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Immune, stress, and redox status biomarkers in newborn calves: Dynamics in serum and saliva during the first week of life

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ABSTRACT

This study investigated serum and saliva immunity, stress, and redox status biomarkers in dairy calves during the first week of life. Blood and saliva samples were collected from 20 calves on days 0, 1, 2, and 7 for the analysis of biomarkers related to innate immunity (myeloperoxidase, calprotectin), cellular (adenosine deaminase), humoral immunity (gamma-glutamyl transferase), stress (α-amylase) and redox status (ferric reducing ability). Colostrum was given to all calves (3.8 ± 0.64 L) within 96 ± 73 min of birth. On day 7, some calves showed signs of neonatal calf diarrhea, most likely due to nutritional factors. All biomarkers were measurable in both fluids from day 0. Myeloperoxidase, Calprotectin and adenosine deaminase were higher in saliva than in serum and significantly increased after colostrum intake. Biomarkers of humoral immunity were higher in serum, increasing with colostrum intake. A positive correlation was found between gamma-glutamyl transferase and IgG and IgA in saliva. Biomarkers of stress and redox status did not appear to change with colostrum intake. Neonatal calf diarrhea led to increased concentrations of some of the biomarkers analyzed, such as myeloperoxidase and α-amylase. These results highlight the effect of colostrum on the cellular and humoral immunity of calves and provide insights into the potential use of saliva to monitor immune and stress status.

1. Introduction

During the first days of life, the calf experiences intense changes associated with the hypothalamus-pituitary-adrenal and inflammatory axis (HPA-immune axis; Hulbert and Moisá, 2016). These changes are triggered by parturition, dam-to-calf microbial transmission, and the acquisition of passive immunity through colostrum intake (Hulbert and Moisá, 2016). Despite the common misconception that calves are born without immunity (Hulbert and Moisá, 2016), it has been demonstrated that they are indeed protected by an innate immune system, possessing all the cellular components required for an adaptive immune response (Wilson et al., 1996), and humoral components such as the complement system (Chase et al., 2008). However, newborn calves have a naïve immune system and exhibit hypogammaglobulinemia, making them reliant on the transfer of passive immunity from colostrum (Barrington and Parish, 2001; Chase et al., 2008). Indeed, colostrum enhances humoral immunity and supports cellular immune responses (Silva et al., 2024). Nevertheless, stress and redox status during late gestation and at birth can influence immune cell function, such as neutrophils and macrophages (Barrington and Parish, 2001). Thus, identifying biomarkers for both cellular and humoral immunity, as well as stress and redox status, is important for monitoring and improving the welfare of newborn calves.

The immune system is a complex of cells and molecules that work

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together to defend the organism against infection. This defense can be organized into two fundamental responses, the innate and the acquired (adaptative) responses (Delves and Roitt, 2000). The innate immune system serves as the organism's first line of defense, relying on physical barriers such as the skin and mucosal surfaces (e.g., the digestive and respiratory tracts), as well as on immune cells, including leukocytes such as macrophages, neutrophils, eosinophils, and basophils (Hulbert and Moisá, 2016). However, newborn calves have a reduced number of immune cells and reduced phagocytic activity, which gradually increases and improves with age (Chase et al., 2008). Myeloperoxidase (Mpx) is an enzyme primarily expressed by neutrophils that exhibits antimicrobial properties by catalyzing the reaction between Cl− and H_2O_2 to produce hypochlorous acid (HOCl), a potent oxidant (Lin et al., 2024). Increased levels of this enzyme have been shown to be associated with inflammatory processes (Lin et al., 2024), and has been used as a biomarker of intramammary infection by estimating neutrophil activity (Cooray, 1994). Another key pro-inflammatory protein is calprotectin or S100 A8-A9, which is involved in cytokine production and leukocyte regulation (Yang et al., 2021). Calprotectin is highly expressed in the cytosolic content of neutrophils and macrophages (Karakus et al., 2023). Recent studies have shown increased salivary calprotectin levels in pigs with experimentally induced sepsis via lipopolysaccharide inoculation (López-Martínez et al., 2023) and elevated serum calprotectin in calves with neonatal diarrhea (Karakus et al., 2023). Additionally, higher serum concentrations have been suggested in cases of viral diseases (Aydin et al., 2022).

The acquired immunity is divided into cellular immunity, which is characterized by T-cell responses, and humoral immunity which involves the production of specific antibodies against pathogens. Adenosine deaminase (ADA) is an enzyme that plays a critical role in the growth and differentiation of T cells and macrophages/monocytes, and serves as an indicator of cellular immune response activity (Schaaf, 2009). Gamma-glutamyl transferase (GGT) is an enzyme involved in the transpeptidation of gamma-glutamyl groups to receptor molecules and is widely used as a biomarker for liver disease (Brennan et al., 2022). In newborn calves, serum GGT activity has been associated with serum IgG levels, providing an indirect method to evaluate the transfer of passive immunity (Parish et al., 1997; Sala et al., 2023). Additionally, GGT showed potential as a biomarker for oxidative stress due to its role in glutathione metabolism, which supports intracellular antioxidant defenses (Anupam et al., 2025). Beyond oxidative stress, GGT has been linked to various health conditions, including cardiovascular diseases (Brennan et al., 2022) and higher mortality risk (Cho et al., 2023).

The calving process can be a stressful event for both cow and calf (Murray and Leslie, 2013), thus stress biomarkers are also valuable to assess post-partum physiological responses. Alpha-amylase activity is a biomarker indicative of the sympathetic nervous system activity (Fujisawa et al., 2021; Pagen et al., 2021). Salivary alpha-amylase (sAA) has been shown to increase in cows during painful and stressful events, at calving (Contreras-Aguilar et al., 2021) and in cows with mastitis (Contreras-Aguilar et al., 2019). It has also been found at higher concentrations in colostrum compared to milk in cows (Yang et al., 2014) and in sows (Botía et al., 2024).

