

EFFECTS OF BORON SUPPLEMENTATION ON DAIRY CALVES' HEALTH: A METABOLOMICS STUDY

Running Title: Boron affects calves' health: A metabolomics evaluation

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ABSTRACT

Boron supplementation has been demonstrated to exert many beneficial effects on animals. The first metabolomic investigation into the effects of boron on dairy calf health is covered in this study. For two months, healthy newborn calves were enrolled in one control group and three experimental groups. Boron was added to the milk of the experimental groups in increasing doses. Biochemical profile, Nuclear Magnetic Resonance (NMR) metabolomic profiles on days 0, 30, and 60, and daily health scores were determined. Worsening of health score parameters was more common in the control calves, and they were prone to getting sick. Enzyme increases due to birth stress were remarkable. Significant increases in the passive colostral adequacy parameter γ -glutamyl transferase were also not maintained with boron addition. A total of 33 water-soluble and 17 lipid-soluble serum metabolites were determined. Increases in glucose, fructose, alanine, cholate, betaine, and 3-hydroxybutyrate levels, and decreases in lactate, isovaleric acid, valine, leucine, tyrosine, and 2-hydroxybutyrate levels were observed. Phosphoglyceride levels were increased, while the levels of different cholesterol types were found to be decreased. From our data, it emerged that better growth performance, increased gluconeogenesis, and liver development were associated with boron supplementation. To interpret the effects of boron supplementation on the health of dairy calves, NMR-based metabolomic assessment has outperformed biochemical analysis.

Keywords: Boron, Health profile, Metabolomics, Neonatal calf

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INTRODUCTION

The mineral element boron is widely distributed and naturally occurs in combination with oxygen in freshwater, saltwater, rocks, and soil. Boron is essential for plants and, in recent studies, it has been determined to be essential for humans and animals as well (Khalig *et al.*, 2018). A wide variety of inorganic borates are found throughout nature in low concentrations and are employed for both industrial and domestic purposes. Limited but valuable evidence has been established by more than 50 years' worth of reports on toxicity to direct the appropriate boron applications (Bulakhova and Ray, 2023).

Borate supplementation has been demonstrated to exert many beneficial effects on animals (Abdelnour *et al.*, 2018, Kabu *et al.*, 2015). Moreover, in adult humans, it has been demonstrated that boron improves calcium retention (Nielsen, 2008). and increases osteoblast production activity (Nielsen *et al.*, 1987). increases the amount of vitamin D that remains in circulation, improves Mg absorption (Nielsen *et al.*, 1990) and lowers the levels of CRP and TNF- α (Jones *et al.*, 2012; Naghii *et al.*, 2011). While boron-focused drug design is still a relatively new field, current discoveries and applications have demonstrated boron as a viable candidate with considerable potential for future development (Messner *et al.*, 2022). In our previous study, we demonstrated an improvement in energy metabolism in peripartum dairy cows with oral boron supplementation (Basoglu *et al.*, 2017). Boron is characterized as a hypolipidemic agent on its lipid profile (Kan and Kucukkurt, 2023).

Boric acid supplementation up to a dose equivalent to 200 ppm B has an optimum effect in growing calves and further, supplementation of supranutritional levels of boric acid equivalent to 600 ppm B had neither harmful health nor any additional beneficial effect on calves' bone health (Singh *et al.*, 2021) During the neonatal period, calves are at high risk of suffering from various diseases (Singh *et al.*, 2009). Due to delayed genetic progress, fewer replacements available for voluntary culling of the lactating herd, and increased cost of replacement, neonatal calf diseases are the primary causes of economic losses, particularly in dairy herds. Studying the effect of boron supplementation in neonatal calves could be important to improve dairy calf health and survival, enhance dairy heifer long-term performance, and satisfy consumer interests in farm animal welfare (Lorenz, 2021).

