J. Dairy Sci. 109:10043–10056
https://doi.org/10.3168/jds.2025-28193

Exploring the sources of variation in the serum metabolic profile of Italian Mediterranean buffaloes

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ABSTRACT

This cross-sectional study investigated the main sources of variation in key blood metabolites related to energy, protein, and hepatic-muscular profiles in lactating Italian Mediterranean buffaloes, focusing on the effects of parity, DIM, season, and ECM. Blood samples were collected from 855 clinically healthy lactating buffaloes for serum analysis. Metabolites were grouped into 3 functional profiles: energy profile, including glucose, cholesterol (CHOL), triglycerides (TRI), nonesterified fatty acids (NEFA), and BHB; protein profile, including total protein, albumin (ALB), globulin (GLO), urea, creatinine (CREA), and albumin-to-globulin ratio (A/G); and hepatic-muscular profile, including alanine aminotransferase (GPT), aspartate aminotransferase (GOT), alkaline phosphatase, lactate dehydrogenase (LDH), γ-glutamyl transferase (GGT), creatine kinase (CK), total bilirubin, high-density lipoproteins (HDL), and low-density lipoproteins (LDL). Mixed linear models were used to assess metabolites variation, with parity, DIM, season, and ECM classes as fixed effects and herd sampling day as a random effect. Overall, most metabolites showed limited variability and remained within physiological ranges, confirming the metabolic stability of the species. Parity significantly affected several indicators. Cholesterol exhibited a nonlinear pattern, peaking in third-parity animals (126 mg/dL). Total protein and GLO increased progressively with parity (from 6.69 to 6.95 g/dL and from 3.44 to 3.80 g/dL, respectively), whereas ALB and A/G were lowest in animals with parity ≥5 (3.16 g/dL and 0.82, respectively). Several hepatic-muscular biomarkers, including GOT, LDH, CK, and HDL, generally decreased with increasing parity. Days in milk were significantly associated with metabolic variation. Nonesterified fatty acids and BHB peaked during early lactation (≤75 DIM) and decreased thereafter (0.39–0.25 mmol/L, 0.40–0.35 mmol/L, respectively), reflecting progressive restoration of energy balance. Cholesterol increased after 50 DIM, whereas TRI reached the lowest concentrations between 51 and 75 DIM before increasing in late lactation. Total protein, ALB, and GLO were lowest at 116 to 140 DIM and subsequently increased, whereas CREA and urea rose progressively in late lactation. Aspartate aminotransferase peaked within the first 50 DIM (136 U/L) and decreased thereafter, whereas GPT and LDL increased after 75 and 50 DIM, respectively. Season significantly influenced only CREA, which was slightly higher during the cold season than the hot season (1.65 vs. 1.61 mg/dL). However, significant DIM × season interactions were observed for CHOL, NEFA, and GGT, whereas parity × season interactions affected urea and LDL, indicating context-dependent metabolic modulation. Increasing ECM was associated with coordinated metabolic upregulation, including increases in CHOL (from 118 to 126 mg/dL), BHB (0.37 to 0.40 mmol/L), GOT (120 to 126 U/L), GGT (27.5 to 30.2 U/L), and CK (185 to 210 U/L). In conclusion, buffaloes in the present study exhibited a stable and well-regulated metabolic profile, with most variation reflecting physiological adaptations to parity, DIM, and productive performance. These findings provide valuable information for clinical interpretation and herd management, although further studies under different environmental conditions are needed to confirm broader applicability.