Redox status, defined as the balance between the generation of reactive oxygen species (ROS) and antioxidant molecules, is vital for the organism's homeostasis. During the first weeks of life, calves can be particularly prone to oxidative stress (Abuelo et al., 2019). Therefore, it is important to assess redox balance during this period. Antioxidant capacity can be assessed using several biomarkers (Rubio et al., 2019). Among these, the ferric reducing ability (FRA) of saliva has been shown to increase in calves during stressful situations in response to oxidative stress, and was also negatively correlated with oxytocin and positively correlated with cortisol (Rubio et al., 2021).

This study hypothesizes that serum and saliva immune, stress, and redox status biomarkers undergo significant changes during the first days of life in calves, particularly in response to colostrum intake. Therefore, this study had the objective of measuring serum and saliva immunity, stress, and redox status biomarkers in dairy calves after colostrum intake and during the first week of life. Blood and saliva samples were collected from 20 calves on days 0, 1, 2, and 7 post-birth for quantification of biomarkers related to innate immunity (myeloperoxidase, calprotectin), cellular immunity (adenosine deaminase), humoral immunity (gamma-glutamyl transferase), stress (α-amylase) and redox status (ferric reducing ability). At day 7 some calves had diarrhea most likely due to nutritional factors and their changes were compared with the calves without clinical signs.

2. Materials and methods

2.1. Animals and management

The study was approved by the Ethics Committee for Animal Welfare (ORBEA) at Universidade Trás-os-Montes e Alto Douro (UTAD, Portugal) under the reference 2664-e-DZ-2023. A full description of animal selection, colostrum management, and health assessment is provided in a previous publication (Silva et al., 2025a). Briefly, twenty dairy calves (14 Friesian and 6 cross-Friesian) from a commercial dairy farm located in Évora (Portugal) were included in this study. Calves were delivered by cows that required no assistance or easy assistance (i.e., simple hand traction from the caretaker without any obstetric device) and were not allowed to suck colostrum from the dam. Calves were allocated in individual straw-bedded pens before colostrum feeding. Colostrum, from the farm colostrum bank, was fed (3.8 ± 0.64 L) within 96 ± 73 min after birth through a bottle with a nipple or with an esophageal tube when the calf was reluctant to suckle the amount established by the farm personnel. Colostrum had an IgG concentration of 44.7 ± 16.6 g/L (mean ± SD), evaluated by ELISA in a previous study (Silva et al., 2025a). Calves were then fed transition milk for 1–3 days, followed by milk replacer (Bovimilk, Vetlima, Vila Nova da Rainha, Portugal), provided twice daily (i.e., 3 L at 0700 and 3 L at 1600 h) during the trial period. Calves had free access to fresh water and health treatments were provided by farm personnel. Calves' health assessment was performed on D0, D1, D2, and D7 with an adapted version of the Wisconsin calf health scoring system, scoring each parameter from 0 (normal) to 3 (very abnormal) (McCarthy et al., 2021; McGuirk and Peek, 2014; Renaud et al., 2018; Steerforth and Van Winden, 2018). At the first sign of neonatal calf diarrhea, calves were provided twice daily with an oral electrolyte solution (ReVital™; R2 Agro A/S, Hedensted, Denmark) and antibiotic treatment, according to the farm protocol. On D7, 9 calves (45 %) showed signs of neonatal calf diarrhea (NCD), with the first signs (changes in fecal consistency) appearing between D4 and D7. These calves had watery feces that sifted through the bedding, and most (n = 7) showed signs of dehydration, ranging from 6 to 8 % (n = 5) to 8–10 % (n = 2), as assessed by the skin elasticity test and visual observation of the degree of enophthalmos (Renaud et al., 2018) and were generally less responsive than the healthy calves. However, these calves did not show signs of systemic illness, as they had rectal temperatures within the normal range (38.64 ± 0.13 ºC; always < 39.4 ºC), no blood in the feces, and no inappetence (McGuirk, 2008). However, one calf was very depressed, had a weak sucking reflex and was moderately dehydrated (8–10 %) on examination, but still had a normal rectal temperature (38.6 ℃). There were no cases of respiratory disease or omphalitis during the study.

2.2. Sample collection

From each calf, saliva and serum samples were collected 30 min before colostrum intake (D0), 24 h (D1), 48 h (D2), and 168 h after birth (D7). Saliva samples were collected using Salivette cotton swabs (Sarstedt GmbH, Nümbrecht, Germany) held alternately on both sides of the mouth for 1 min or until the cotton was saturated. To avoid contamination with milk residues, samples were collected at least 2 h after the

previous meal. Blood samples were then collected from the jugular vein and transferred into serum collection tubes (4.9 mL, Primavette, Germany). Samples were transported refrigerated on the same day of collection to the Laboratory of Oral Biology and Salivary Proteomics at the Universidade de Évora, Portugal. Saliva samples were centrifuged (Z 323 K, Hermle LaborTechnik GmbH, Wehingen, Germany) at 9000 × *g* for 5 min at 4 ºC (Lamy et al., 2017). Serum tubes were centrifuged at 1660 × *g* for 10 min at 24 ºC. Aliquots of saliva and serum were prepared and stored at −80 ºC until analysis.

2.3. Laboratory analysis

Immune and stress biomarkers were analyzed in saliva and serum samples with an automated chemistry analyzer (Olympus Diagnostics GmbH, Hamburg, Germany) at the University of Murcia, Spain.