Metabolomics is a powerful research tool that has the potential and ability to improve understanding of the relationship between dietary intake patterns, nutrients, physical activity, and health status (Chowdhury *et al.*, 2023). Metabolomics is concerned with the identification, quantification, and characterization of the comprehensive ensemble of endogenous and exogenous metabolites present in biological specimens (van Niekerk *et al.*, 2021; Vignoli *et al.*, 2019). Metabolomics is a rapidly evolving science and has many applications in neonatal medicine (Noto *et al.*, 2016). Calves with sepsis and bronchopneumonia were the subjects of our previous metabolomics studies (Basoglu *et al.*, 2014, 2016, 2018). Metabolomics, although in its early stages in veterinary research, is a promising tool regarding diagnosis, biomarker dis-

covery, and uncovering new insights into disease pathophysiology (Tran *et al.*, 2020). Post-genomic technologies are becoming more accepted in veterinary studies, but due to many problems related to their feasibility, cost, and practicality the use of these technologies in practice is still limited (Marcato *et al.*, 2018).

Prematurity, perinatal asphyxia, and diarrhea, neonatal sepsis are diseases associated with particularly high morbidity and mortality, and metabolomics may provide unique insight into these conditions (Basoglu *et al.*, 2020; Huang *et al.*, 2020; Noto *et al.*, 2016).

In light of the numerous potential health-related functions of boron supplementation, but the lack of comprehensive neonatal studies, we designed this study to evaluate the effects of boron supplementation on the health of dairy calves via NMR-based metabolomics.

MATERIALS AND METHODS

Animals and Boron Supplementation

The Committee on the Use of Animals in Research of Selcuk University's Faculty of Veterinary Medicine approved the experimental design (Approval No: 2021/86).

In the study, 28 (1 day-old, weight: ≥ 40 kg) Holstein male calves were used. The calves were randomly split into four groups (one control and three experimental groups, each with seven calves). Colostrum, milk, drinking water, and calf starter diets were provided to the calves (Table 1). For 60 days, the milk of three of the experimental groups was supplemented with 15, 30, and 45 mg of boron

(135, 265, and 400 mg of borax, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) each day.

Calf Health Score

Nasal discharge, ocular discharge, ear position or head carriage, appetite, attitude, coughing, rectal temperature, fecal consistency, naval characteristics, and joint appearance were the clinical parameters considered in the health score. As the clinical sign progresses from normal to very abnormal, assigned scores range from 0 to 3 (Table 2) (Kara, 2020).

Serum Biochemistry

Jugular vein puncture was used to obtain blood samples from calves on days 0 (20th h after birth), 30, and 60 d of the trial into tubes without anticoagulant for biochemical and NMR analyses. Serum samples were obtained by centrifugation at 5000 rpm for 20 min after coagulation of non-anticoagulant blood samples and stored at -80°C until analysis.

Total protein, albumin, glucose, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine phosphate kinase (CPK), γ -glutamyl transferase (GGT), Ca, Mg, and P were all measured in serum samples using standard spectrometric methods.

The statistical software SPSS 25.00 (IBM for Windows) was used for the analysis. For every set of data, p -values < 0.05 were considered statistically significant. The Kolmogorov-Smirnov test was used to examine the data's normality (null hypothesis) and revealed a non-parametric distribution. The data were presented as the median (minus the max-

imum). The groups were compared using the one-way ANOVA test.

NMR analysis

Samples preparation for ¹H-NMR spectroscopy

Sample extraction was performed at Selcuk University, Faculty of Veterinary Medicine, Konya, Turkey (Stringer *et al.*, 2016) and NMR measurements were executed at the CERM/CIRMMP facility of the ESFRI Institute at the University of Florence (Florence), Italy.

The dried water-soluble serum samples were dissolved in 700 μ L of ²H₂O (99.9 atom % D, Sigma Aldrich, St. Louis, Missouri, USA), then samples were homogenized by whirling for 1 min. A total of 70 μ L of potassium phosphate buffer (pH 7.4; 1.5 M K₂HPO₄, 100% (v/v) ²H₂O, 2 mM NaN₃, 5.8 mM deuterated 3-(Trimethylsilyl) propionic-2,2,3,3-d₄ acid (TMSP) were added to 630 μ L of each sample. For the NMR analysis, 600 μ L of each mixture was put into 5 mm NMR tubes (Bruker BioSpin s.r.l.).