Key words: biomarkers, dairy herd management, health, metabolism

INTRODUCTION

More than 204 million water buffaloes (*Bubalus bubalis*) are raised worldwide, underscoring the importance of this species within the global dairy industry. Buffaloes possess a remarkable ability to convert low-quality, fibrous feedstuffs into milk and meat while maintaining productivity even under hot and humid climate condi-

Received December 21, 2025.
Accepted May 5, 2026.
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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

tions, where many cattle breeds would experience substantial performance limitations (Borghese et al., 2022). In Italy, buffalo farming is deeply integrated into traditional agricultural systems, with the iconic product Mozzarella di Bufala Campana generating more than half a billion euros annually and supporting an extensive network of farms and dairies (Alterisio et al., 2021; Carraturo et al., 2022; ISMEA, 2023). Southern Italy alone accounts for ~95% of the entire European buffalo population, comprising nearly 400,000 animals (Pisanu et al., 2019; Puggioni et al., 2020; BDN 2025). Over the past decade, national buffalo milk production has consistently exceeded 2 million metric tonnes, largely due to improvements in feeding strategies, reproduction management, and increased attention to animal welfare (Carraturo et al., 2022; Matera et al., 2025).

Despite its substantial economic and cultural importance, current knowledge of buffalo health and physiology remains considerably more limited than that of dairy cattle (Guccione et al., 2016). This knowledge gap is particularly evident in the understanding of mastitis, metabolic disorders, and broader physiological adaptations (Fiore et al., 2023). A comprehensive understanding of buffalo metabolism is essential, as metabolic imbalances may reduce milk yield, impair reproductive performance, and compromise animal welfare (Ghanem and El-Deeb, 2010; Sundrum, 2015). Therefore, the early detection of metabolic and physiological alterations is relevant for supporting preventive interventions before clinical signs become evident, with potential benefits for animal health and productivity (LeBlanc, 2010). To assess these conditions, veterinarians and researchers commonly rely on blood metabolites, liver enzymes, and hormonal biomarkers, which represent practical and reliable indicators of metabolic status (Esposito et al., 2014). For example, BHB and nonesterified fatty acids (**NEFA**) are widely used to identify subclinical ketosis and hepatic lipid mobilization (Van Saun, 2006). Similarly, biomarkers such as aspartate transaminase (**GOT**), γ-glutamyl transferase (**GGT**), cholesterol (**CHOL**), and total bilirubin (**TBIL**) provide valuable information regarding hepatic function (Puppel and Kuczyńska, 2016). Although these biomarkers are well established in dairy cattle, information remains limited regarding the physiological and environmental factors influencing blood metabolite variation in buffaloes (Campanile et al., 1997; Ciaramella et al., 2005; Fiore et al., 2023). Previous studies in cattle and other ruminant species have demonstrated that both animal-related and environmental factors, including parity, lactation stage, and seasonal conditions, significantly influence metabolic profiles (Drackley et al., 2001; Kessel et al., 2008; Guccione et al., 2016). Therefore, the present study aimed to evaluate the associations of parity, lactation stage, and season with key blood metabolites reflecting energy, protein, and hepatic-muscular metabolism in lactating Italian Mediterranean buffaloes.

MATERIALS AND METHODS

All clinical procedures adhered to the most recent Good Clinical Practices standards (EMA, 2025) and received formal approval from the Ethical Animal Care and Use Committee of the University of Naples Federico II, Italy (PG/2022/0025539). All farmers were informed about the aims and procedures of the study and provided verbal consent.

Farms and Animals

The study was conducted between December 2021 and August 2023 on 19 commercial buffalo farms located in central and southern Italy (Lazio, Campania, Calabria, and Puglia), each housing between 200 and 450 lactating animals. Before study initiation, all potentially eligible buffaloes underwent a comprehensive clinical examination following the procedures described by Ciaramella and Guccione (2023). Animals were excluded if any pathological condition was detected, as defined according to diagnostic guidelines for dairy cattle (Fadul et al., 2022; Supplemental Table S1, see Notes). Consequently, only clinically healthy animals were enrolled. Within each farm, buffaloes were selected to ensure balanced representation across key variables, including parity, DIM, and sampling season. Approximately 50% of the farms were sampled during 2 distinct seasons (winter and summer), requiring a structured longitudinal selection of animals. This strategy was designed to generate homogeneous and comparable groups across farms and environmental conditions. Overall, 855 lactating Italian Mediterranean buffaloes were enrolled, averaging 45 animals per farm. Monthly milk samples were collected during official test-day recordings performed by the National Buffalo Breeder Association (**ANASB**). Milk samples were analyzed using the FT3 MilkoScan system (FOSS Analytical, Hillerød, Denmark), providing individual milk yield and composition traits (fat and protein content). Average milk yield per lactation was 2,653 ± 424 kg, with mean fat and protein contents of 7.92% and 4.61%, respectively. Individual milk yield across lactation was subsequently converted to ECM using the buffalo-specific equation described by Campanile et al. (2003):