2.3.1. Biomarkers related with the immune system

Cellular immunity biomarkers: the Mpx activity was analyzed with an automated assay based on a spectrophotometric manual assay (Krueger et al., 1990). The calprotectin concentration was analyzed with the BÜHLMANN fCal Turbo® assay kit (BÜHLMANN, Laboratories AG, Schönenbuch, Switzerland). The ADA activity was measured with a commercially spectrophotometric automated assay (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA).

Humoral immunity biomarkers: the GGT activity was measured with a commercial assay (OSR6020, Beckman Coulter Inc., Fullerton, CA, USA). The IgG and IgA concentrations were analyzed with commercial ELISA kits (Bethyl Laboratories, Montgomery, TX, USA), as previously described (Silva et al., 2025a).

2.3.2. Biomarkers related with stress

The α-amylase activity was measured using a commercial kit (α-Amylase, OSR6182, Beckman Coulter).

2.3.3. Biomarkers related with redox status

The FRA was used as a measure of oxidative stress, following the method of Benzie and Strain (1996).

The calprotectin, Mpx, and FRA analyses were performed using an Olympus AU400® Chemistry Analyzer (Olympus Diagnostics GmbH, Hamburg, Germany), and the ADA, GGT, and α-amylase with an Olympus Diagnostica GmbH AU 600 (Beckman Coulter, Ennis, Ireland).

2.4. Statistical analysis

Statistical analyses were performed with R (R Core Team, 2024). Graphics were created with Graph Pad (GraphPad Prism, version 9 for Windows, Graph Pad Software Inc., San Diego, CA, USA). A Linear Mixed-Effects Model (LMM) was applied with time (D0, D1, D2, and D7) and sample type (serum and saliva) as fixed effects, calf as a random factor, and Mpx, calprotectin, ADA, GGT, FRA, and α-amylase as dependent variables, using the lme4 package (Bates et al., 2015). Only calves without any sign of disease (n = 20 on D0, D1, and D2, and n = 11 on D7) were included. The Restricted Maximum Likelihood (REML) method was used for model fitting. Post-hoc comparisons were conducted with Tukey adjustment. Correlations between analytes were assessed using Spearman's rank coefficients. The concentration of the analytes in the serum and saliva was compared between healthy calves (HC) and calves with NCD at D7 using a Linear Model and at D0, D1, and D2 using a LMM. The IgG and IgA data reported in a previous publication (Silva et al., 2025b) was used in the correlation analysis.

The Shapiro-Wilk test and normal probability plots were used to evaluate the normality of continuous variables. Homoscedasticity was assessed by visual inspection of scatterplots of residuals against predicted values. When the abovementioned assumptions were not met, variables were log10-transformed. The results from the transformed variables were back-transformed and reported as geometric Mean [CI at 95 %]. The results from the non-transformed variables were reported as least square means (LSM) ± standard error of the mean (SEM). Statistical differences were considered when $P < 0.05$.

3. Results

3.1. Immune biomarkers

Among the immunity-related biomarkers (calprotectin, Mpx, and ADA; Fig. 1), Mpx activity did not show significant changes over time in either serum (27.4 [19.4, 38.5] UI/L; $P \geq 0.994$) or saliva (101.6 [72.3, 142.9] UI/L; $P \geq 0.055$). The Mpx concentrations were higher ($P < 0.001$) in saliva than in serum on D0 (159.19 [91.50, 276.94] and 26.22 [14.86, 46.26] UI/L, respectively) and on D1 (173.47 [99.72, 301.81] and 32.31 [18.57, 56.21] UI/L, respectively) but were similar on D2 (88.94 [51.13 [154.73] and 30.63 [17.61, 53.29] UI/L, respectively; $P = 0.100$) and on D7 (43.42 [20.63, 91.38] and 21.60 [10.26, 45.46] UI/L, respectively; $P = 0.869$). Serum calprotectin concentration on D7 (0.04 [0.027, 0.059] mg/mL) was lower than on D0 (0.109 [0.081, 0.148] mg/mL), D1 (0.145 [0.108, 0.194] mg/mL), and D2 (0.087 [0.065, 0.116] mg/mL; $P \leq 0.022$). Saliva calprotectin concentration was higher on D1 (0.507 [0.379, 0.680] mg/L) and on D7 (0.384 [0.261, 0.565] mg/L) compared to D0 (0.151 [0.112, 0.204] mg/L; $P < 0.001$). Calprotectin concentrations were similar in serum and saliva on D0 ($P = 0.720$) but were significantly higher in saliva on all subsequent days ($P < 0.001$).

Serum ADA concentration was higher at D1 (10.44 [7.470, 14.600] UI/L; $P < 0.001$) compared to D0 (2.860 [2.030, 4.030] UI/L), D2 (3.060 [2.190, 4270] UI/L), and D7 (2.420 [1.550, 3.790] UI/L). Saliva ADA concentration was significantly higher on D1 (18.29 [13.09, 25.57] UI/L) and on D2 (20.54 [14.69, 28.71] UI/L) compared to D0 (7.49 [5.31, 10.56] UI/L; $P \leq 0.004$). ADA activity was similar between serum and saliva on D1 ($P = 0.212$) but was significantly higher in saliva on D0, D2, and D7 ($P < 0.001$).