The lipid serum dried extracts were dissolved in 700 μ L of Deuterated chloroform, CDCl₃, (99.8 atom % D, Sigma Aldrich, St. Louis, Missouri, USA), and vortexed for 1 min to homogenize the mixture. For the analysis, an aliquot of 600 μ L from each sample was put into 5 mm NMR tubes (Bruker BioSpin s.r.l.).

A 600 MHz spectrometer (Bruker BioSpin s.r.l.; Rheinstetten, Germany) operating at 600.13 MHz proton Larmor frequency was used to obtain one-dimensional ¹H-NMR spectra for each sample. Each sample was held within the NMR probe head for at least 5 min to reach the equilibrium temperature at 310

K prior to starting the measurements. The Nuclear Overhauser Effect Spectroscopy (NOESY)-pulse sequence (NOESY 1Dpresat; noesygprr1d.com; Bruker BioSpin) was used to acquire a one-dimensional ¹H-NMR spectrum for each water- and lipid-soluble sample using 98,304 data points, a spectral width of 18,028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, a mixing time of 0.01 s and 64 scans.

For details regarding instrument configuration, and setting we refer the readers to our previous publications (Basoglu *et al.*, 2016).

Spectral processing

Before the use of the Fourier transform the free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor. Transformed spectra were automatically corrected for baseline and phase distortions through TopSpin 3.6.2 (Bruker BioSpin software). All spectra of serum water-soluble extracts were calibrated to the anomeric glucose doublet at δ 5.24 ppm, whereas lipid-soluble samples were aligned to the chloroform singlet at δ 7.20 ppm. Prior to pattern recognition, total integral normalization was applied to the spectra.

Statistical analysis

All data analyses were performed in the “R” statistical environment (R Core Team, 2014). Metabolites, whose signals in the NOESY spectra of serum water-soluble extracts were well defined and resolved, were assigned using the Bovine Metabolome Database (Foroutan *et al.*, 2020), an ¹H NMR spectra library of pure organic compounds (BBIOREF-CODE, Bruker BioSpin), and literature

when available. A total of 33 metabolites were unambiguously assigned and quantified in all spectra.

Assignments of NMR signals in the NOESY spectra of serum lipid-soluble extracts were based on the literature (Oostendorp *et al.*, 2006) and the Bovine Metabolome Database (Forountan *et al.*, 2020). A total of 17 lipid features were assigned and quantified in all spectra.

The relative quantification of the metabolites and the lipid features (concentrations in arbitrary units) was performed by integrating NMR peaks using an R script, in-house developed, based on standard line-shape analysis methods.

The non-parametric Wilcoxon Rank-Sum test was used to infer differences between the four groups (control, and experimentals 1, 2, 3) at the three-time points (day 0, 30, 60). Whereas the non-parametric Wilcoxon signed-rank test was used to investigate pairwise differences between the three-time points within each group of interest (control, and experimentals 1, 2, 3). In this pilot study, we did not adjust our analysis for multiple testing to reduce the risk of missing promising biomarkers, but this also increased our risk of a type I error.

RESULTS

Health Score

Considering the health score parameters, the tendency to be sick was higher in the control group calves. In the experimental groups, growth performance and the lowest score parameters were dominant, recorded disease events were typically brief and mild, and the calves were generally in good health. However, neonatal

calf diarrhea was diagnosed in three of the control calves during their first week of life (with a fecal score of 2), and bovine respiratory disease was diagnosed in two of the control calves at one month of age (with a cough and nose score of 3). The risk of a health event was the same for all boron treatment groups.

