

# Investigation of Metabolites and Mineral Content in Blood Serum of Dairy Cows of the Simmental Breed in Samsun Province During the Transition Period

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## Research Article

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# Abstract

This study evaluated the metabolic and mineral profiles in the blood serum of Simmental dairy cows during the transition period to assess their energy, protein, and mineral status. A total of 78 cows from 10 farms in Samsun Province were grouped based on farm size (medium, large), altitude (sea level, mid-altitude), and dietary energy level (low, optimum). Blood samples were collected three weeks before calving, at calving, and three weeks postpartum. Farm size and altitude had no significant effect on serum glucose and NEFA levels; however, dietary energy levels had a significant impact ( $P = 0.004$ ). Cows fed optimum energy diets had significantly higher prepartum serum glucose concentrations ( $P < 0.008$ ). NEFA and BHBA levels remained below risk thresholds, indicating no severe negative energy balance. Postpartum, serum total cholesterol and HDL levels increased significantly ( $P < 0.001$ ), while LDL levels remained stable, reflecting mild liver stress and effective NEFA utilization. LDL levels were higher in cows on low-energy diets ( $P < 0.001$ ), but without adverse hepatic effects. Total protein levels were lowest prepartum and increased postpartum, particularly in cows fed optimum energy content. Liver enzymes (AST and ALT) were influenced by both feed and period but remained within normal physiological limits. Triglyceride levels remained within reference ranges, suggesting no hepatic fat accumulation. Altitude had a significant effect on MDA levels, which were elevated at mid-altitudes. In contrast, SOD levels showed no significant variation. Serum calcium and magnesium were influenced by dietary energy levels, while phosphorus levels remained unaffected. In conclusion, both dietary energy level and physiological period significantly influenced metabolic and mineral parameters in Simmental dairy cows during the transition period. Conversely, farm size and altitude had minimal effects. Feeding cows an optimal-energy diet supported overall metabolic health throughout the transition period.

## INTRODUCTION

The transition period in dairy cattle—defined as the three weeks before and after parturition—is a critical physiological phase marked by major metabolic and hormonal changes. During this time, cows undergo fetal growth, mammary gland development, the onset of lactation, and uterine involution, making them highly susceptible to metabolic and infectious diseases [1, 2]. Successful adaptation requires a precise balance of energy intake, protein metabolism, and mineral availability to maintain homeostasis, support immune function, and sustain productive performance [3]. Blood serum biomarkers—such as albumin, globulins, and liver enzymes—serve as reliable indicators of nutritional status, metabolic health, and organ function. For instance, reduced serum protein concentrations may reflect inadequate protein intake, hepatic dysfunction, or overall malnutrition [4]. Adequate dietary intake of energy, protein, macro minerals, trace elements, and electrolytes is essential for maintaining metabolic stability [5, 6]. Mineral imbalances during this period are associated with increased disease risk, reduced milk yield, and poor reproductive efficiency [7–9].

Negative energy balance (NEB) is a common occurrence in the postpartum period due to a mismatch between peak milk production (5–8 weeks) and peak feed intake (10–14 weeks) [10]. This leads to mobilization of body fat and protein reserves, elevating NEFA and BHBA levels and predisposing cows to conditions like ketosis, fatty liver, and hypocalcemia [11]. Therefore, monitoring blood parameters—such as NEFA, BHBA, total cholesterol, HDL, LDL, triglycerides, AST, ALT, and total protein—is essential for assessing energy status and liver function. Additionally, oxidative stress markers like MDA and SOD provide valuable insight into redox balance during this physiologically demanding phase. While many studies have explored nutrition and management strategies during the transition period [12, 13], limited research has examined the effect of environmental factors such as altitude. Altitude may directly or indirectly affect the physiology and performance of dairy cows by altering atmospheric pressure, temperature, solar radiation, feed quality, and seasonal availability [14, 15]. Evidence from other ruminant species suggests that altitude can alter milk composition and mineral content [16], but its impact during the transition period in dairy cows is not well understood.

Therefore, the objective of this study was to evaluate the effects of altitude, dietary energy level, and farm size on serum metabolite and mineral profiles in Simmental dairy cows during the transition period in Samsun Province. This investigation seeks to provide a better understanding of how environmental and nutritional factors interact to influence the health and productivity of dairy cows.

## MATERIALS AND METHODS

### Ethical approval

This study was approved by the local ethics board for animal experiments Directorate of Samsun Veterinary Control Institute, Ministry of Food, Agriculture and Livestock, Türkiye with following No:2018-2, dated:22/02/2018.

### **Animal Material**

The study was conducted on 78 clinically healthy Simmental dairy cows from 10 medium- and large-scale enterprises located in Samsun Province, Türkiye. The cows were categorized based on three main criteria: enterprise size (medium vs. large), altitude (sea level vs. medium altitude), and dietary energy level (low vs. optimum energy). Enterprises with 5 to 30 lactating cows were classified as medium scale, while those with more than 30 lactating cows were considered large-scale. In terms of altitude, farms situated at elevations up to 500 meters above sea level were classified as sea level, while those located between 500 and 1000 meters were categorized as mid-altitude. Dietary energy levels were determined based on the metabolizable energy (ME) content of the total mixed ration (TMR). Enterprises where the TMR had an energy value below 2400 kcal/kg were grouped as low energy, while those with energy values above 2400 kcal/kg were categorized as optimum energy. All cows included in the study were between their second and fifth lactation stages.

### **Feed Material**

Total mixed rations (TMR) used in each enterprise were collected and subjected to chemical analysis. Prior to analysis, the samples were ground using a laboratory mill equipped with a 1 mm sieve. Dry matter (DM) content was determined by oven drying at 105°C for 4 hours. Crude ash was measured by incineration in a muffle furnace at 550°C for 4 hours. Nitrogen (N) content was analyzed using the Kjeldahl method, and crude protein was calculated using the conversion factor  $N \times 6.25$ . Crude fat was determined according to AOAC (1984) procedures. Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF), which represent the cell wall fractions, were analyzed using an ANKOM 200 Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA) following the method described by Van Soest et al. (1991). The metabolizable energy (ME) of the TMR was estimated using the equation provided by Adams (1994). The average nutrient and energy levels of the TMR used in the study are presented in Table 1, and the mineral content is shown in Table 2.

### **Collection and Analysis of Blood Samples**

Artificial insemination records of the animals were reviewed, and blood samples were collected during the final three weeks of pregnancy. Sampling was performed at three time points: the beginning of the prepartum period, at parturition, and three weeks postpartum. For serum analysis, blood was collected into tubes without an anticoagulant. The serum was then separated by centrifugation at 3000 rpm for 10 minutes at +4°C and subsequently stored at -80°C until analysis. For the analysis of superoxide dismutase (SOD), blood samples were collected into tubes containing anticoagulants, washed three times with physiological saline, and prepared accordingly.

Serum concentrations of unesterified fatty acids, beta-hydroxybutyric acid, triglycerides, cholesterol, LDL, HDL, glucose, total protein, AST, ALT, and phosphorus were measured using commercial assay kits in accordance with the manufacturers' instructions. Malondialdehyde (MDA) and SOD levels were analyzed spectrophotometrically using established chemical methods. SOD catalyzes the dismutation of toxic superoxide radicals ( $O_2^{\cdot-}$ ), generated during oxidative metabolism, into hydrogen peroxide and molecular oxygen. The SOD activity assay was based on measuring the optical density (OD) at 560 nm of the blue-colored formazan dye formed by the reaction of nitroblue tetrazolium (NBT) with superoxide radicals generated by xanthine and xanthine oxidase (XOD). SOD inhibits the formation of formazan by scavenging superoxide radicals, and one unit of SOD activity is defined as the amount causing 50% inhibition of NBT reduction under the specified assay conditions [17]. MDA, a secondary product of lipid peroxidation and a key biomarker of oxidative stress, was measured using the method described by Cordiano et al. [18]. MDA forms a pink complex when reacts with thiobarbituric acid at 95°C and pH 3.4 under aerobic conditions. The amount of MDA was quantified spectrophotometrically at 532 nm.