$$\text{ECM} = \{[\text{fat (g/kg)} - 40 + \text{protein (g/kg)} - 31] \times 0.01155\} + 1 \times \text{milk yield (kg/d)}.$$

All farms operated under comparable management conditions, using open-yard housing systems that provided ~15 m^2/head and 80 cm/head at the feed bunk, with ad libitum access to water. At each sampling date, TMR samples were collected from each farm and stored at −20°C until chemical analysis. Samples were dried in a forced-air oven at 103°C overnight for OM determination and ground using a Retsch mill type SK (Bauknecht, Stuttgart, Germany) to pass through a 1 mm screen. Ash content was determined by combustion at 550°C for 4 h, and ether extract (**EE**) was analyzed according to European Commission Regulation No. 152/2009 (European Commission, 2009). Nitrogen content was determined using the Dumas combustion method (AOAC International, 2023), involving sample combustion at 900°C in excess oxygen with a Dumatherm analyzer (Gerhardt GmbH & Co., Königswinter, Germany), as described by Mihaljev et al. (2015). Crude protein was calculated as nitrogen × 6.25. Fiber fractions, including amylase-treated NDF exclusive of residual ash (**aNDFom**) and ADF exclusive of residual ash (**ADFom**), were determined according to Mertens et al. (2002), using heat-stable amylase without sodium sulfite and corrected for ash content. The boiling and filtration steps were carried out using a semi-automated system (FIWE Raw Fiber Extractor, VELP Scientifica, Usmate Velate, Italy). Hemicellulose (**HC**) and cellulose (**CEL**) were calculated through sequential fiber fraction analysis following Robertson and Van Soest (1981). Starch content was determined enzymatically (McCleary et al., 2019). Undigested NDF (**uNDF**) was assessed after 240 h of in vitro fermentation according to Raffrenato et al. (2018), using rumen fluid collected from slaughtered buffaloes and processed as described by Simoni et al. (2021). Three consecutive runs were performed to analyze all samples. Rumen fluid was maintained at 39°C under anaerobic conditions, homogenized, and filtered through 4 layers of cheesecloth. It was then mixed with buffer solution at a 1:4 ratio in flasks containing 0.50 g of sample and incubated in an in vitro batch fermentation system. On average, diet composition was as follows: OM 94.5% ± 1.20%, ash 7.42% ± 1.54%, CP 14.3% ± 2.45%, EE 5.20% ± 2.00%, aNDFom 42.9% ± 3.00%, ADFom 24.4% ± 3.60%, HC 14.5% ± 4.02%, CEL 20.7% ± 2.58%, starch 21.7% ± 3.10%, and uNDF 14.7% ± 1.50% (Supplemental Table S2, see Notes).