Chemistry Profile

As compared with all the other time points, some parameters (T bilirubin, T protein, Ca, and P) on day 0 showed higher levels in the control group. The most notable increases were in GGT levels in serum samples taken on day 0; GGT levels returned to their normal values on days 30 and 60 in all the groups. Other enzymes' levels were increased on day 0 in all the groups. Although some differences in other biochemical parameters (BUN, creatinine, Mg, albumin, and ALT) were observed, they were within reference limits (Figure 1, Supplemental Tables 1, 2, and 3).

NMR-Based Metabolomic Analysis

Serum water-soluble extracts

A total of 33 metabolites were quantified in all NOESY spectra of water-soluble extracts (Supplemental Table 4).

Univariate analysis of the metabolites' variations between the four groups (inter-individual variability) at the three-time points (Figure 2, Supplemental Table 5) was performed: the control group seems the most differentiated at each of the three-time points. The analysis of the variations between the three-time points (intraindividual variability) in each of the four groups (Supplemental Table 6) pointed out several statistically significant variations in all groups ($p < 0.05$).

Serum lipid-soluble extracts

A total of 17 lipid signals were quantified in all NOESY spectra of lipid-soluble extracts (Figure 3 and Supplemental Table 4). Univariate analysis of the lipid variations between the four groups at the three-time points (Supplemental Table 7) was performed: the control group seems the most differentiated and the differ-

ences were more marked on day 60. The analysis of the variations between three-time points in each of the four groups (Supplemental Table 8) pointed out several statistically significant variations ($p < 0.05$). For all the four groups the day 0 point showed to be the most differentiated with respect to both days 30 and 60.