For elemental analysis, sample pre-treatment was carried out using a Cem Brand Mars Xpress microwave wet incinerator. Feed samples (0.5 g) and serum samples (1 ml, thoroughly homogenized) were placed into Teflon containers. Each sample was treated with 5 ml of concentrated  $HNO_3$  (65%) and 2 ml of  $H_2O_2$  (30%), sealed, and subjected to microwave digestion. After digestion, vessels were allowed to cool in a fume hood for 15 minutes with lids closed. The lids and inner walls were rinsed with

deionized water, and the contents were filtered through Whatman No. 42 filter paper into 15 ml polyethylene tubes. The final volume was adjusted to 15 ml with ultrapure water, and samples were stored at +4°C until analysis [19].

A collaboration protocol was established with the Black Sea Agricultural Research Institute and the Provincial Control Laboratory for elemental analyses. Phosphorus content was determined using ICP-OES at the Black Sea Agricultural Research Institute, while other elements were measured using an ICP-MS system at the Provincial Control Laboratory. Prepared samples and calibration standards were placed in the autosampler of the ICP-MS device. To convert the detected signal into concentrations, a calibration curve consisting of at least five standard points was constructed for each element. The instrument drew the sample via a probe and delivered it to the nebulizer, where it was atomized and introduced into the high-temperature plasma (6000–10000 K). In the plasma, compounds dissociate, and elements become ionized. The ionized sample passed through the sampler and skimmer cones and entered the ion lenses, where non-ionized and irrelevant substances were removed. The desired ions were sequentially sent to the detector based on their mass-to-charge ratio. The ion count per second (cps) was recorded. After establishing the calibration graph, cps values for each element in the sample were compared to the curve, and results were reported as concentrations in ppb or ppm [20].

### **Statistical Analysis**

Data were analyzed using IBM SPSS Statistics Version 23 (Ondokuz Mayıs University, Samsun). The Shapiro–Wilk test was used to assess the normality of data distribution. Serum concentrations of glucose, NEFA, total cholesterol, HDL, LDL, total protein, AST, ALT, SOD, Ca, P, Mg, Cu, Zn, and Mn were analyzed using the General Linear Model (GLM), considering the main effects of farm management, altitude, dietary energy level, sampling period, and their interactions (altitude × period and feed × period). Significant main effects were compared using pairwise comparisons with Bonferroni correction. Nutrient and mineral contents of the TMR were compared between optimum and low energy groups using the independent samples t-test. Quantitative data were expressed as mean ± standard error of the mean (SEM) for normally distributed variables, and as median (min–max) for non-normally distributed variables. Non-parametric data were analyzed using the Mann–Whitney U test for comparisons between groups within each period and the Wilcoxon or Friedman test to evaluate temporal changes within groups.

## **RESULTS**

### **Serum Glucose and NEFA Levels**

Results of the serum glucose and NEFA levels according to farm, altitude, feed, and period have been presented in Table 3 as the main effect and interaction effect in Table 5. Serum glucose levels showed variation depending on altitude, feed type, and physiological period. At sea level, glucose levels decreased from prepartum to postpartum, whereas at medium altitude, they slightly increased during the postpartum period. The feed type significantly influenced glucose concentrations, with cows receiving an optimum energy diet maintaining higher glucose levels compared to those fed a low energy diet ( $P = 0.001$ ). Moreover, a significant interaction between feed and period was found ( $P = 0.008$ ), indicating that energy intake modulated glucose dynamics across the periparturient period. Serum NEFA levels were significantly affected by feed ( $P = 0.004$ ) and period ( $P < 0.001$ ), peaking at calving. Although altitude alone did not significantly alter NEFA concentrations, a significant altitude × period interaction was detected ( $P < 0.001$ ), suggesting that energy mobilization patterns differed with altitude during the transition period.

### **Serum BHBA Levels**

Table 3 and Table 4 compare the serum BHBA values according to farm, altitude, feed, and period as main and interaction effects, respectively. BHBA concentrations varied with farm type, altitude, and feed. Large farms exhibited significant changes in BHBA levels over time ( $P = 0.002$ ), whereas medium farms did not show significant variation. Sea-level farms showed significantly higher BHBA prepartum and postpartum compared to medium altitude farms ( $P < 0.001$  and  $P = 0.029$ , respectively). Cows fed with an optimum energy diet had significantly higher BHBA concentrations, particularly in the postpartum period (0.56 mmol/L), compared to those on a low energy diet ( $P < 0.001$ ). Period also had a significant effect ( $P = 0.009$ ), with a marked increase in BHBA levels during postpartum.

### **Serum Total Cholesterol, HDL, and LDL Levels**

Table 3 and Table 4 present the results for cholesterol, HDL, and LDL levels as main and interaction effects, respectively. Total cholesterol concentrations increased significantly postpartum ( $P < 0.001$ ), with the highest levels recorded in medium altitude farms. Although feed did not have a significant main effect on total cholesterol, there were observable differences across periods. HDL levels were significantly influenced by altitude and feed type, with higher concentrations seen in cows at sea level and those receiving optimum energy diets ( $P < 0.001$ ). The feed  $\times$  period interaction was significant ( $P = 0.045$ ), indicating different HDL responses across energy groups. LDL concentrations were significantly reduced at high altitude and in cows fed an optimum energy diet ( $P < 0.001$ ), demonstrating the role of environmental and nutritional factors in lipid metabolism.

### **Serum Triglyceride Levels**

Table 3 and Table 4 present the comparison of serum triglyceride (TG) values during the prepartum, parturition, and postpartum periods according to farm size, altitude, diet, and time period, as main and interaction effects, respectively. Triglyceride levels declined significantly from prepartum to calving and remained lower during the postpartum period ( $P < 0.001$ ). Sea-level farms showed lower overall triglyceride values compared to medium altitude farms. Cows receiving optimum energy feed maintained higher triglyceride levels across the transition period ( $P = 0.037$ ), while those fed low energy diets exhibited a more marked decrease, especially postpartum.

### **Serum Total Protein, AST, and ALT Levels**

Table 5 and Table 6 summarize the changes in serum total protein, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels in relation to altitude, diet, and physiological period (prepartum, parturition, and postpartum), as main and interaction effects, respectively. Serum total protein increased significantly during the postpartum period, particularly in cows fed an optimum energy diet ( $P < 0.001$ ). AST levels were significantly elevated at calving and postpartum in cows kept at sea level and those receiving optimum feed ( $P < 0.001$ ). Significant interactions were noted between altitude and period ( $P = 0.036$ ) and between feed and period ( $P = 0.005$ ). ALT activity also changed across the period, with the highest levels observed in postpartum cows at sea level. ALT was significantly influenced by feed and altitude ( $P = 0.039$  and  $P = 0.027$ , respectively), indicating liver function changes in relation to both environmental and dietary factors.

### **Serum Superoxide Dismutase (SOD) Activity**

Table 5 and Table 6 present SOD activity level according to farm, altitude, feed, and period as main and interaction effects, respectively. SOD activity was significantly influenced by feed type ( $P < 0.001$ ), with optimum energy diets resulting in higher enzyme levels postpartum. The feed  $\times$  period interaction was significant ( $P < 0.001$ ), while altitude and period alone did not show significant effects. This suggests that dietary energy intake plays a pivotal role in oxidative stress regulation.