Serum GGT concentration was lower on D0 (11.90 [9.63, 14.70] UI/L; $P < 0.001$) compared to D1 (1727.90 [1405.57, 2124.00] UI/L), but then decreased on D2 (983.60 [800.13, 1209.10] UI/L; $P = 0.003$) and further on D7 (383.50 [290.48, 506.20] UI/L; $P < 0.001$; Fig. 2). Saliva GGT concentration on D1 (107.80 [87.65, 132.50] UI/L) was significantly higher than the concentration on D2 (63.00 [51.24, 77.40] UI/L; $P = 0.006$) and D7 (55.50 [74.60, 42.79] UI/L; $P = 0.005$), but the concentration on D0 (88.10 [71.26, 108.80] UI/L) did not differ from the concentrations on any of the days ($P \geq 0.170$). The GGT concentrations were generally higher in serum than in saliva ($P < 0.001$), except on D0, where the GGT concentrations were higher in saliva than in serum ($P < 0.001$). Serum and saliva IgG and IgA concentrations were particularly increased at D1, as detailed in a previous publication (Silva et al., 2025b). Changes in these concentrations in calves without signs of disease are shown in Fig. 3. Serum IgG concentration increased from D0 (0.54 ± 0.99 mg/mL) to D1 (18.24 ± 0.97 mg/mL) and then decreased on D2 (16.93 ± 0.97 mg/mL) and on D7 (14.27 ± 1.30 mg/mL; $P < 0.001$). Saliva IgG concentration increased from D0 (0.004 ± 0.01 mg/mL) to D1 (0.12 ± 0.01 mg/mL) and then decreased on D2 (0.05 ± 0.01 mg/mL) without further changes on D7 (0.03 ± 0.01 mg/mL; $P < 0.001$). Serum IgA concentration increased from D0 (2.91 ± 0.51 μg/mL) to D1 (2256.64 ± 386.41 μg/mL) and then decreased on D2 (1285.27 ± 220.08 μg/mL) and again on D7 (206.87 ± 47.77 μg/mL; $P < 0.001$). Saliva IgA concentration increased from D0 (36.89 ± 4.42 μg/mL) to D1 (69.56 ± 8.12 μg/mL) and then decreased on D2 (35.19 ± 4.11 μg/mL) without further changes on D7 (34.0 ± 5.32 μg/mL; $P < 0.001$).

3.2. Stress and redox status biomarkers

Regarding the stress biomarkers (Fig. 4), the α-amylase activity in serum was significantly lower on D7 (125.12 ± 8.74 UI/L) compared to

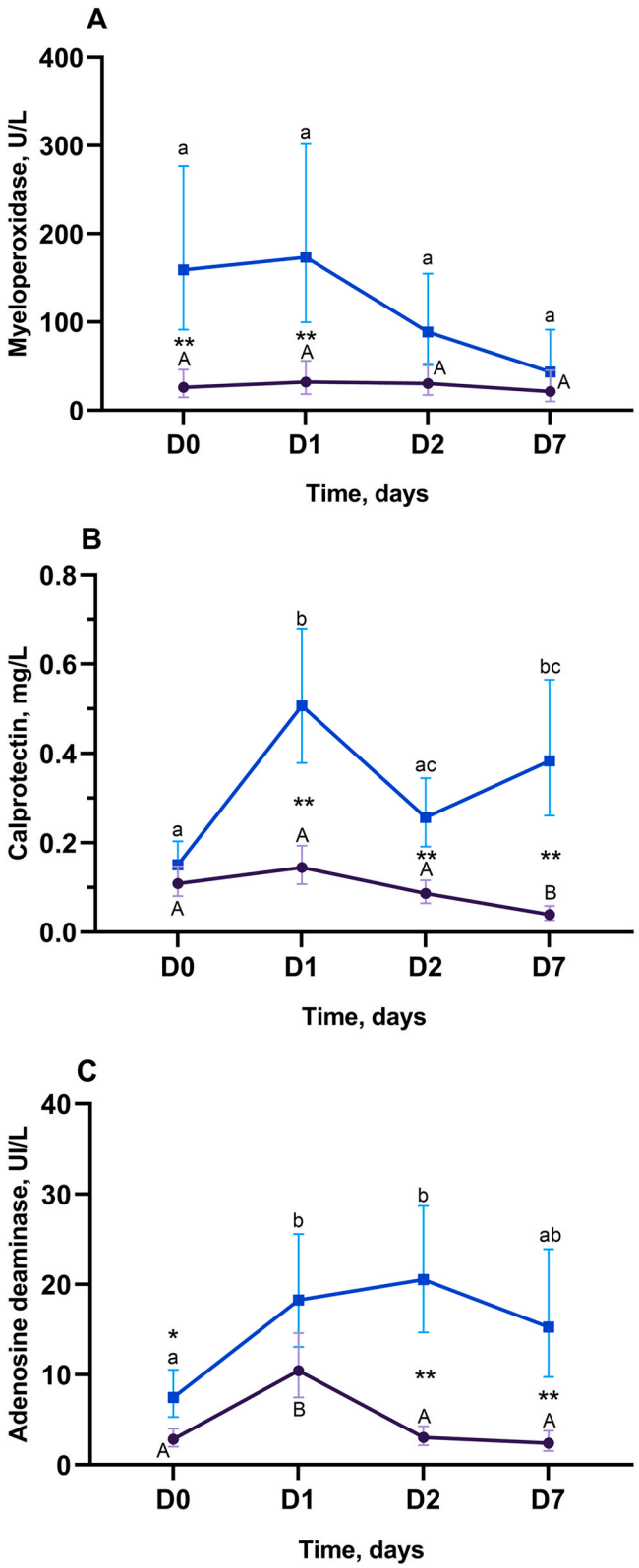

Fig. 1. Changes in Mpx (**A**), calprotectin (**B**), and ADA (**C**) in serum (●) and saliva (■) across time (i.e., D0, D1, D2, and D7; expressed as geometric mean ± CI at 95%). Upper- and lower-case letters represent significant differences ($P < 0.05$) across time in serum and saliva, respectively. Significant differences between sample fluids (i.e., serum and saliva) are indicated on each day with * ($P < 0.05$) and ** ($P < 0.01$).