Blood Sampling and Analysis

Blood samples were collected by caudal venipuncture into 10-mL vacuum tubes without anticoagulant (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). Approximately 50% of farms were visited twice, at 6-mo intervals (winter and summer), whereas the remaining farms were sampled once during either the cold or hot season. This design was adopted to capture more variability while maintaining balanced seasonal representation despite logistical constraints. Samples were centrifuged immediately after collection at 800 × *g* for 5 min at room temperature to obtain serum, which was stored at −20°C until analysis. Serum metabolites were measured using an automated clinical analyzer (SAT 450 AMS Alliance, KPM Analytics, Westborough, MA). Biochemical variables were grouped into 3 metabolic profiles according to Puppel and Kuczyńska (2016): energy profile consisted of glucose (**GLU**), CHOL, triglycerides (**TRI**), NEFA, BHB; the protein profile consisted of total protein (**TP**), albumin (**ALB**), globulins (**GLO**), albumin/globulin ratio (**A/G**), urea, creatinine (**CREA**); the hepatic-muscular profile consisted of alanine aminotrasferase (**GPT**), GOT, alkaline phosphatase (**ALP**) lactate dehydrogenase (**LDH**), GGT, creatine kinase (**CK**), TBIL, high-density lipoproteins (**HDL**), and low-density lipoproteins (**LDL**). All parameters were measured using commercial kits from Spinreact (Barcelona, Spain), except for NEFA and BHB, which were analyzed using Randox kits (Randox, Crumlin, UK).

Data Editing and Statistical Analysis

Buffaloes with DIM ≤5 were excluded from the analysis. Serum metabolite distributions were evaluated for normality. For normally distributed variables, outliers exceeding ±3 SD from the mean were removed. For non-normally distributed variables, outliers were identified using the interquartile range (**IQR**) method, excluding values below quartile 1 (**Q1**) − 1.50 × IQR or above quartile 3 (**Q3**) + 1.50 × IQR. After data editing, descriptive statistics were calculated for all metabolites (Table 1). Days in milk were categorized into 9 classes (≤50, 51–75, 76–90, 91–115, 116–140, 141–165, 166–190, 191–215, and >216 d) based on the average lactation curve of the study population derived from ANASB test-day data. This classification also reflected the main physiological phases of lactation and ensured a balanced number of animals per class, allowing robust statistical comparisons. Parity was grouped into 5 classes (1, 2, 3, 4, and ≥5). Sampling season was classified as hot (spring–summer) or cold (autumn–winter). Milk yields, converted into ECM, were divided into 3 balanced classes: class 1: (ECM ≤4,052 kg), class 2 (4,052 kg < ECM ≤ 4,967 kg), and class 3 (ECM >4,967 kg). Sources of variation in serum metabolites were investigated using repeated linear mixed models implemented with PROC GLIMMIX in SAS software (version 9.4, SAS Institute Inc., Cary, NC), as follows:

$$y_{ijklm} = \mu + \text{DIM}_i + \text{parity}_j + \text{season}_k + \text{ECM class}_l + (\text{DIM} \times \text{parity})_{ij} + (\text{DIM} \times \text{season})_{ik} + (\text{parity} \times \text{season})_{jk} + \text{HSD}_m + e_{ijklm},$$

where y_{ilklm} represents the serum metabolite; μ is the population mean for the evaluated trait; DIM_i is the fixed effect of the ith DIM class (i = from 1 to 9); parity_j is the fixed effect of the jth parity class (j = 1, 2, 3, 4, ≥5); season_k is the fixed effect of the kth sampling season (k = hot [spring and summer] or cold [autumn and winter]); ECM class_l is the fixed effect of the lth ECM class (l = class 1: ≤4,052 kg; class 2: 4,052–4,967 kg; class 3: >4,967 kg); $(\text{DIM} \times \text{parity})_{ij}$ is the fixed interaction effect between DIM_i and parity_j classes; $(\text{DIM} \times \text{season})_{ik}$ is the fixed interaction effect between DIM_i and season_k classes; $(\text{parity} \times \text{season})_{jk}$ is the fixed interaction effect between parity_j and season_k classes; HSD_m is the random effect of the mth herd sampling day (**HSD**) $\sim N\left(0, \sigma^2_{\text{HSD}}\right)$, where σ^2_{HSD} is the variance associated with the herd-sampling-day effect; and e_{ijklm} is the random residual $\sim N\left(0, \sigma^2_e\right)$, where σ^2_e is the error variance. The HSD effect accounted for shared herd-level environmental, nutritional, and management factors specific to each sampling date. Due to right-skewed distributions, NEFA, CK, ALP, TBIL, LDL, and BHB were analyzed using generalized linear mixed models with gamma distribution and log-link functions (Santinello et al., 2024). Results are presented as LSM ± SEM, and multiple comparisons were performed using Bonferroni-adjusted post hoc tests with significance declared at $P < 0.05$.