### **Serum Malondialdehyde (MDA) Concentration**

Table 5 and Table 6 present the results of MDA values according to farm, altitude, feed, and period as main and interaction effects, respectively. MDA levels varied significantly with farm type, altitude, and feed. Cows in medium farms and at medium altitude exhibited higher MDA values during postpartum. A significant effect of feed was noted, with low-energy diets associated with increasing MDA levels across time ( $P = 0.021$ ), indicating elevated lipid peroxidation. The period did not have a significant effect overall ( $P = 0.448$ ), but interaction terms and subgroup analyses showed considerable variation.

### **Serum Cu, Zn, and Mn Levels**

The results of the serum levels of copper (Cu), zinc (Zn), and manganese (Mn) according to farm, altitude, feed, and period are presented in Tables 7 and 8 as main and interaction effects, respectively. Copper levels increased postpartum and were significantly affected by altitude ( $P = 0.047$ ) and feed ( $P = 0.030$ ), with optimum energy diets yielding higher concentrations. Zinc levels were relatively stable across the periods, though cows on optimum energy diets had slightly higher values ( $P = 0.034$ ). Manganese concentrations were significantly influenced by altitude ( $P < 0.001$ ) and feed ( $P < 0.001$ ), with the highest value observed in cows at high altitude receiving optimum energy feed.

### **Serum Ca, P, and Mg Levels**

Serum levels of calcium (Ca), phosphorus (P), and magnesium (Mg) according to farm, altitude, feed, and period have been presented in Tables 7 and 8 as main and interaction effects, respectively. Serum calcium levels increased postpartum and were significantly higher in cows fed low energy diets ( $P = 0.002$ ). Phosphorus concentrations increased steadily during the periparturient period and were significantly affected by altitude ( $P < 0.001$ ). Magnesium levels were also significantly affected by altitude ( $P = 0.043$ ) and feed ( $P < 0.001$ ), with higher concentrations in cows receiving low-energy diets, particularly during the postpartum period.

## DISCUSSION

Dairy cows undergo significant metabolic adaptations during the transition period, which spans the weeks before and after calving. During this time, the body faces a dramatic shift in energy demands, primarily due to fetal growth and the onset of lactation. Consequently, feeding strategies during this period must be designed to anticipate and support the rapid metabolic changes that occur [21]. Glucose serves as the principal fuel for metabolism and plays a central role in the energy balance of dairy cows. However, because blood glucose is tightly regulated by homeostatic mechanisms, it is not a reliable standalone indicator for assessing metabolic disturbances or herd-level issues [22]. During negative energy balance (NEB), which arises either due to insufficient dietary energy intake or reduced dry matter consumption, glucose deficits are typically compensated through gluconeogenesis in the liver. In this process, glucose can be synthesized from various precursors, including propionate, glycerol, lactate, and branched-chain amino acids such as isoleucine, leucine, and valine [23]. When gluconeogenesis alone cannot meet glucose demands, the body compensates by mobilizing fat reserves, leading to the production of ketone bodies like BHBA ( $\beta$ -hydroxybutyric acid) through hepatic oxidation of NEFA (non-esterified fatty acids) [24]. Therefore, evaluating blood glucose in isolation provides limited insight; a more accurate assessment of energy status requires the combined analysis of related metabolites such as NEFA, BHBA, and cholesterol.

In the present study, the main effects of management and altitude were not statistically significant for serum glucose and NEFA levels. However, the type of feed showed a significant influence, particularly with a notable feed  $\times$  period interaction for glucose levels. The relatively consistent blood glucose values across all periods observed in this study further support the concept that glucose concentrations are maintained within a narrow range due to strong homeostatic control [25]. Nonetheless, some previous studies [26, 27] have reported that cows fed high-energy diets during the prepartum period tend to have higher blood glucose levels than those fed low-energy diets. Consistent with these findings, the current study observed that cows receiving an optimum energy diet exhibited significantly higher prepartum glucose levels. This indicates a more favorable energy balance in cows-fed optimum energy rations compared to those on low-energy diets. NEFA concentrations reflect the degree of triacylglycerol mobilization from adipose tissue, while BHBA indicates the liver's efficiency in oxidizing NEFA. For this reason, both analytes are widely used as indicators of energy status and to help identify the risk of metabolic disorders during the periparturient period [28]. It is important to note that an increase in NEFA and BHBA levels during the transition period is expected, as the body adapts to energy deficits through fat mobilization. However, not all elevations in these metabolites should be interpreted as pathological [29]. Typically, NEFA concentrations begin to rise two to four days before calving and reach their peak around three days postpartum [30].

In the present study, the levels of NEFA and BHBA measured both prenatally and postnatally were below the threshold values typically associated with metabolic diseases. For these metabolites to be linked to metabolic disorders, the magnitude and rate of increased need to be greater and more severe than what is considered normal. In dairy cows, BHBA levels  $>0.6$  mmol/L and NEFA levels  $>0.3$  mEq/L measured prepartum, and BHBA levels  $>1.0$  mmol/L and NEFA levels  $>0.7$  mEq/L measured postpartum, are considered risky for the development of subclinical ketosis, fatty liver syndrome, or other transitional metabolic disorders [31]. The fact that the levels in this study remained below these thresholds suggests that negative energy balance (NEB) was not severe in any of the groups, and the liver's oxidation functions were likely intact. However, an increase in NEFA levels was observed at parturition. Increased lipolysis during birth, along with hormonal changes, catecholamine release, and heightened sympathetic nervous system activity, can lead to a significant rise in NEFA concentrations around calving [32]. Cows fed with optimum or high-energy diets during the prepartum period are generally not expected to experience significant increases in NEFA levels, as their higher energy intake supports a more favorable energy balance. This aligns with findings from several studies, which report that NEFA levels tend to rise more markedly in cows fed low-energy diets during the prepartum period [26, 27, 33]. Contrary to these studies, the present study observed higher NEFA levels across all periods in animals fed optimum energy diets. This could be attributed to an increased capacity for gluconeogenesis in cows fed low-energy rations during the prepartum period. Efficient

gluconeogenesis is a critical pathway for dairy cows to ensure a steady supply of glucose to mammary tissue, especially during periods of high milk production [34, 35]. When gluconeogenesis is insufficient, the body mobilizes its reserves to meet the energy demands of lactation [33]. Serum BHBA levels, as influenced by farm, altitude, feed, and period, also showed interesting patterns. Animals fed with optimum energy diets exhibited statistically significant differences in their prepartum, parturition, and postpartum BHBA values, whereas cows on low-energy rations did not show significant differences across periods. Some studies [26, 36] have suggested that prepartum diet does not affect BHBA concentrations. However, in this study, a significant effect of prepartum energy intake on BHBA levels was observed, highlighting the impact of diet on energy metabolism during the transition period. Furthermore, the period itself had a statistically significant effect on BHBA concentrations, reinforcing the dynamic nature of metabolic changes during the transition period. Significant physiological changes occur in lipid and cholesterol metabolism in dairy cows during the transition from pregnancy to lactation. Negative energy balance (NEB), which begins with a reduction in dry matter intake during the prepartum period, intensifies postpartum with the onset of lactation and the associated increase in energy demands [37]. In response, body fat reserves are mobilized, leading to elevated levels of non-esterified fatty acids (NEFA) in the bloodstream. These NEFAs are metabolized in the liver through  $\beta$ -oxidation or re-esterified into triglycerides (TG). The re-esterified TGs are either stored in the liver or packaged into very low-density lipoproteins (VLDL) by hepatocytes and secreted into circulation [37]. However, when the liver's capacity to export TGs as VLDL is exceeded, triglycerides accumulate within hepatic tissue, potentially leading to fatty liver syndrome and disrupting lipid and cholesterol metabolism [38, 39]. Consequently, low cholesterol levels observed during the transition period are often associated with impaired hepatic VLDL synthesis due to fat infiltration in the liver [40]. Supporting this, Van den Top et al. [41] reported that plasma TG, HDL, and LDL concentrations decreased from four weeks prepartum to the first week postpartum, likely due to hepatic TG accumulation, followed by a gradual increase until the twelfth week postpartum. The reduction in VLDL concentrations immediately after parturition may result from a constant rate of hepatic VLDL synthesis despite increased catabolism by the mammary gland [42] or from impaired VLDL secretion due to hepatic lipid overload (Herdt et al., 1988). Since the liver has a limited capacity to process excessive NEFA into VLDL, this imbalance can lead to a transient decline in cholesterol transport components. In the same context, the increase in LDL and cholesterol levels observed in the weeks following calving may reflect a new balance between hepatic NEFA uptake and VLDL output [41]. Similarly, Kessler et al. [39] found that LDL, HDL, and total cholesterol concentrations declined from the third week antepartum to the first week postpartum, then began to rise again from the fourth week postpartum, reaching a new equilibrium. Consistent with these findings, the current study's plasma total cholesterol and HDL concentrations were significantly higher in the third week postpartum compared to the prepartum and calving periods, regardless of altitude and dietary energy level. According to Puppione [43], the exchange of surface components between VLDL, LDL, and HDL is interrelated, such that cholesterol exported from the liver via VLDL is metabolized to LDL, which serves as the primary carrier of cholesterol to peripheral tissues. HDL, in turn, returns excess cholesterol from peripheral tissues to the liver, a process that maintains lipid homeostasis. Therefore, plasma concentrations of HDL and LDL often change in parallel [39, 41].