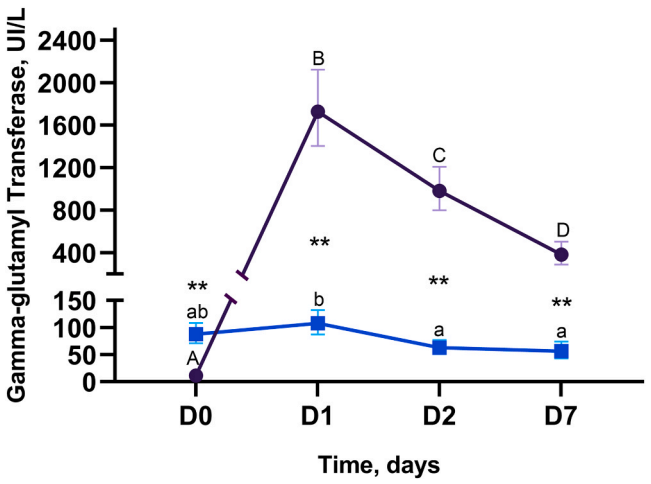

Fig. 2. Changes in GGT in serum (●) and saliva (■) across time (i.e., D0, D1, D2, and D7; expressed as geometric mean ± CI at 95%). Upper- and lower-case letters represent significant differences ($P < 0.05$) across time in serum and saliva, respectively. Significant differences between sample fluids (i.e., serum and saliva) are indicated on each day with ** ($P < 0.01$).

D2 (158.31 ± 6.90 UI/L; $P = 0.014$), but was similar to the activity on D0 (151.55 ± 7.09 UI/L; $P = 0.109$) and on D1 (150.84 ± 6.96 UI/L; $P = 0.124$), without any significant differences between D0, D1, and D2 ($P \geq 0.979$). The α-amylase activity in saliva (8.75 ± 5.25 UI/L) was similar across time ($P \leq 0.626$). Likewise, serum FRA concentrations were similar across the experimental period (0.629 ± 0.01 mmol/L; $P \geq 0.974$). In saliva, FRA concentration was higher on D1 (0.251 ± 0.02) compared to D0 (0.159 ± 0.02; $P = 0.046$) and D2 (0.145 ± 0.02; $P = 0.011$), while the concentration on D7 (0.201 ± 0.03) was similar to the other days ($P \geq 0.771$). Alpha-amylase and FRA concentrations were consistently higher in serum than in saliva throughout the experimental period ($P < 0.001$).

3.3. Correlations among serum and saliva biomarkers

In saliva, calprotectin was correlated with ADA activity ($\rho = 0.59$; $P < 0.001$) and with IgG concentration ($\rho = 0.61$; $P < 0.001$). In serum, the ADA activity was correlated with GGT ($\rho = 0.53$; $P < 0.001$), IgG ($\rho = 0.40$; $P < 0.001$), and IgA ($\rho = 0.44$; $P < 0.001$), while in saliva, ADA activity was only correlated with IgA ($\rho = 0.45$; $P < 0.001$). Serum GGT was correlated with serum IgA ($\rho = 0.71$; $P < 0.001$) and IgG ($\rho = 0.83$, $P < 0.001$), and saliva GGT was also correlated with saliva IgA ($\rho = 0.63$; $P < 0.001$) and IgG ($\rho = 0.37$; $P = 0.002$). Despite showing similar temporal dynamics, no direct correlations were found between serum and saliva biomarkers. Interestingly, serum GGT activity was strongly correlated with saliva IgG ($\rho = 0.76$; $P < 0.001$). In serum, FRA concentration was positively correlated with Mpx activity ($\rho = 0.51$; $P < 0.001$), whereas in saliva it was negatively correlated ($\rho = -0.24$; $P = 0.048$), although the latter showed only a weak and borderline significant association.

3.4. Neonatal calf diarrhea

The HC group was compared with the NCD group on D7, when clinical signs of diarrhea were present in all affected calves (Table 1). Serum Mpx activity was significantly higher in NCD calves (73.8 [33.6, 162.2]) than in HC (20.5 [10.0, 41.7]; $P = 0.021$). In saliva, no significant differences in Mpx activity were observed between groups ($P = 0.140$). Serum calprotectin concentration showed a tendency to be higher in the NCD group (0.075 [0.044, 0.126]) than in the HC group (0.039 [0.025, 0.063]; $P = 0.072$). A similar pattern was observed in saliva ($P = 0.111$).