RESULTS

Association Between Parity and Serum Metabolites

The LSM of serum metabolites according to parity are reported in Table 2. Within the energy profile, parity was significantly associated with CHOL concentrations ($P < 0.05$), which increased up to the third parity (from 123 to 126 mg/dL) and subsequently decreased in buffaloes with parity ≥5 (115 mg/dL).

Within the protein profile, parity was significantly associated with ALB, GLO, A/G, and TP concentrations ($P < 0.05$). Primiparous buffaloes exhibited the highest ALB and A/G values (3.26 g/dL and 0.95, respectively), which progressively decreased with increasing parity. Conversely, primiparous animals showed the lowest TP and GLO concentrations (6.69 g/dL and 3.44 g/dL, respectively), both of which increased with parity.

Within the hepatic-muscular profile, GPT, GOT, ALP, LDH, CK, and HDL were significantly associated with parity ($P < 0.05$). High-density lipoprotein concentrations were highest during the first 3 parities, whereas the lowest value was observed in buffaloes with parity ≥5 (60.9 mg/dL). Creatine kinase progressively decreased with increasing parity, from 215 U/L in primiparous buffaloes to 179 to 189 U/L in animals with parity ≥4. The lowest GPT, GOT, and LDH values were observed in fourth-parity buffaloes. In contrast, ALP was lowest in primiparous animals (207 U/L) and increased up to the third parity ($P < 0.05$).

Table 1. Descriptive statistics of serum metabolites in Italian Mediterranean buffaloes (n = 855)

Serum metabolite[1]	Mean ± SD	Median	IQR[2]
Energy profile			
GLU (mg/dL)	68.0 ± 13.8	69.0	18.0
CHOL (mg/dL)	122.0 ± 33.3	120	40.0
TRI (mg/dL)	28.4 ± 13.1	30.0	16.0
NEFA (mmol/L)	0.43 ± 0.47	0.26	0.25
BHB (mmol/L)	0.40 ± 0.13	0.40	0.20
Protein profile			
TP (g/dL)	6.88 ± 0.77	6.90	1.00
ALB (g/dL)	3.24 ± 0.28	3.20	0.40
GLO (g/dL)	3.64 ± 0.68	3.60	0.90
A/G	0.87 ± 0.23	0.90	0.28
CREA (mg/dL)	1.64 ± 0.27	1.60	0.30
Urea (mg/dL)	55.7 ± 16.0	54.0	21.0
Hepatic-muscular profile			
GPT (U/L)	42.5 ± 9.75	42.0	11.0
GOT (U/L)	123 ± 24.2	120	33.0
ALP (U/L)	281 ± 187	234	191
LDH (U/L)	1,342 ± 241	1,320	317
GGT (U/L)	18.7 ± 7.10	28.0	8.40
CK (U/L)	203 ± 123	175	82.0
TBIL (mg/dL)	0.14 ± 0.12	0.10	0.07
HDL (mg/dL)	65.6 ± 16.5	65.0	22.0
LDL (mg/dL)	31.3 ± 18.0	28.0	14.0

[1]GLU = glucose; CHOL = cholesterol; TRI = triglyceride; NEFA = nonesterified fatty acid; TP = total protein; ALB = albumin; GLO = globulins; A/G = albumin-to-globulin ratio; CREA = creatinine; GPT = alanine aminotransferase; GOT = aspartate aminotransferase; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; GGT = γ-glutamyl transferase; CK = creatine kinase; TBIL = total bilirubin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.
[2]IQR = interquartile range.