However, in the present study, while plasma HDL and total cholesterol levels increased significantly postpartum, LDL concentrations remained relatively stable across all periods. This finding aligns with the observations of Yildiz et al. [44], who also reported unchanged LDL levels despite increases in other lipid components. The stable LDL levels alongside rising HDL and total cholesterol may indicate that hepatic fat accumulation was mild and that the extent of NEFA mobilization and hepatic overload was not severe enough to impair VLDL synthesis markedly. The energy level of rations provided during the transition period plays a crucial role in regulating lipid metabolism and the overall health of dairy cows [45]. During this period, the cow inevitably experiences negative energy balance (NEB) due to rising energy demands for fetal growth and lactation. As a result, body fat reserves are mobilized to meet this energy deficit. The primary nutritional strategy during this time is to minimize excessive fat mobilization and reduce the metabolic burden of NEFA on the liver by increasing dry matter intake and enhancing the energy density of the ration. Newman et al. [45] demonstrated that dairy cows fed high-energy diets during the transition period had significantly higher plasma total cholesterol and HDL levels compared to those on medium- or low-energy diets, while LDL levels remained largely unchanged. In line with these findings, the present study showed that cows fed optimum energy diets had higher levels of total cholesterol and HDL compared to those on low-energy diets. However, in contrast to previous reports, LDL concentrations were higher in cows fed low-energy diets in this study. This may suggest that the extent of NEB-induced fat mobilization in the low-energy group did not impair hepatic VLDL synthesis, thereby maintaining normal LDL output from the liver. Nevertheless, plasma LDL concentrations are not determined solely by liver synthesis. They are also tightly regulated by the expression of LDL receptors on peripheral tissues such as muscle, adipose tissue, and the liver itself [46]. For this reason, LDL

levels should be interpreted cautiously and not regarded as direct indicators of hepatic VLDL production. When considering the elevated total cholesterol levels and relatively low LDL concentrations observed in cows fed optimum energy diets, it may be inferred that these animals had a higher net HDL level. Since HDL is commonly recognized as an anti-inflammatory lipoprotein, its elevation may also reflect a more favorable systemic inflammatory profile in cows receiving better nutrition [47]. Moreover, due to the dynamic nature of lipoprotein metabolism, HDL concentrations have been proposed to be a more reliable indicator of liver function in transition cows compared to triglycerides or LDL [45]. The ability of the liver to maintain adequate HDL synthesis may therefore reflect its metabolic resilience during NEB.

In the present study, a comprehensive evaluation of liver and energy-related metabolites, namely glucose, triglycerides, total cholesterol, LDL, HDL, AST, ALT, NEFA, and BHBA, suggests that there was no evidence of severe adipose tissue mobilization or liver dysfunction (such as clinical ketosis or fatty liver) in any of the dietary groups or time points. Instead, the cows maintained healthy gluconeogenic activity and liver oxidative functions throughout the transition period. The relatively low milk yield (10–15 liters/day) of the animals used in the study is likely a key factor contributing to this outcome, as lower-producing cows are under less metabolic stress and thus have a reduced risk of transition-related disorders. Total serum protein is composed of the sum of albumin and globulin. In addition to their structural functions, serum proteins play critical roles in metabolism, including the transport of lipids, fatty acids, copper, iron, and hemoglobin, as well as maintaining osmotic pressure [48]. In ruminants, total protein (TP) levels are considered more indicative of nitrogen utilization efficiency, organ function (especially kidney and liver), and general nutritional health, rather than a reflection of metabolic protein stores. Due to the strong homeostatic regulation of protein metabolism, moderate declines in protein absorption, except under prolonged fasting, rarely result in significant changes in serum protein levels [49]. In recent years, the measurement of total protein and its fractions (albumin and globulin) has gained importance in metabolic profile tests to evaluate the protein status of dairy cows during the transition period. However, research on the diagnostic use of these fractions remains limited. Piccione et al. [50] observed that serum TP concentrations decreased from the late prepartum period to the first week postpartum, followed by an increase in the third and fourth weeks. They attributed the initial decrease primarily to a reduction in globulin levels. In the present study, TP values remained within the established reference ranges [51] and were consistent with those reported in other studies examining blood metabolites in transition cows [50, 52, 53]. Unlike some previous studies, the lowest TP levels in our study were observed during the prepartum period, with a significant increase in the postpartum period. This prepartum decrease may be linked to a specific energy deficit affecting hepatic protein synthesis [53] or to transient immunosuppression that impairs neutrophil and lymphocyte functions during late gestation [54]. The higher postpartum TP levels in cows fed the optimum energy ration, compared to those on the low energy diet, may reflect a better energy-protein balance, promoting more effective microbial protein synthesis in the rumen.

During the transition period, dairy cows undergo major physiological adaptations, and these are often accompanied by oxidative stress, which can negatively affect health and productivity [55]. The intense growth of mammary tissue, rapid differentiation of secretory cells, and high milk synthesis substantially increase the body's oxygen demand [56]. This increased demand leads to elevated production of reactive oxygen species (ROS). Under normal conditions, ROS are neutralized by the antioxidant defense system. However, during the periparturient period, an imbalance between ROS production and antioxidant capacity may occur, increasing oxidative stress and potentially contributing to metabolic disorders [57]. Several studies have documented oxidative stress during this period and its association with metabolic diseases [58, 59]. Lipid peroxidation is a major consequence of oxidative stress, characterized by a chain reaction that results in the formation of malondialdehyde (MDA), a measurable end-product of this process. In contrast, superoxide dismutase (SOD), a key antioxidant enzyme, plays a crucial role in neutralizing ROS. Therefore, assessing both MDA and SOD levels provides insight into oxidative stress status and potential links with metabolic health.

In the present study, MDA concentrations were higher in the postpartum period compared to prepartum and calving, consistent with findings by Sharma et al. [55], who also reported an increase in MDA after parturition. In contrast, Turk et al. [60] found the highest MDA levels during calving, attributing this to lipid peroxidation driven by NEFA increases under negative energy balance. It is well established that excessive NEFA oxidation generates high levels of ROS, initiating lipoperoxidative processes [61]. However, in our study, the NEFA concentrations before and after calving did not indicate excessive lipid mobilization, suggesting that fat mobilization was moderate. Still, the parallel increases in both NEFA and MDA in the postpartum period support the connection between fat metabolism and oxidative stress. Altitude is another factor that may influence oxidative stress. Exposure to medium altitude, with its reduced oxygen pressure, has been associated with increased ROS formation and oxidative damage to lipids,

proteins, and DNA [62]. Supporting this, our study found higher MDA levels in cows at medium altitude, possibly reflecting hypoxia-induced oxidative stress. The energy level of the ration, on the other hand, did not significantly affect MDA levels. This may be attributed to the absence of severe negative energy balance in either diet group, as previously discussed.