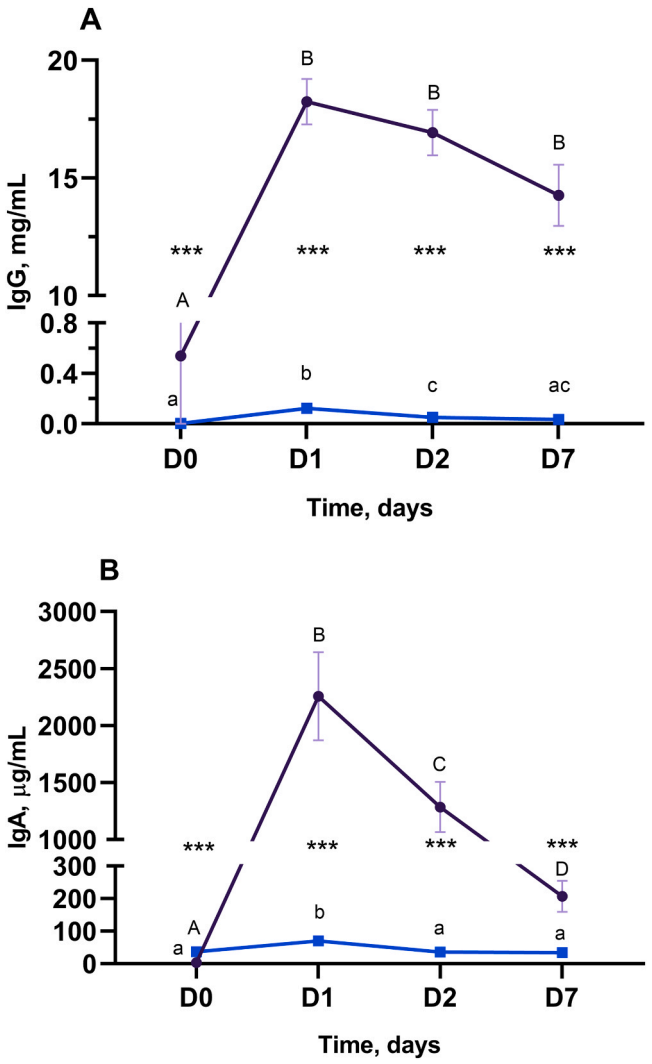

Fig. 3. Changes in immunoglobulin G (**A**) and in immunoglobulin A (**B**) in serum (●) and saliva (■) across time (i.e., D0, D1, D2, and D7; expressed as LSM ± SEM). Upper- and lower-case letters represent significant differences ($P < 0.05$) across time in serum and saliva, respectively. Significant differences between sample fluids (i.e., serum and saliva) are indicated on each day with *** ($P < 0.001$).

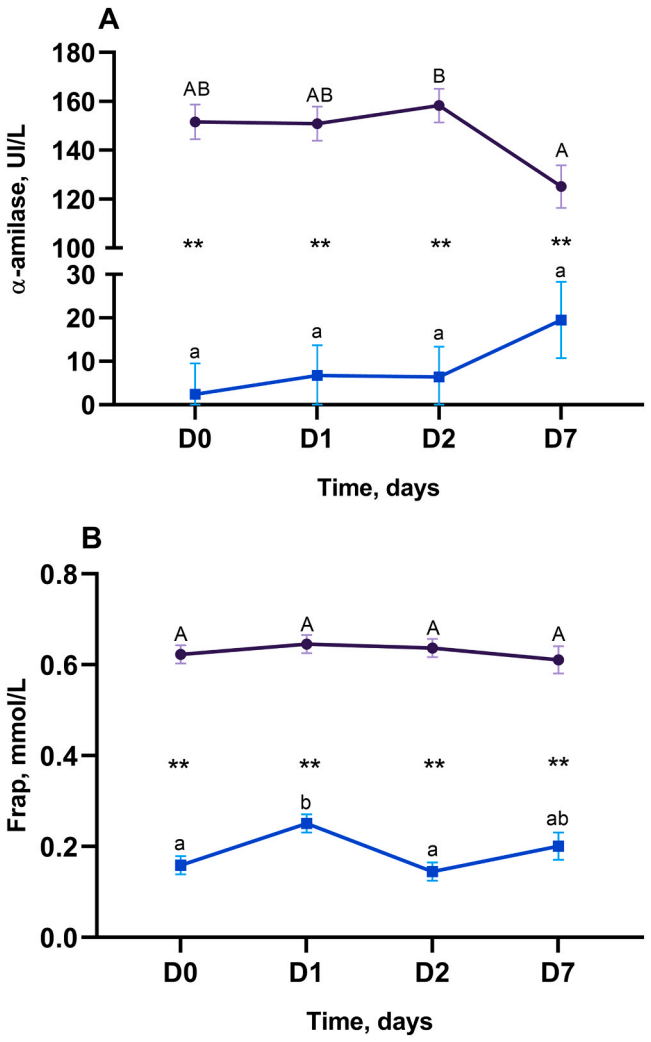

Fig. 4. Changes in α-amylase (**A**) and in FRA (**B**) in serum (●) and saliva (■) across time (i.e., D0, D1, D2, and D7; expressed as LSM ± SEM). Upper- and lower-case letters represent significant differences ($P < 0.05$) across time in serum and saliva, respectively. Significant differences between sample fluids (i.e., serum and saliva) are indicated on each day with ** ($P < 0.01$).

In saliva, the GGT activity showed a tendency to be higher in calves from the NCD group (82.0 [60.1, 112.0]) compared to HC (57.4 [43.3, 76.0]; $P = 0.090$), but the same was not observed in serum ($P = 0.824$). Serum α-amylase activity was also increased in NCD calves (182 ± 14.2) compared to HC (115.0 ± 12.9; $P = 0.002$), but not salivary α-amylase ($P = 0.472$). In saliva, the FRA concentration showed a tendency to be higher in HC (0.197 [0.138, 0.280]) compared to NCD (0.118 [0.08, 0.175]; $P = 0.059$), but not in serum ($P = 0.729$).

Changes in these biomarkers could have occurred before clinical signs were identified, therefore the same analyte concentrations were tested for D0, D1, and D2 in serum and saliva, but no differences were observed, except for serum α-amylase ($P > 0.05$; Table S1 in the supplementary file). Serum α-amylase was higher in NCD than in HC from D0 to D7 ($P = 0.002$; Table 2 and Figure S1 in the supplementary file).

4. Discussion

This study shows that serum and saliva biomarkers undergo significant changes during the first days of life in newborn calves. While innate (calprotectin, Mpx) and cellular (ADA) immunity biomarkers were found at higher concentrations in saliva, humoral immunity (GGT), stress, and redox status biomarkers were higher in serum.