Association Between DIM and Serum Metabolites

The LSM of serum metabolites according to DIM classes are presented in Table 3. Within the energy profile, DIM significantly influenced CHOL, TRI, NEFA, and BHB concentrations ($P < 0.05$), whereas GLU was unaffected ($P > 0.05$). Cholesterol concentrations increased after 50 DIM and remained relatively stable throughout lactation. Triglyceride values were generally stable, with the lowest concentrations observed between 51 and 75 DIM (25.9 mg/dL) and the highest after 216 DIM (32.3 mg/dL). Nonesterified fatty acid concentrations peaked within the first 50 DIM (0.39 mmol/L) and

Table 2. Least squares means and SEM of serum metabolites according to the effect of parity (n = 855)

	Parity					
Serum metabolite[1]	1	2	3	4	≥5	SEM
Energy profile						
GLU (mg/dL)	71.5	70.7	68.7	68.1	70.2	1.88
CHOL (mg/dL)	123^{ab}	125^{a}	126^{a}	116^{ab}	115^{b}	3.38
TRI (mg/dL)	29.5	28.9	30.2	28.1	27.9	1.78
NEFA (mmol/L)	0.30	0.32	0.28	0.26	0.28	0.04
BHB (mmol/L)	0.37	0.40	0.39	0.40	0.38	0.02
Protein profile						
TP (g/dL)	6.69^{b}	6.85^{ab}	6.86^{ab}	6.84^{ab}	6.95^{a}	0.09
ALB (g/dL)	3.26^{a}	3.25^{a}	3.24^{ab}	3.18^{ab}	3.16^{b}	0.03
GLO (g/dL)	3.44^{c}	3.60^{bc}	3.61^{abc}	3.66^{ab}	3.80^{a}	0.08
A/G	0.95^{a}	0.89^{ab}	0.90^{ab}	0.86^{bc}	0.82^{c}	0.03
CREA (mg/dL)	1.65	1.62	1.62	1.62	1.65	0.03
Urea (mg/dL)	54.4	54.9	53.1	51.3	52.6	2.09
Hepatic-muscular profile						
GPT (U/L)	41.9^{a}	43.7^{a}	42.6^{ab}	39.6^{b}	41.4^{ab}	1.12
GOT (U/L)	127^{a}	127^{a}	121^{ab}	116^{b}	123^{ab}	2.92
ALP (U/L)	207^{b}	238^{ab}	267^{a}	232^{ab}	238^{ab}	5.32
LDH (U/L)	1,385^{a}	1,367^{ab}	1,327^{bc}	1,262^{d}	1,278^{cd}	29.1
GGT (U/L)	27.9	28.9	29.6	27.4	29.2	0.74
CK (U/L)	215^{a}	198^{ab}	194^{ab}	179^{b}	189^{ab}	9.58
TBIL (mg/dL)	0.10	0.10	0.11	0.11	0.11	0.01
HDL (mg/dL)	66.8^{a}	68.0^{a}	67.0^{a}	63.4^{ab}	60.9^{b}	1.89
LDL (mg/dL)	28.1	27.7	28.4	26.7	26.3	1.50

$^{a–d}$Means with different superscript letters within a row differ significantly ($P < 0.05$).

[1]GLU = glucose; CHOL = cholesterol; TRI = triglyceride; NEFA = nonesterified fatty acid; TP = total protein; ALB = albumin; GLO = globulins; A/G = albumin-to-globulin ratio; CREA = creatinine; GPT = alanine aminotransferase; GOT = aspartate aminotransferase; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; GGT = γ-glutamyl transferase; CK = creatine kinase; TBIL = total bilirubin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

progressively decreased as lactation advanced, reaching the lowest values after 216 DIM (0.25 mmol/L). β-Hydroxybutyrate concentrations were highest between 51 and 75 DIM (0.42 mmol/L) and between 116 and 140 DIM (0.41 mmol/L), with slight decreases observed between 191 and 215 DIM.