In the present results regarding antioxidant defense, SOD levels did not significantly differ with altitude, dietary energy level, or physiological period. All groups had similar SOD concentrations. Despite the increase in MDA postpartum, SOD levels remained unchanged, a finding also reported by Sharma et al. [55]. These results suggest that while lipid peroxidation may increase after calving, the antioxidant capacity, at least in terms of SOD, remains relatively stable. The mean serum SOD and MDA levels before, during, and after parturition are presented in Tables 10 and 11, respectively.

The activity of cellular antioxidant systems is directly influenced by the availability of trace minerals such as copper (Cu), zinc (Zn), and manganese (Mn) in the body [62]. These elements are essential cofactors of superoxide dismutase (SOD), a critical antioxidant enzyme that detoxifies superoxide radicals, a key component of reactive oxygen species (ROS) in immune cells. The continuous production and activity of endogenous antioxidants such as SOD during the transition period, when oxidative stress increases due to intense physiological demands, relies heavily on adequate levels of these trace elements. Numerous studies have shown that reductions in Cu, Zn, and Mn concentrations during the transition period can exacerbate oxidative stress in dairy cows [63, 64]. According to Herdt and Hoff [65], reference values for blood serum trace elements in adult dairy cows are: Cu, 0.6–1.1 µg/mL; Zn, 0.6–1.9 µg/mL; and Mn, 0.9–6.0 ng/mL. In the present study, mean values of these minerals across all groups and time points (Cu: 0.65–0.78 µg/mL, Zn: 1.13–1.40 µg/mL, Mn: 1.52–2.15 ng/mL) fell within these reference ranges regardless of altitude, feed level, or physiological period. Additionally, changes in Cu and Mn levels over time paralleled the trends observed in SOD levels, reinforcing their role in supporting antioxidant activity.

One of the most common disorders during the transition period is downer cow syndrome [66], which is characterized by disturbances in the homeostasis of macrominerals such as calcium (Ca), phosphorus (P), magnesium (Mg), and potassium (K). These four macrominerals are vital for normal nerve and muscle function, and imbalances or deficiencies can impair mobility and predispose cows to other transition-related diseases, including ketosis, fatty liver, and retained placenta. Although homeostatic mechanisms are usually effective in maintaining normal blood concentrations of these minerals, they can fail under the stress of parturition and early lactation, leading to conditions like milk fever [67].

In adult dairy cows, normal blood Ca concentration ranges from 8.5 to 10 mg/dL [54]. During the transition period, significant calcium is lost through colostrum (1.7–2.3 g Ca/kg) and milk (1.1 g Ca/kg). This loss is typically compensated by increased calcium absorption from the intestine, mediated by 1,25-dihydroxyvitamin D, and mobilization from bone stores, stimulated by parathyroid hormone (PTH). Therefore, it is also important to monitor other macrominerals, especially P and Mg, that influence these compensatory pathways. For instance, plasma phosphorus concentrations normally range from 4 to 8 mg/dL (1.3 to 2.6 mmol/L). If phosphorus levels rise above 6 mg/dL (1.9 mmol/L), it may inhibit the renal conversion of 25-hydroxyvitamin D to its active form, thereby impairing calcium absorption from the intestine despite PTH stimulation.

Magnesium also plays a crucial role in calcium homeostasis. Normal blood Mg concentration in cows is between 1.8 and 2.4 mg/dL (0.75–1.0 mmol/L). Hypomagnesemia interferes with calcium regulation in two ways: by reducing PTH secretion in response to hypocalcemia and by decreasing tissue sensitivity to PTH [68]. Blood Ca levels may drop below 5 mg/dL under such conditions. Potassium, another important macromineral, is essential for maintaining intracellular osmotic balance and neuromuscular excitability. Although K deficiency is relatively uncommon, it may occur during prolonged anorexia or diseases like ketosis. Normal plasma K levels range from 3.9 to 5.8 mEq/L, and concentrations below 2.5 mmol/L are often observed in clinical hypokalemia and downer cow syndrome [69, 70]. Intracellular potassium also regulates calcium release from the sarcoplasmic reticulum, and deficiency can impair muscle contractility by reducing calcium signaling.

In the current study, we evaluated the serum concentrations of Ca, P, Mg, and K, macrominerals associated with metabolic diseases, during the transition period. Monitoring these minerals, especially during the dry period, is critical for the early detection and prevention of periparturient metabolic disorders. Notably, deficiencies do not always present with clinical signs. Subclinical imbalances, such as subclinical hypocalcemia, defined by reduced Ca levels without obvious clinical symptoms, can still reduce productivity. Thresholds for subclinical hypocalcemia typically range between 8.0 and 8.8 mg/dL [71, 72]. In the present study, calcium concentrations remained within reference ranges for healthy dairy cows throughout all periods and exceeded the

thresholds for subclinical hypocalcemia. Interestingly, serum Ca levels were lower in the group fed the optimum energy ration compared to those on the low energy diet. This may reflect the low calcium content of the low energy ration, which could have stimulated PTH activity, thereby enhancing calcium mobilization and absorption. Feeding low-calcium diets during the dry period is a known strategy to stimulate PTH and support calcium homeostasis around calving [65]. Magnesium concentrations in our study also remained within the normal range [73], similar to findings by Jeong et al. [74], who investigated Mg levels in cows during the transition period. Mild hypomagnesemia, defined as serum Mg between 1.3 and 1.8 mg/dL, is common and often accompanied by mild hypophosphatemia (serum P between 2 and 4 mg/dL) and mild hypokalemia (serum K between 1.8 and 2.6 mmol/L) in transition cows [75]. In our study, plasma P levels ranged from 4 to 8 mg/dL, consistent with reference values for healthy dairy cattle [73]. The highly correlated values of serum P, Ca, Mg, and K observed in this study align well with those reported by Tsiamadis et al. [76], who investigated macromineral dynamics during the transition period.

## CONCLUSION

Metabolic disorders resulting from inadequate nutrition during the transition period in dairy cows can lead to substantial economic losses. This study aimed to evaluate the energy-protein status, oxidative stress markers, and mineral profiles in Simmental dairy cows during the transition period, with consideration of farm size, altitude, and dietary energy levels in the total mixed ration (TMR). Serum glucose and NEFA concentrations were not significantly influenced by farm size or altitude; however, dietary energy level had a significant effect ( $p < 0.001$ ), with higher prepartum glucose levels observed in cows fed optimum energy diets ( $p = 0.008$ ). Glucose concentrations remained stable across physiological periods, confirming the tight homeostatic regulation of blood glucose. NEFA and BHBA levels remained below clinically relevant thresholds, and prepartum BHBA concentrations were not significantly affected by dietary energy levels. Postpartum, serum total cholesterol and HDL concentrations increased significantly ( $p < 0.001$ ), peaking in the third week, while LDL levels remained stable across time points, suggesting mild fat mobilization and preserved hepatic function. Nevertheless, dietary energy level significantly influenced LDL concentrations ( $p < 0.001$ ), with higher values observed in cows receiving low-energy diets. Total protein (TP) levels were within reference ranges throughout the study but were lowest during the prepartum period and increased significantly postpartum, particularly in cows fed optimum energy diets. This finding may reflect enhanced microbial protein synthesis in the rumen supported by better energy balance. Serum AST and ALT activities were influenced by both diet and period, suggesting a variable metabolic load but no evidence of hepatic damage. Serum triglyceride concentrations remained within physiological limits, indicating adequate hepatic VLDL synthesis capacity and the absence of fatty liver syndrome. Collectively, the results for key energy and lipid metabolism indicators (glucose, triglycerides, cholesterol, LDL, HDL, AST, ALT, NEFA, and BHBA) suggest that the cows were not in a state of negative energy balance, likely due to their relatively low milk yield. Oxidative stress evaluation revealed significantly higher MDA concentrations in cows housed at medium altitude, possibly reflecting hypoxia-induced oxidative stress. SOD activity did not vary significantly with altitude, dietary energy level, or physiological stage, suggesting preserved antioxidant defense mechanisms across all groups. Serum Cu and Mn levels were significantly influenced by altitude and period, while Zn was affected by feed. Cu, Zn, and Mn levels were higher in cows fed optimum energy. Ca and Mg concentrations were significantly affected by dietary energy, though P levels were not. The maintenance of macromineral homeostasis during the transition period likely contributed to the absence of metabolic disorders. In conclusion, the findings suggest that the cows maintained adequate metabolic and mineral balance throughout the transition period, likely supported by moderate milk production levels and well-managed nutritional strategies.