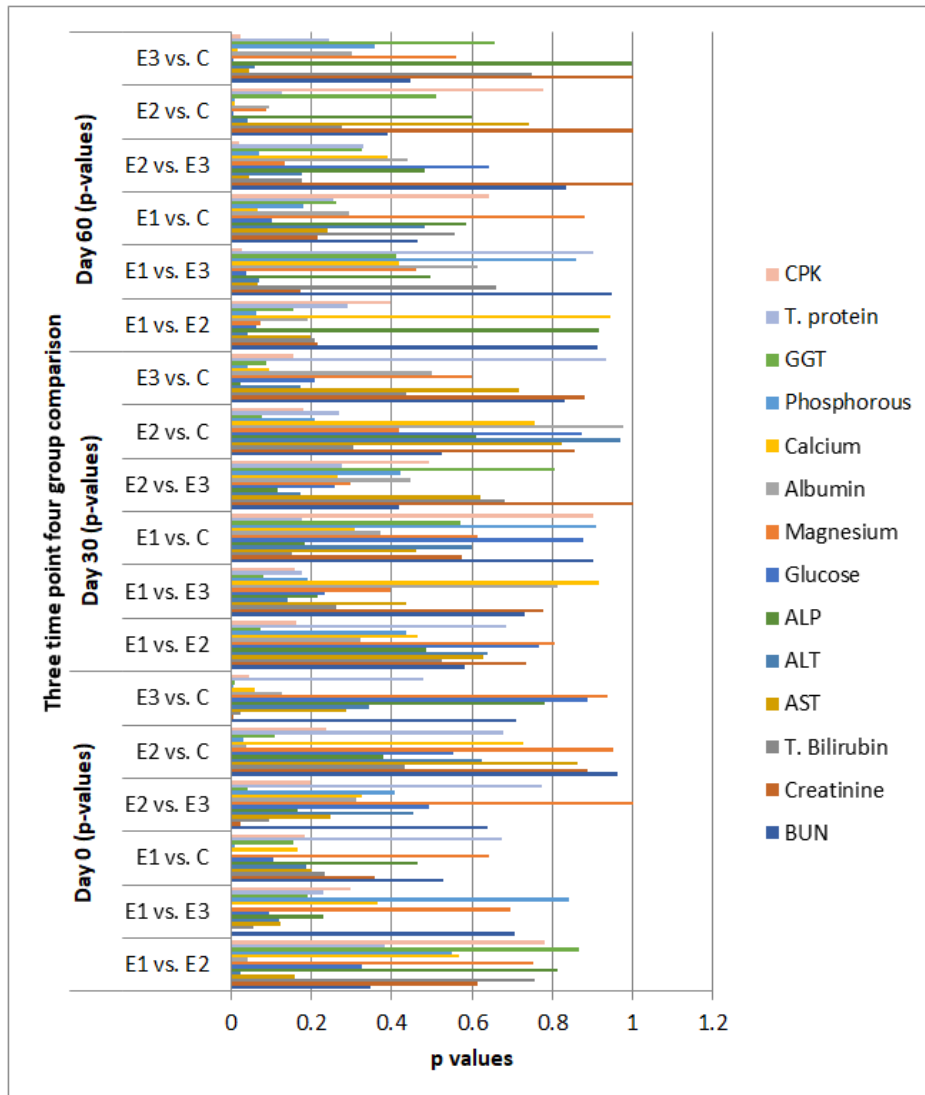


Fig. 1: Bar chart of the serum biochemistry parameters’ variations between the four groups at the three-time points (univariate analysis), E1: Experimental group 1, E2: Experimental group 2, E3: Experimental group 3, C: Controls.

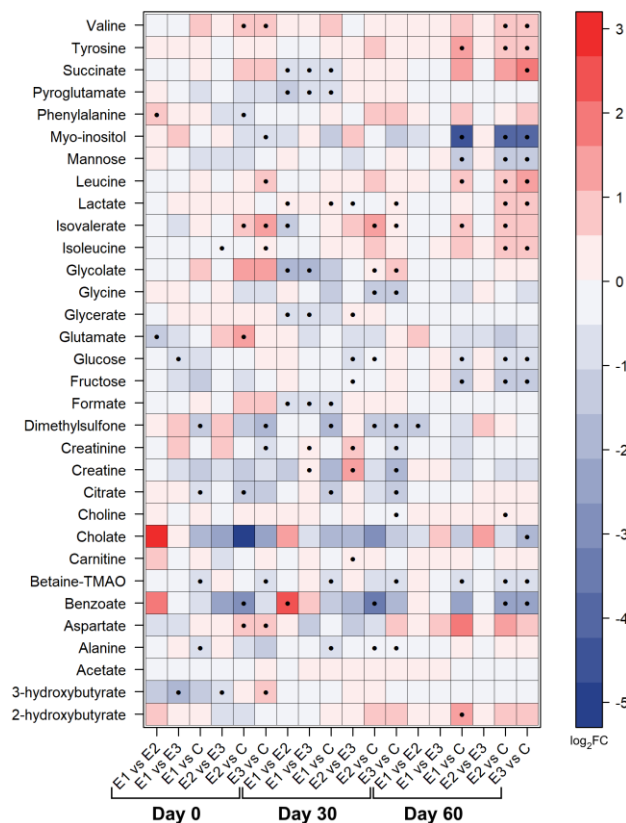


Fig. 2: Heatmap of the quantified metabolites identified by 1H NMR in serum water-soluble extracts (relative concentrations) for the comparison among experimental groups and controls at the three-time points (day 0, day 30, day 60). Colors indicate the Log₂ of the fold change (FC) value (right-side bar). Dots have been used to depict the significant comparisons (Wilcoxon test, *p*-value ≤ 0.05). E1: Experimental group 1, E2: Experimental group 2, E3: Experimental group 3, C: Controls.