Within the protein profile, TP, ALB, GLO, CREA, and urea were significantly associated with DIM ($P < 0.05$). Total protein, ALB and GLO remained relatively stable throughout lactation, with the lowest values observed between 116 and 140 DIM (6.57 g/dL, 3.14 g/dL and 3.44g/dL, respectively). Creatinine concentrations were also relatively stable but increased after 191 DIM (1.72–1.73 mg/dL). Urea concentrations were lowest during early lactation (≤50 DIM; 48.7 mg/dL) and progressively increased as lactation progressed (from 54.8 to 55.3 mg/dL).

Within the hepatic-muscular profile, DIM significantly influenced GPT, GOT, GGT, TBIL, and LDL ($P < 0.05$). Alanine aminotransferase concentrations were lowest within the first 50 DIM (37.8 U/L; $P < 0.05$) and remained relatively stable thereafter. Aspartate aminotransferase concentrations peaked within the first 50 DIM (136 U/L) and declined after 116 DIM ($P < 0.05$). γ-Glutamyltransferase values were lowest during the first 75 DIM and highest between 141 and 165 DIM ($P < 0.05$). Total bilirubin decreased after 50 DIM and remained stable thereafter (approximately 0.11 mg/dL). In contrast, LDL concentrations were lowest during the first 50 DIM and increased subsequently ($P < 0.05$). The significance levels of fixed effects and their interactions are summarized in Supplemental Table S2. The interaction between DIM and parity was not significant for any analyzed serum metabolite ($P > 0.05$).

Association Between Season of Sampling and Serum Metabolites

The LSM of serum metabolites according to sampling season are reported in Table 4. No significant seasonal differences were observed for energy or hepatic-muscular profiles ($P > 0.05$). Within the protein profile, CREA was the only metabolite significantly affected by sampling season ($P < 0.05$), with higher concentrations observed during the cold season than the hot season (1.65 vs. 1.61 mg/dL, respectively).

The interaction between parity and sampling season was significant only for urea ($P = 0.0215$) and LDL ($P = 0.0052$), whereas the interaction between DIM and sampling season was significant for CHOL, NEFA, and GGT

Table 3. Least squares means and SEM of serum metabolites according to the effect of DIM (n = 855)