## Declarations

**Author Contribution** NO, MS, BB, RP: data collection, data analysis, and writing. MS, MW, NO, DT, AV: conceptualization, methodology, and supervision. AV, MS, MW, RP: methodology, review, and editing. NO, MS, DT, MW: data analysis, review, and editing. NO, BB, MS, MW: writing, review, and editing.

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**Data Availability:** Data is provided within the manuscript or supplementary information files.

**Declarations** The animal care procedures were approved and carried out in accordance with the specified regulations of the Institutional Animal Ethics Committee (IAEC).

**Conflict of Interest** The authors declare no competing interests.

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## Tables

Table 1. Mean nutrient (%) and energy levels (kcal/kg) in feeds according to energy levels in the total ration

|                 | Optimum Energy | Low Energy    | P      |
|-----------------|----------------|---------------|--------|
| DM              | 52.34±0.66     | 64.04±3.95    | 0.009  |
| CA              | 8.24±0.12      | 8.07±0.23     | 0.529  |
| CP              | 16.05±0.39     | 13.76±0.32    | <0.001 |
| ADF             | 24.88±0.4      | 33.73±0.75    | <0.001 |
| NDF             | 42.18±1.2      | 48.12±0.59    | <0.001 |
| ME              | 2454.75±14.89  | 2131.04±25.74 | <0.001 |
| NE <sub>L</sub> | 1.54±0.01      | 1.32±0.02     | <0.001 |

DM: Dry matter; CA: Crude ash; CP: Crude protein; ADF: Acid detergent fibre; NDF: Neutral detergent fibre; ME: Metabolic energy  
NE<sub>L</sub>: Net energy lactation

Table 2. Average mineral levels in feeds according to energy levels in the total ration

|           | Optimum Energy | Low Energy   | P     |
|-----------|----------------|--------------|-------|
| Ca        | 0.61±0.037     | 0.57±0.065   | 0.566 |
| P, %      | 0,23±0,007     | 0,23±0,026   | 0.961 |
| Mg        | 0.2±0.008      | 0.21±0.009   | 0.456 |
| K, %      | 1.02±0.041     | 0.97±0.049   | 0.438 |
| Na,%      | 0,19±0.031     | 0.19±0.029   | 0.876 |
| Fe, mg/kg | 313.13±13.58   | 269.09±24.77 | 0.131 |
| Zn, mg/kg | 98.71±36.15    | 37.45±3.54   | 0.119 |
| Mn, mg/kg | 52.39±2.47     | 59.88±3.8    | 0.110 |
| Mo, mg/kg | 0.56±0.06      | 0.7±0.05     | 0.089 |

*Table 3. Investigation of mean serum glucose, NEFA, BHBA, total cholesterol, HDL, LDL, and triglycerides levels according to the main effect of altitude, feed and period*

| Factors<br>Parameters | Glucose    | NEFA                   | BHBA                         | Total<br>Cholesterol     | HDL                     | LDL        | Triglycerides                |
|-----------------------|------------|------------------------|------------------------------|--------------------------|-------------------------|------------|------------------------------|
| Sea level             | 70.25±1.29 | 0.39±0.02              | 0.45<br>(0.24:1.07)          | 119.35±3.58              | 79.51±2.04              | 25.62±1.73 | 24,5 (10,4:56,6)             |
| Medium<br>altitude    | 69.89±1.3  | 0.38±0.01              | 0.38 (0.21:0.80)             | 126.93±4.08              | 70.63±1.65              | 16.93±0.41 | 36,9(19,5:80,6)              |
| P                     | 0.457      | 0.452                  | <0.001                       | 0.010                    | <0.001                  | <0.001     | <0.001                       |
| Pre-partum            | 71.67±1.73 | 0.26±0.01 <sup>c</sup> | 0,44(0,21:0,75) <sup>a</sup> | 112.66±4.13 <sup>b</sup> | 71.58±1.94 <sup>b</sup> | 23.57±1.76 | 34(18,1:80,7) <sup>a</sup>   |
| Partum                | 69.07±1.45 | 0.48±0.02 <sup>a</sup> | 0,4(0,24:0,62) <sup>b</sup>  | 109.94±4.01 <sup>b</sup> | 69±2.01 <sup>b</sup>    | 19.19±1.48 | 27,3(12,2:53,1) <sup>b</sup> |
| Post-<br>partum       | 69.52±1.6  | 0.4±0.02 <sup>b</sup>  | 0,43(0,22:0,92) <sup>a</sup> | 145.66±4.76 <sup>a</sup> | 85.99±2.68 <sup>a</sup> | 22.41±1.96 | 28,7(10,4:56,6) <sup>b</sup> |
| P                     | 0.264      | <0.001                 | 0.009                        | <0.001                   | <0.001                  | 0.145      | <0.001                       |
| Optimum<br>energy     | 73.26±1.43 | 0.41±0.02              | 0.46<br>(0.24:1.07)          | 120.06±3.88              | 79.56±2.38              | 16.55±0.31 | 31,3(15:56,6)                |
| Low energy            | 67.64±1.16 | 0.36±0.01              | 0.39 (0.21:0.8)              | 124.83±3.73              | 72.4±1.55               | 25.72±1.7  | 28,6(10,4:80,6)              |
| P                     | 0.001      | 0.004                  | <0.001                       | 0.935                    | 0.263                   | <0.001     | 0,27                         |

<sup>a,b,c</sup>: There is no difference between the main effects of altitude, period and feed with the same letter.

