentation performance decreases, thereby increasing methane production. However, if vitamin, mineral and nitrogen sources that increase the fermentation performance are added to these feeds, there will be a decrease in methane production due to the increased fermentation performance. Choosing alfalfa instead of straw reduces methane production by 33% (Haque et al., 2001). Therefore, one of the minerals used to increase the fermentation performance can be boron. As a matter of fact, this information supports our study.In an *in vitro* rumen study, the use of B mineral at a dose of 300 ppm reduced cellulose digestion (Martinezand Church, 1970). In an in vitro rumen study in which zeolite and a plant extract were added to alfalfa grass in vitro, Ece and Avcı (2018) reported that the use of high doses reduced the production of CH_4 methane gas. Secondly, in this context, Sharma et al. (2022) reported that they used increasing doses (200 to 400 ppm) of B in the pregnant buffalo diet and had no effect on N use and N balance in the postpartum phase. In a study in which increasing doses (0, 200, 300 and 400 mg/kg) of boron were added to the ration of Merino rams, the ammonia concentration in the rumen fluid tended to be lower in all boron-treated groups compared to the control (Sızmaz et al 2017). Kabu and Civelek (2012) reported that sodium borate ($Na_2B_4O_7$ $5H_2$, 30 g/day) administered orally to 12 pregnant cows did not affect blood urea nitrogen

(BUN) levels.

Borax administration to Austrian Simmental (Fleckvieh) cows increased blood urea nitrogen (BUN) concentrations (Kabu and Uyarlar, 2012). In a study using zeolite, bentonite and sepiolite clay group minerals in in vitro ruminal fermentation, rumen ammonia concentration decreased. The use of zeolite stabilized the ammonia concentration in the early stages of fermentation. It has been observed that the buffering effect of bentonite and sepiolite is less and their use together reduces microbial fermentation depending on the concentrate feed rate (Amanzougarene et al., 2022). Due to the complexity of the rumen microbial ecosystem, the available evidence for the effects of boron supplementation in ruminant animals is extremely limited. Therefore, more in vitro research is needed to determine whether boron has positive effects on rumen fermentation in ruminants.

Saponins suppress methane production by reducing the number of rumen protozoa or methanogens. Having high antioxidant properties, alfalfa is rich in antinutritional factors such as cellulose, saponin, β-glucan and xylan (Mutlu and Yıldız, 2020). Our study investigated whether there is a significant change in the number of protozoa and methane gas value of saponin by using alfalfa as a substrate in in vitro ruminal fermentation. In our study, the total number of protozoa was affected by the addition of increasing doses of boron. The presence of protozoa in Merino rams fed with different boron concentrations (0, 200, 300 and 400 ppm) increased in other groups compared to the control group and high dose group (Sızmaz et al., 2017). The in vitro organic matter digestibility value was affected by the addition of boron. In another study using five different roughages as substrates in the in vitro gas production technique, the IVOMD value was significantly increased (Sallam et al., 2007). In an in vitro study using alfalfa hay with acorn and zeolite, IVOMD was increased (Ece and Avcı, 2018).More in vitro research is needed to determine whether boron has positive effects on ruminal profile in in vitro fermentation in ruminants.

Substances such as malate, fumarate and acrylate increase propionate production, and their mode of action is similar to ionophore antibiotics. These are propionate precursors and they cause a decrease in methane formation by enabling the use of hydrogen in propionate production (Martin, 1998). When we look at reductive acetogenesis, here hydrogen ions are used by acetogenic bacteria for the formation of acetic acid. This metabolic pathway has been detected so far in termites, the large intestine of humans, rabbits, pigs, and the rumen of newborn lambs (Breznack and Kene 1990). Adult ruminants also have reductive acetogenic bacteria in their rumen. However, they only convert glucose, and cellobiose into acetic acid (Greening and Leedle, 1989). There is a very important correlation between SCFA and gas production in the rumen. Gas is mainly produced when carbohydrates in feedstuffs are fermented to acetate and butyrate by fermentation of propionate, which only gives gas from buffering of acid. Therefore, feeds that produce higher amounts of propionate produce lower gas volumes (Blümmel and Ørskov, 1993).In our study, acetic acid and total short chain fatty acid values were linearly and cubically affected. The effective dose for acetic acid and total short chain fatty acid is B4 (200 ppm). With increasing doses of boron, propionic acid, isobutyric acid, butyric acid and valeric acid values were linearly affected. The number of articles investigating SCFA data on the use of boron as a feed ingredient or feed additive in in vitro rumen fermentation is either absent or limited. Therefore, this part of the discussion will discuss SCFA values over different minerals or feedstuffs. In a study investigating the differences in in vitro and in vivo ruminal fermentation of different feed ingredients in ruminants, acetate, propionate, valerate, butyrate, and total SCFA values showed a significant increase in in vitro and in vivo fermentation (Brown wt al., 2002). In another study using five different roughages as substrate in in vitro gas production technique, SCFA value was significantly affected (Sallam et al., 2007). In a study investigating the effect of boron supplementation on the performance of peripartum Murrah Buffaloes, plasma non esterified fatty acids (NEFA) were decreased (Sharma et al., 2022). In an in vivo study, Merino rams fed different boron concentrations (0, 200, 300 and 400 ppm) did not increase the total VFA concentration in the rumen fluid in the other groups compared to a control group (Sızmaz et al., 2017). The use of the clay mineral palygorskite to investigate the effect on rumen fermentation in an in vitro gas production system increased acetate and total volatile fatty acids concentrations (Zeng et al., 2017). These studies support our study. Many current studies are needed to understand the effect of SCFA value with boron addition in in vitro gas production technique.

CONCLUSION

In vitro gas production technique shows that us-

ing alfalfa as substrate in fermentation and different doses of boron as feed additive can improve ruminal microbial fermentation, promote SCFA formation and nitrogen utilization. With this study, it can be said that boron has the potential to reduce enteric methane emission. However, current in vitro and in vivo studies are needed to understand whether boron has an effect on many parameters, especially on enteric methane emission.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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