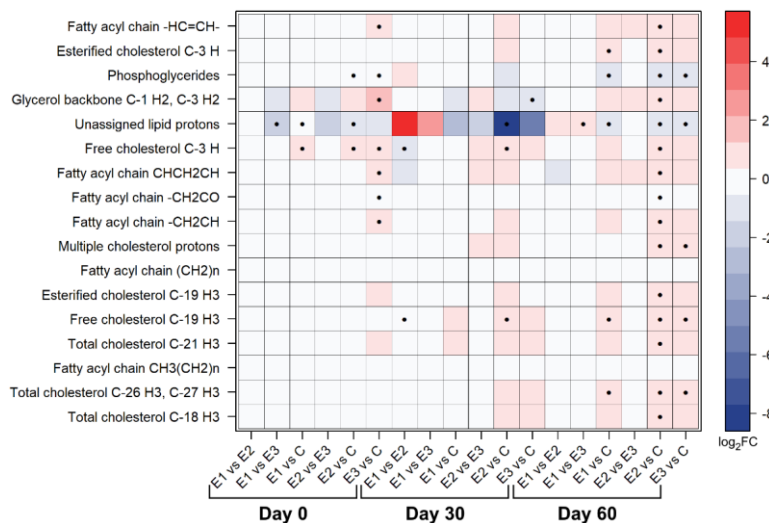


Fig. 3: Heatmap of the quantified metabolites identified by 1H NMR in serum lipid-soluble extracts (relative concentrations) for the comparison among experimental groups and controls at the three-time points (day 0, day 30, day 60). Colors indicate the Log₂ of the fold change (FC) value (right-side bar). Dots have been used to depict the significant comparisons (Wilcoxon test, *p*-value ≤ 0.05). E1: Experimental group 1, E2: Experimental group 2, E3: Experimental group 3, C: Controls.

Table 1: Calves' feeding diet.

| | |
|------------------|---|
| Day 1 | Colostrum: 2 L within the first hour following parturition, 1 L 6 h later, 1 L 12 h later |
| Days 2-3 | Colostrum: two meals 3 L/d |
| Days 4-7 | Normal milk: two meals 3 L/d Calf starter feed, ad libitum Water, ad libitum |
| Days 8-60 | Normal milk: two meals 4 L/d Calf starter feed, ad libitum Water, ad libitum |

Table 2: Calf health scoring chart^a.

| Scoring criteria | 0 | 1 | 2 | 3 |
|-------------------------|-------------------------|---|---|--|
| Cough score | None | Induce single cough | Induced repeated or occasional spontaneous coughs | Repeated spontaneous coughs |
| Nose score | Normal serous discharge | Small amount of unilateral cloudy discharge | Bilateral cloudy or excessive mucus discharge | Copious bilateral mucopurulent discharge |
| Eye score | Normal | Small amount of ocular discharge | Moderate amount of bilateral discharge | Heavy ocular discharge |
| Ear score | Normal | Ear flick or head shake | Slight unilateral droop | Head tilt or bilateral droop |
| Fecal score | Normal | Semi-formed pasty | Loose, but stays on top of bedding | Water sifts through bedding |

^aAdapted from the University of Wisconsin–Madison calf health scoring chart that can be found at http://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf.

DISCUSSION

Boron is considered a prebiotic chemical element with a role in both the origin and evolution of life, as well as an essential micronutrient for some bacteria, plants, fungi, and algae (Biță *et al.*, 2022). Boron may have clinically significant systemic effects beyond that of its putative and more subtle essentiality functions (Calabrese *et al.*, 2023). For the first time, dairy calves supplemented with boron were subjected to NMR-based metabolomics tests at set intervals for two months. In this study, although increased enzyme levels in the first samples due to birth stress in the

biochemical profile were remarkable, no significant changes were found with the effect of boron. Immunoglobulin G and GGT are important parameters to understand whether the colostrum passive transfer is sufficient for passive immunity following birth. The immature immune system and poor self-regulation ability of calves make them the main subjects of diarrheal diseases (Huang *et al.*, 2020). Although the increase in GGT was expected to continue with the addition of boron, this evidence did not occur in our population study. In addition, an increase in total protein, Ca, and P levels of control group animals on day 0 was similar to

the results already shown in our previous studies (Baspinar *et al.*, 2017; Basoglu *et al.*, 2011). The same issue has been also shown by McGuirk (2020).

The control group seems the most differentiated at each of the three-time points regarding both water-soluble and lipid-soluble metabolites. This indicates that calves' metabolism following parturition is unstable and boron appears to contribute to their metabolic balance. It will be a promising approach to restore the metabolic balance and alleviate the clinical complications of diseases on the basis of the metabolomic profile of calves (Huang *et al.*, 2020).