HDL: high density lipoproteins; LDL: low density lipoproteins; NEFA; on-esterified fatty acids; BHBA: beta hydroxybutyrate

*Table 4. Investigation of mean serum glucose, NEFA, BHBA, total cholesterol, HDL and LDL and tryglycerides levels according to interaction effect of altitude. feed and period*

| Factor                    | Category        | Pre-partum                   | Partum                       | Post-partum                  |
|---------------------------|-----------------|------------------------------|------------------------------|------------------------------|
| Glucose (mg/dl)           |                 |                              |                              |                              |
| Altitude                  | Sea level       | 73.79±2.51                   | 69.53±1.79                   | 67.43±2.31                   |
|                           | Medium altitude | 69.07±2.26                   | 68.51±2.39                   | 72.09±2.1                    |
| Feed                      | Optimum energy  | 79.66±2.37 <sup>a</sup>      | 71.28±1.91 <sup>b</sup>      | 68.83±2.76 <sup>b</sup>      |
|                           | Low energy      | 65.49±2.02 <sup>b</sup>      | 67.37±2.08 <sup>b</sup>      | 70.05±1.88 <sup>b</sup>      |
| NEFA (mmol/L)             |                 |                              |                              |                              |
| Altitude                  | Sea level       | 0.23±0.02 <sup>c</sup>       | 0.45±0.03 <sup>a</sup>       | 0.48±0.03 <sup>a</sup>       |
|                           | Medium altitude | 0.31±0.01 <sup>b</sup>       | 0.51±0.02 <sup>a</sup>       | 0.31±0.02 <sup>b</sup>       |
| Feed                      | Optimum energy  | 0.28±0.02                    | 0.49±0.02                    | 0.47±0.04                    |
|                           | Low energy      | 0.25±0.02                    | 0.47±0.03                    | 0.36±0.02                    |
| BHBA (mmol/L)*            |                 |                              |                              |                              |
| Altitude                  | Sea level       | 0.5(0.29:0.75) <sup>a</sup>  | 0.41(0.24:0.62) <sup>b</sup> | 0.49(0.24:0.92) <sup>a</sup> |
|                           | Medium altitude | 0.36(0.21:0.69)              | 0.4(0.25:0.59)               | 0.39(0.22:0.68)              |
| Feed                      | Optimum energy  | 0.47(0.29:0.75) <sup>b</sup> | 0.41(0.24:0.62) <sup>c</sup> | 0.56(0.25:0.92) <sup>a</sup> |
|                           | Low energy      | 0.37(0.21:0.69)              | 0.4(0.25:0.59)               | 0.37(0.22:0.68)              |
| Total Cholesterol (mg/dl) |                 |                              |                              |                              |
| Altitude                  | Sea level       | 110.93±4.9                   | 106.21±4.96                  | 140.9±7.2                    |
|                           | Medium altitude | 114.78±7.02                  | 114.51±6.55                  | 151.5±5.81                   |
| Feed                      | Optimum Energy  | 103.36±5.62                  | 108.54±5.01                  | 148.27±6.68                  |
|                           | Low Energy      | 119.84±5.71                  | 111.02±6.02                  | 143.63±6.72                  |
| HDL (mg/dl)               |                 |                              |                              |                              |
| Altitude                  | Sea level       | 72.16±2.31                   | 73.23±3.02                   | 93.14±4.12                   |
|                           | Medium altitude | 70.87±3.29                   | 63.8±2.25                    | 77.21±2.54                   |
| Feed                      | Optimum Energy  | 69.65±2.43 <sup>b</sup>      | 75.23±3.55 <sup>b</sup>      | 93.81±4.9 <sup>a</sup>       |
|                           | Low Energy      | 73.08±2.88 <sup>b</sup>      | 64.18±2.02 <sup>b</sup>      | 79.95±2.58 <sup>a</sup>      |
| LDL (mg/dl)               |                 |                              |                              |                              |
| Altitude                  | Sea level       | 27.19±3.06                   | 22.52±2.54                   | 27.15±3.34                   |
|                           | Medium altitude | 19.13±0.59                   | 15.1±0.62                    | 16.57±0.75                   |
| Feed                      | Optimum Energy  | 17.46±0.55                   | 14.84±0.44                   | 17.36±0.53                   |
|                           | Low Energy      | 28.3±2.91                    | 22.56±2.5                    | 26.31±3.34                   |
| Triglycerides (mg/dl)*    |                 |                              |                              |                              |
| Altitude                  | Sea Level       | 27.8(18.1:52) <sup>a</sup>   | 21(12.2:44) <sup>b</sup>     | 21(10.4:56.6) <sup>b</sup>   |
|                           | Medium Altitude | 41.3(26.6:80.7) <sup>a</sup> | 34.4(19.5:53.1) <sup>b</sup> | 33.3(21:51.2) <sup>b</sup>   |

|      |                |                              |                              |                              |
|------|----------------|------------------------------|------------------------------|------------------------------|
| Feed | Optimum energy | 33.5(18.1:52) <sup>a</sup>   | 27.6(15.6:44) <sup>b</sup>   | 32.3(15:56.6) <sup>a</sup>   |
|      | Low energy     | 36.5(18.2:80.7) <sup>a</sup> | 26.3(12.2:53.1) <sup>b</sup> | 27.7(10.4:51.2) <sup>b</sup> |

<sup>a-b</sup>: No difference between altitude\*period and feed\*period interactions with the same letter, Shows the median (minimum:maximum) values.

*Table 5. Investigation of mean serum total protein, ALT, AST SOD and MDA levels according to main effect of altitude, feed and period*

| Factors         | Parameters | Total Protein           | AST                     | ALT                     | SOD        | MDA             |
|-----------------|------------|-------------------------|-------------------------|-------------------------|------------|-----------------|
| Sea level       |            | 85.79±1.37              | 47.06±1.11              | 21.63±0.77              | 50.28±0.71 | 4.89 (2.5:16.6) |
| Medium altitude |            | 81.63±1.38              | 38.11±0.97              | 19.29±0.56              | 42.73±1    | 9.29 (2.6:21.7) |
| P               |            | 0.205                   | 0.665                   | 0,917                   | 0,192      | <0.001          |
| Pre-partum      |            | 81.69±1.32 <sup>b</sup> | 36.08±0.95 <sup>b</sup> | 21.59±0.79 <sup>a</sup> | 46.65±1.11 | 6.1(2.7:16.3)   |
| Partum          |            | 82.78±1.72 <sup>b</sup> | 46.9±1.32 <sup>a</sup>  | 18.34±0.81 <sup>b</sup> | 47.07±1.06 | 6.2(2.8:16.1)   |
| Post-partum     |            | 87.31±1.97 <sup>a</sup> | 46.14±1.51 <sup>a</sup> | 21.8±0.92 <sup>a</sup>  | 46.95±1.17 | 6(2.5:21.7)     |
| P               |            | 0,007                   | <0.001                  | 0,008                   | 0,644      | 0.448           |
| Optimum energy  |            | 91.02±1.43              | 49.08±1.28              | 19.93±0.79              | 49.13±0.92 | 5.2 (2.5:16.6)  |
| Low energy      |            | 78.44±1.15              | 38.38±0.83              | 21.08±0.63              | 45.16±0.86 | 7.2 (2.6:21.7)  |
| P               |            | <0.001                  | <0.001                  | 0,039                   | 0,155      | <0.001          |

<sup>a-b</sup>: There is no difference between the main effects of altitude, period and feed with the same letter.

ALT: alanine aminotransferase; AST: aspartate aminotransferase; SOD; superoxide dismutase; MDA; malondialdehyde

*Table 6. Investigation of mean serum total protein AST and ALT , SOD and MDA levels according to interaction effect of altitude. feed and period*