The finding that biomarkers related to innate and cellular immunity were higher in saliva than in serum suggests that immune defense mechanisms at mucosal surfaces are more active than those associated with systemic immune responses at this early age. This pattern was observed for both innate immunity cellular mechanisms (i.e., calprotectin and Mpx) and acquired immunity mechanisms (i.e., ADA). A possible explanation is that the calf is exposed early to microorganisms from both the dam and the environment (Owens et al., 2021), with initial colonization occurring mainly through the nasal and oral mucosa (Stilling et al., 2014). It is also important to consider the dynamic shifts in leukocyte populations that occur in newborn calves during this period. Neonates typically exhibit rapid changes in white blood cell counts, including a transient neutrophilia (Tennant et al., 1974), alongside significant maturation and redistribution of lymphocyte populations (Morita et al., 2022; Panousis et al., 2018). Nevertheless, the precise nature of these variations during the first week remains somewhat unclear and appears to be influenced by hormonal changes post-birth, including corticosteroids (Panousis et al., 2018; Tennant et al., 1974), and potentially other molecules related to colostrum intake and metabolic adaptation (Grigaleviciute et al., 2023). These

Table 1
Immune (MPx, calprotectin, ADA, and GGT), stress (α-amylase), and redox status (FRA) biomarkers in one week old (D7) healthy calves (HC; n = 11) and calves with neonatal diarrhea (NCD; n = 9). Results are expressed as geometric mean [CI at 95 %] unless specified.

Analyte	Fluid	HC	NCD	*P*-value*
MPx	Serum	20.5 [10.0, 41.7]	73.8 [33.6, 162.2]	0.021*
	Saliva	41.2 [15.5, 109.0]	108.0 [36.7, 318]	0.140
Calprotectin	Serum	0.039 [0.025, 0.063]	0.075 [0.044, 0.126]	0.072
	Saliva	0.379 [0.217, 0.661]	0.734 [0.396, 1.359]	0.111
ADA	Serum	2.5 [1.98, 3.20]	2.7 [2.06, 3.51]	0.697
	Saliva	15.9 [9.9, 25.4]	21.4 [12.7, 36.0]	0.385
GGT	Serum	389.0 [289.0, 525.0]	371.0 [267.0, 516.0]	0.824
	Saliva	57.4 [43.3, 76.0]	82.0 [60.1, 112.0]	0.090
α-amylase	Serum[a]	115.0 ± 12.9	182 ± 14.2	0.002*
	Saliva	6.64 [4.19, 10.5]	8.44 [5.08, 14.0]	0.472
FRA	Serum[a]	0.621 ± 0.02	0.633 ± 0.02	0.729
	Saliva	0.197 [0.138, 0.280]	0.118 [0.08, 0.175]	0.059

[a] Results expressed as LSM ± SEM; *significancy (*t*-test) between groups (HC and NCD). MPx, myeloperoxidase; ADA, adenosine deaminase; GGT, gamma-glutamyltransferase; FRA, ferric reducing ability.

Table 2
Serum α-amylase concentrations across the experimental period (D0, D1, D2, and D7) in healthy calves (HC = 11) and in calves with neonatal diarrhea (NCD = 9). Results are expressed as geometric mean [CI at 95 %].

	Fixed factor, Health Status		
Time	HC	NCD	*P*-value
D0	123.0 [105.0, 144.1]	175.2 [146.7, 209.2]	0.082
D1	120.1 [102.5, 140.7]	181.5 [142.4, 216.2]	0.025
D2	129.3 [110.4, 151.5]	186.3 [156.4, 221.9]	0.064
D7	111.6 [95.3, 130.7]	175.4 [147.2, 208.9]	0.011

physiological shifts in white blood cells relative abundance and activity likely contribute to the observed concentrations and varying patterns of innate and cellular immunity biomarkers.

Although no significant differences were observed across days in salivary Mpx activity, the inflammatory response associated with the calving process (Murray and Leslie, 2013) may explain the pattern observed. In contrast to Mpx, salivary calprotectin concentration and ADA activity increased following colostrum intake, which may be attributed to the presence of these proteins in colostrum. Previous studies have shown higher ADA concentrations in colostrum compared to milk in sows (Botía et al., 2024) and cows (Sato et al., 2010). After absorption, colostrum leukocytes can migrate to Peyer's patches and mesenteric lymph nodes or may reach other organs, such as the spleen or liver (Liebler-Tenorio et al., 2002; Reber et al., 2006), which may explain the decrease of ADA concentrations in serum after D1. Nevertheless, the present results support that colostrum intake influences the regulation of cellular immunity.

In the present study, GGT activity increased in both serum and saliva following colostrum intake and was correlated with IgG and IgA concentrations in both fluids. Serum GGT has been used in previous studies to evaluate the transfer of passive immunity, showing moderate correlations (Parish et al., 1997; Sala et al., 2023). To the authors' knowledge, the relationship between salivary GGT activity and the transfer of passive immunity has not been previously studied, although serum GGT in this study correlated with salivary IgG concentrations, suggesting a potential link that warrants further investigation.

The calf's oxidative/antioxidative profile can be influenced by the redox balance of colostrum (Abuelo et al., 2014), which may explain the higher FRA concentration observed in saliva. Furthermore, a positive correlation between FRA and IgG concentration has been reported in sheep colostrum ($r = 0.256$; $P < 0.05$; Guiso et al., 2022), supporting the antioxidant proprieties of colostrum.

Salivary α-amylase is a recognized biomarker of the autonomic nervous system (dys)function (Ali and Nater, 2020), and has been used to assess stress in several species. Examples include pain-related stress in horses (Contreras-Aguilar et al., 2018a) and dogs (Kang et al., 2022), as well as acute stress in pigs induced by a nasal snare restraining technique (Contreras-Aguilar et al., 2018b). In the present study, serum α-amylase concentration decreased by D7 to levels similar to those reported in healthy 2–4-month-old calves (Zendehbad et al., 2013), suggesting that postpartum stress pressisted for at least 2 days and stabilized by the end of the first week of life.