Alanine and valine are very important glucose precursors. The effects of alanine on productive traits in ruminants are still little discussed in the literature (Schalch Junior *et al.*, 2022). When glucose is deficient in the body, animals require gluconeogenesis to produce it, and gluconeogenesis from glucogenic amino acids is quantitatively important. By increasing glucose absorption, the first feeding of colostrum improved the glucose status of newborn calves, resulting in elevated plasma glucose (Guo and Tao, 2018). In neonatal calves, dietary fructose supplementation may change carbohydrate metabolism and may affect the expression of mRNA and the activity of the small intestine carbohydrase. Decreased blood glucose, L-lactate, and increased NEFA concentrations may support altered carbohydrate metabolism in response to dietary fructose supplementation (Ronald *et al.*, 2020). In this study, glucose and fructose increases on day 60 were significant in all the experimental groups. The increase was signif-

icant in experimental group 2 compared to the control group on day 30. This data indicates that boron increases gluconeogenesis in a dose-dependent manner. In this study, alanine was found to be high on day 30 in the experimental groups. The fact that alanine was not found to be high on day 60 can be attributed to the higher activation of gluconeogenesis, depending on the dose of boron. It is observed that alanine levels, measured at intervals, decreased gradually in calves with neonatal sepsis in our previous study (Basoglu *et al.*, 2018).

Cholate is a bile salt that is present in the body and has a pool size of one to three grams. It is synthesized in the liver from cholesterol and is protected by several hepatic and intestinal transporters. Cholate can predict liver blood flow and perfusion and has a high first-pass hepatic extraction (80 to 90%), and is not metabolized (Helmke, 2015; Ikeda *et al.*, 2017). Elevated milk supply preweaning feeding led to a reduction in cholate, glycocholate, chenodeoxycholate, deoxycholate, glycodeoxycholate, lithocholate, glycolithocholate, glycolithocholate sulfate, 3-dehydrocholate, and 7-ketodeoxycholate (Leal *et al.*, 2021). In this study, it was observed that the cholate level was significantly higher on day 60 in experimental group 3 compared to the control group. In addition, the fact that different cholesterol types were low in experimental group 2, and two types were lower in experimental group 3 on day 60 confirms the dose-dependent efficacy of boron in synthesizing cholate from cholesterol.

Isovaleric acid supplements can promote rumen development and subsequent growth of calves by improving the morphology and function of the rumen mucosa (Szczeniak *et al.*, 2016; Vockley and Ensenauer, 2006; Liu *et al.*, 2018). Waste milk did not affect the pH value, the concentrations of VFA (acetic acid, propionic acid, butyric acid, isovaleric acid, valeric acid), and $\text{NH}_3\text{-N}$ in dairy calves compared to feeding milk replacer (Zhang *et al.*, 2023). In this study, isovaleric acid was found to be lower in the experimental groups on day 30 (experimental group 3) and day 60 (experimental groups 1 and 2). This can be attributed to the effects of boron in increasing energy metabolism and synthesis reactions.

The calves' growth performance, feed efficiency, and antioxidant capacity are all enhanced by sodium butyrate supplementation prior to weaning up to day 60. To comprehend how butyrate boosts calves' growth and antioxidant capacity, mechanistic studies using physiological, immunological, transcriptomic, and proteomic approaches are also required (Liu *et al.*, 2021). In this metabolomic study, decreases in 2-hydroxybutyrate levels were noted in the experimental groups, while a significant decrease was noted in experimental group 1 on day 60. Increases in 3-hydroxybutyrate levels were also noted, and these increases were less marked in accordance with the boron dose. This indicates that boron impacts ketone bodies' metabolism and increases gluconeogenesis.