| Factor               | Category               | Pre-partum                  | Partum                   | Post-partum                |
|----------------------|------------------------|-----------------------------|--------------------------|----------------------------|
| Total protein (g/L)  |                        |                             |                          |                            |
| Altitude             | Sea level              | 82.61±1.82                  | 84.88±2.61               | 89.88±2.54                 |
|                      | Medium altitude        | 80.55±1.91                  | 80.2±2.07                | 84.15±3.05                 |
| Feed                 | Optimum energy         | 85.5±1.87 b                 | 89.76±2.5b               | 97.8±2.55a                 |
|                      | Low energy             | 78.73±1.72c                 | 77.39±2.02c              | 79.19±2.23c                |
| AST (U/L)            |                        |                             |                          |                            |
| Altitude             | Sea level              | 37.39±1.14b                 | 51.1±1.76a               | 52.69±1.89a                |
|                      | Medium altitude        | 34.47±1.55b                 | 41.74±1.65b              | 38.1±1.64b                 |
| Feed                 | Optimum Energy         | 38.47±1.49b                 | 52.92±2.02a              | 55.86±1.89a                |
|                      | Low Energy             | 34.24±1.16c                 | 42.26±1.41b              | 38.64±1.47b                |
| ALT (U/L)            |                        |                             |                          |                            |
| Altitude             | Sea level              | 21.9±1.03 <sup>b</sup>      | 17.62±1.35 <sup>c</sup>  | 25.36±1.33 <sup>a</sup>    |
|                      | Medium altitude        | 21.22±1.25 <sup>b</sup>     | 19.23±0.69 <sup>b</sup>  | 17.41±0.78 <sup>c</sup>    |
| Feed                 | Optimum Energy         | 20.59±1.21                  | 17.03±1.39               | 22.17±1.38                 |
|                      | Low Energy             | 22.37±1.05                  | 19.36±0.93               | 21.51±1.25                 |
| SOD (U/g protein)    |                        |                             |                          |                            |
| Altitude             | Sea level              | 48.41±1.28                  | 50.47±1.22               | 51.96±1.14                 |
|                      | Medium altitude        | 44.5±1.87                   | 42.89±1.58               | 40.8±1.73                  |
| Feed                 | Optimum Energy         | 44.27±1.76 <sup>c</sup>     | 49.44±1.37 <sup>a</sup>  | 53.69±1.21 <sup>a</sup>    |
|                      | Low Energy             | 48.49±1.38 <sup>ab</sup>    | 45.24±1.5 <sup>bc</sup>  | 41.75±1.44 <sup>c</sup>    |
| MDA (nmol/g protein) |                        |                             |                          |                            |
| Altitude             | Sea level (n=49)       | 5.3(3.1:16.3)               | 5.2(2.8:16.1)            | 5.2(2.5:16.7)              |
|                      | Medium altitude (n=29) | 8.2(2.7:14.6)               | 9.2(3.5:12.9)            | 9.2(2.9:21.7)              |
| Feed                 | Optimum energy (n=34)  | 5.3(3.1:9.2)                | 5.3(2.8:11.5)            | 4.6(2.5:16.6)              |
|                      | Low energy (n=44)      | 7.3(2.7:16.3) <sup>ab</sup> | 7(3.5:16.1) <sup>b</sup> | 7.7(2.9:21.7) <sup>a</sup> |

<sup>a-b</sup>: No difference between altitude\*period and feed\*period interactions with the same letter

*Table 7. Investigation of mean serum micro (Cu, Zn, and Mn) and macro minerals (Ca, P, and Mg) levels according to the main effect of altitude. feed and period*

| Item            | Cu                      | Zn          | Mn        | Ca                      | P         | Mg        |
|-----------------|-------------------------|-------------|-----------|-------------------------|-----------|-----------|
| Sea level       | 73.69±1.25              | 138.14±3.83 | 1.68±0.05 | 11.44±0.17              | 4.88±0.1  | 2.83±0.05 |
| Medium altitude | 67.04±1.29              | 121.92±2.2  | 1.78±0.04 | 11.58±0.16              | 4.91±0.1  | 2.64±0.04 |
| P               |                         |             |           |                         |           |           |
| Pre-partum      | 68.15±1.28 <sup>B</sup> | 135.48±3.55 | 1.71±0.06 | 11.84±0.19 <sup>B</sup> | 4.77±0.13 | 2.67±0.04 |
| Partum          | 69.61±1.65 <sup>B</sup> | 125.37±4.6  | 1.67±0.06 | 10.95±0.21 <sup>A</sup> | 4.82±0.11 | 2.74±0.06 |
| Post-partum     | 73.9±1.75 <sup>A</sup>  | 131.46±4.08 | 1.8±0.06  | 11.74±0.2 <sup>B</sup>  | 5.1±0.12  | 2.82±0.06 |
| P               |                         |             |           |                         |           |           |
| Optimum energy  | 74.24±1.54              | 132.96±4.06 | 1.95±0.04 | 11.19±0.18              | 5.07±0.11 | 2.62±0.04 |
| Low energy      | 67.9±1.08               | 129.13±2.83 | 1.56±0.04 | 11.76±0.15              | 4.76±0.09 | 2.84±0.04 |

<sup>a-b</sup>: No difference between altitude\*period and feed\*period interactions with the same letter, <sup>A-B</sup>: There is no difference between the main effects of period with the same letter. Cu: copper; Zn: Zinc; Mn: manganese; Ca: calcium, P: phosphorus; Mg: magnesium

*Table 8. Investigation of mean serum Cu, Zn, Mn, Ca, P and Mg levels according to interaction effect of altitude, feed and period*

| Factor     | Category        | Pre-partum             | Partum                 | Post-partum            |
|------------|-----------------|------------------------|------------------------|------------------------|
| Cu (µg/dl) |                 |                        |                        |                        |
| Altitude   | Sea level       | 70.29±1.3              | 71.35±2.31             | 78.93±2.29             |
|            | Medium altitude | 66.01±2.17             | 67.52±2.32             | 67.65±2.29             |
| Feed       | Optimum Energy  | 71.89±1.94             | 71.37±2.88             | 79.51±2.78             |
|            | Low Energy      | 65.5±1.6               | 68.23±1.89             | 69.86±2.06             |
| Zn (µg/dl) |                 |                        |                        |                        |
| Altitude   | Sea level       | 139.25±5.58            | 135.01±7.63            | 140.17±6.68            |
|            | Medium altitude | 130.95±3.98            | 113.8±3.54             | 121.02±3.41            |
| Feed       | Optimum Energy  | 139.4±6.27             | 125.18±7.42            | 134.29±7.32            |
|            | Low Energy      | 132.54±4.08            | 125.51±5.89            | 129.35±4.63            |
| Mn (µg/dl) |                 |                        |                        |                        |
| Altitude   | Sea level       | 1.6±0.08               | 1.64±0.08              | 1.8±0.09               |
|            | Medium altitude | 1.85±0.08              | 1.71±0.07              | 1.79±0.07              |
| Feed       | Optimum Energy  | 1.84±0.07 <sup>b</sup> | 1.84±0.07 <sup>b</sup> | 2.15±0.07 <sup>a</sup> |
|            | Low Energy      | 1.6±0.08 <sup>c</sup>  | 1.55±0.08 <sup>c</sup> | 1.52±0.06 <sup>c</sup> |
| Ca (mg/dl) |                 |                        |                        |                        |
| Altitude   | Sea level       | 11.76±0.28             | 10.81±0.29             | 11.78±0.28             |
|            | Medium altitude | 11.94±0.26             | 11.11±0.29             | 11.7±0.29              |
| Feed       | Optimum Energy  | 11.52±0.29             | 10.49±0.34             | 11.56±0.28             |
|            | Low Energy      | 12.1±0.25              | 11.31±0.25             | 11.9±0.28              |
| P (mg/dl)  |                 |                        |                        |                        |
| Altitude   | Sea level       | 4.91±0.18              | 4.76±0.15              | 4.97±0.16              |
|            | Medium altitude | 4.6±0.17               | 4.89±0.17              | 5.25±0.17              |
| Feed       | Optimum Energy  | 5.12±0.22              | 4.9±0.17               | 5.19±0.18              |
|            | Low Energy      | 4.5±0.14               | 4.75±0.15              | 5.02±0.16              |
| Mg (mg/dl) |                 |                        |                        |                        |
| Altitude   | Sea level       | 2.69±0.06              | 2.8±0.09               | 2.99±0.08              |
|            | Medium altitude | 2.65±0.05              | 2.66±0.07              | 2.61±0.08              |
| Feed       | Optimum Energy  | 2.56±0.06              | 2.58±0.08              | 2.73±0.08              |
|            | Low Energy      | 2.76±0.05              | 2.86±0.08              | 2.89±0.09              |

<sup>a-b</sup>: No difference between altitude\*period and feed\*period interactions with the same letter