The NCD is a multifactorial disease that can be triggered by both infectious and non-infectious factors. However, without specimen identification, the diagnostic outcome is compromised (Cho and Yoon, 2014). The most likely cause of the NCD observed in this study was non-infectious, potentially triggered by a dietary change associated with the abrupt transition from maternal milk to milk replacer, as the calves showed no signs of systemic illness. Milk was provided in a fixed volume rather than adjusted to the calf's body weight, which may explain why not all calves were affected. Additionally, factors such as peripartum calving management, calf immunity, and environmental stress cannot be excluded as potential factors contributing to the development of neonatal calf diarrhea (Cho and Yoon, 2014; Klein-Jöbstl et al., 2014). Additionally, calves with NCD had lower saliva IgA and IgM concentrations on D1 compared to HC, which may have limited mucosal defense (Silva et al., 2025a). It is not unlikely that concomitant infection occurred after the initial symptoms, with *Cryptosporidium spp*. being a plausible candidate due to its high prevalence on dairy farms and the similarity of its typical symptoms to those observed in this study (Vermunt, 1994). However, since calves were not monitored beyond the first week, and specimen identification was not performed, coupled with the routine administration of antibiotics at the onset of diarrhea as per farm protocol, the exact cause remains uncertain.

Nevertheless, these calves exhibited an inflammatory status, as evidenced by higher MPx and calprotectin activities in serum compared to HC. An increase in serum calprotectin concentrations has been previously documented in calves with NCD (Aydin et al., 2022; Karakus et al., 2023), however, to our knowledge, this has not been reported in calf saliva, although it has been observed in pigs (Ortín-Bustillo et al., 2023). No statistically significant differences were observed for salivary MPx and calprotectin, likely due to the sample size, as the P-values were very close to the significance threshold. Newborn calves have an immature adaptive immune system, as reflected by ADA concentrations. The values reported in this study were considerably lower than those reported by Yarim et al. (2016) in calves aged 10–30 d, in which ADA activity decreased in response to *Cryptosporidium spp*. infection. Additionally, the NCD group showed a wider range of values for innate immunity biomarkers compared to HC, an observation that may be related to the varying degrees of dehydration observed. Calves with enteritis may experience pain (Studds et al., 2018), therefore it would be possible to observe differences in α-amylase between groups. In this study calves with NCD had higher serum α-amylase activity compared to HC, but this was not demonstrated in saliva. To our knowledge, no other studies have investigated α-amylase in association with NCD. Indeed, hyperamylasemia has been related to gastroenteritis in humans (Ben-Horin et al., 2002). However, the NCD group showed higher concentrations of serum α-amylase than the HC group at all time points. While these findings may suggest a possible association with gastrointestinal disorders, they are not sufficient to support the use of serum α-amylase as an early biomarker of enteric disease. The observed differences may instead reflect pre-natal or post-natal physiological conditions that predispose some calves to gastrointestinal problems.

The FRA in serum, which can be considered a measure of the total antioxidant capacity, was not affected by NCD, similar to the results

from Fu et al. (2024). However, the tendency for reduced FRA in saliva in the NCD group could have been related to the oxidative mechanisms of the innate immunity response, which can be corroborated by the negative correlation observed between FRA and Mpx in saliva. A decrease in serum oxidative stress biomarkers was observed as calves recovered from diarrhea (Fu et al., 2024). So, additional measurements in the following days could have shown us additionally details, and a delayed response increasing FRA levels would be expected as stress faded (Rubio et al., 2021).

Future research could strengthen these findings by including a panel of early-life biomarkers, such as intestinal fatty acid–binding protein (I-FABP; enterocyte damage), mucosal repair markers like intestinal alkaline phosphatase and trefoil factor 3 (Durgut and Ok, 2023; Ok et al., 2020), and inflammatory proteins such as IL-8, serum amyloid A, or haptoglobin (Choi et al., 2021; El-Deeb et al., 2022). These biomarkers have shown significant elevations in neonatal calves with diarrhea compared to healthy controls, and are increasingly used in human neonatal research for gut injury detection (Howarth et al., 2022).

It is important to point out that this report has various limitations. First, this is a pilot study, and these results should be confirmed in more animals. In addition, although we followed calves during the first week of life, blood and saliva samples were not taken daily. As a result, we can only infer the trends in analyte variation between D2 and D7. Also, all calves received colostrum, which prevents direct comparison with calves deprived of colostrum, limiting our ability to confirm suggested mechanisms regarding variations of biomarkers due to colostrum intake. The limited research on colostrum biomarkers highlights the need to investigate their variability and influencing factors, to better understand the impact of colostrum intake on early calf immune responses.

5. Conclusions

This study demonstrates that immune and stress biomarkers change in serum and in saliva during the first week of life in newborn calves. Myeloperoxidase, Calprotectin, and adenosine deaminase were higher in saliva than in serum and significantly increased after colostrum intake. Biomarkers of humoral immunity were higher in serum, increasing after colostrum intake. Stress and redox status biomarkers did not appear to change with colostrum intake. These results highlight the effect of colostrum on the calf cellular and humoral immunity and provide information regarding the potential use of saliva to monitor immune and stress status in calves.

CRediT authorship contribution statement

E. Lamy: Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **F.G. Silva:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Conceptualization. **C. Conceição:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **J.J. Cerón:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **A. Muñoz:** Writing – review & editing, Validation, Investigation. **L. Pardo-Marín:** Writing – review & editing, Validation, Investigation. **S.R. Silva:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **J.O.L. Cerqueira:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Ethical statement

The authors confirm that the ethical policies of the Veterinary Journal, as noted on the Journal's author guidelines page, have been adhered to. This study was approved by the Ethics Committee for Animal Welfare (ORBEA) at Universidade Trás-os-Montes e Alto Douro (UTAD, Portugal) under the reference 2664-e-DZ-2023.

Declaration of Generative AI and AI-assisted technologies in the writing process

No Generative AI and AI-assisted technologies were used in the writing process.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Silva reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106524.

Data availability

Data used for this study are available under request to the corresponding author.

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