L-Lactate is a well-established biomarker of tissue hypoxia, sepsis, disease severity, and mortality in critically

ill humans and domestic animals (Kraut and Madias, 2016; Rosenstein *et al.*, 2018). Little information is available about the relevance and prognostic utility of increased plasma L-lactate in calves with acute abdominal emergencies (Lausch *et al.*, 2019). D-lactate accumulation contributes to acidosis and diarrhea in calves, and the gastrointestinal tract appears to be the site of D-lactate production (Omele *et al.*, 2001). In this study, the lactate level was found to be lower in the experimental groups 2 and 3 than in the control group on day 60. This can be attributed to the fact that boron increases gluconeogenesis and glucose synthesis.

In calves that drink colostrum, leucine, valine, and glutamate concentrations are high, and they pass into the bloodstream and benefit the health of neonatal calves (Zhao *et al.*, 2018). Calves' gastrointestinal tract development and trypsin activity in the small intestine can both benefit from leucine supplementation (Cao *et al.*, 2019). Leucine plays a significant role in the metabolism of skeletal muscle, according to studies; it works independently of other branched-chain amino acids like valine and isoleucine to increase protein synthesis and decrease proteolysis. The cell's overall amino acid requirements are affected by changes in the concentration of essential amino acids like leucine, which in turn affect the metabolism of the remaining key amino acids. These findings highlight how amino acid metabolism is affected by leucine metabolism and production (Guo *et al.*, 2020). In this study, leucine and isoleucine levels decreased in boron-given groups and these decrements became more significant on day 60. It is note-

worthy that while leucine and valine levels were high in the first 24 hr after colostrum, they decreased more in groups given boron. This can be attributed to the boron dose-dependent increase in protein synthesis and the positive effect of boron on liver development.

Betaine is important for growth, lactation, protein synthesis, and fat metabolism in animals, however, there are few studies on transitional dairy cows and newborn calves. Betaine-treated calves have higher plasma concentrations of total protein and globulin, indicating enhanced immunity (Wang *et al.*, 2019). Betaine supplementation slightly reduced rectal temperature and circulating hematocrit but did not affect other metrics in the heat-stress experimental calves (Al-Qaisi *et al.*, 2022). Our previous experiment (Basoglu *et al.*, 2017) has shown that boron supplementation enhanced postpartum betaine levels which were decreased at partum in cows. In this study, although betaine levels were high in all boron-administered groups on day 60, and in experimental groups 1 and 3 on day 30, total protein and albumin levels did not show significant alterations. Different parameters (cytokines, acute phase proteins, immune reactants such as D and L-lactate) should be evaluated to understand whether the immune system is affected by betaine.

Potential indicators of oxidative stress in many acute and chronic disorders include the amino acids meta- and ortho-tyrosine (Molnar *et al.*, 2016). Improvement of oxidative status, particularly early in life when oxidative homeostasis is incompletely

established, and reduction of protein tyrosine nitration with mixed tocopherols addition may be responsible for additional nutrients available for growth (Quigley *et al.*, 2021). The low tyrosine levels on day 60 in all the experimental groups may be meaningful depending on the boron dose in terms of oxidative stress.

Glycerol-based phospholipids are phosphoglycerides. They constitute the majority of the lipid bilayers of cell membranes (Opgenorth *et al.*, 2020; Mukhopadhyay and Trauner, 2023). In this study, increases in phosphoglycerides in the experimental groups on day 60 can be interpreted as an important contribution to the development of the calf.

In conclusion, optimal nutrition during the pre-weaning period is essential. Practical calf feeding needs to be improved and communicated by advisors (van Niekerk *et al.*, 2021; Palczynski *et al.*, 2020). Since positive effects of boron supplementation in dairy calves after birth have been observed in the present study, this micro-mineral can get involved in such nutrition. NMR-based metabolomics has been useful in understanding the underlying mechanisms of boron supplementation. These effects are dose-dependent and they can be associated with increased activation of the gluconeogenesis pathway, benefit for liver development, and growth of newborn calves. In the future, more comprehensive studies including other omics technologies and/or the investigation of the immune system and microbiome in neonatal calves could be performed.

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