	DIM									
Serum metabolite[1]	≤50	51–75	76–90	91–115	116–140	141–165	166–190	191–215	≥216	SEM
Energy profile										
GLU (mg/dL)	70.5	69.2	70.5	69.7	67.8	69.5	71.2	68.9	71.0	2.10
CHOL (mg/dL)	103^{b}	122^{a}	123^{a}	129^{a}	121^{a}	125^{a}	120^{a}	124^{a}	121^{a}	3.67
TRI (mg/dL)	28.7^{ab}	25.9^{b}	28.0^{ab}	28.4^{ab}	28.9^{ab}	29.0^{ab}	29.5^{ab}	29.4^{ab}	32.3^{a}	1.90
NEFA (mmol/L)	0.39^{a}	0.32^{ab}	0.27^{ab}	0.30^{ab}	0.26^{ab}	0.28^{ab}	0.27^{ab}	0.29^{ab}	0.25^{b}	0.04
BHB (mmol/L)	0.40^{ab}	0.42^{a}	0.40^{ab}	0.39^{ab}	0.41^{a}	0.38^{ab}	0.37^{ab}	0.35^{b}	0.37^{ab}	0.02
Protein profile										
TP (g/dL)	6.97^{a}	6.73^{ab}	6.92^{a}	6.92^{a}	6.57^{b}	6.82^{ab}	6.79^{ab}	6.87^{ab}	6.97^{a}	0.1
ALB (g/dL)	3.23^{abc}	3.14^{c}	3.17^{bc}	3.23^{abc}	3.14^{c}	3.22^{abc}	3.23^{abc}	3.29^{ab}	3.32^{a}	0.04
GLO (g/dL)	3.75^{ab}	3.58^{ab}	3.74^{a}	3.69^{ab}	3.44^{b}	3.60^{ab}	3.55^{ab}	3.58^{ab}	3.65^{ab}	0.09
A/G	0.86	0.87	0.85	0.87	0.91	0.89	0.90	0.90	0.90	0.03
CREA (mg/dL)	1.65^{ab}	1.56^{b}	1.58^{b}	1.63^{ab}	1.57^{b}	1.62^{b}	1.64^{ab}	1.72^{a}	1.73^{a}	0.03
Urea (mg/dL)	48.7^{b}	51.4^{ab}	51.3^{ab}	54.7^{a}	54.6^{a}	53.7^{ab}	55.3^{a}	54.8^{a}	54.9^{a}	2.20
Hepatic-muscular profile										
GPT (U/L)	37.8^{b}	41.0^{ab}	42.9^{a}	43.6^{a}	42.2^{a}	41.8^{ab}	42.4^{a}	42.9^{a}	42.0^{ab}	1.20
GOT (U/L)	136^{a}	127^{ab}	126^{abc}	125^{abc}	116^{bc}	122^{bc}	120^{bc}	122^{bc}	114^{c}	3.23
ALP (U/L)	212	231	243	224	214	242	239	266	259	25.2
LDH (U/L)	1,326	1,320	1,357	1,356	1,299	1,333	1,307	1,331	1,286	30.5
GGT (U/L)	26.7^{b}	27.0^{b}	28.5^{ab}	29.0^{ab}	29.0^{ab}	30.8^{a}	28.8^{ab}	29.3^{ab}	28.3^{ab}	0.83
CK (U/L)	192	184	188	216	184	186	204	194	207	12.2
TBIL (mg/dL)	0.14^{a}	0.11^{b}	0.10^{b}	0.11^{b}	0.11^{b}	0.10^{b}	0.10^{b}	0.10^{b}	0.11^{b}	0.01
HDL (mg/dL)	60.4	66.5	65.3	67.8	65.6	68.1	65.3	64.9	63.2	2.10
LDL (mg/dL)	21.8^{b}	27.1^{a}	29.0^{a}	27.8^{a}	27.8^{a}	28.6^{a}	28.7^{a}	28.4^{a}	28.4^{a}	1.63

[a–c]Means with different superscript letters within a row differ significantly ($P < 0.05$).

[1]GLU = glucose; CHOL = cholesterol; TRI = triglyceride; NEFA = nonesterified fatty acid; TP = total protein; ALB = albumin; GLO = globulin; A/G = albumin-to-globulin ratio; CREA = creatinine; GPT = alanine aminotransferase; GOT = aspartate aminotransferase; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; GGT = γ-glutamyl transferase; CK = creatine kinase; TBIL = total bilirubin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

($P < 0.05$). Figure 1 illustrates the parity × sampling season interaction for urea and LDL concentrations. Urea concentrations remained relatively stable across parity classes during the cold season, with the lowest value observed in third-parity buffaloes (52.7 mg/dL). In contrast, during the hot season, urea concentrations peaked in second-parity animals (55.1 mg/dL) and decreased after parity 4. Low-density lipoprotein concentrations were highest in buffaloes with parity ≥5 sampled during the cold season (29.3 mg/dL) and lowest in the same parity class during the hot season (23.6 mg/dL).

Figure 2 illustrates the DIM × sampling season interactions for CHOL, NEFA, and GGT. In buffaloes sampled during the cold season, CHOL concentrations were lowest within the first 50 DIM (109 mg/dL) and peaked between 91 and 115 DIM (133 mg/dL). A similar pattern was observed during the hot season, with lower CHOL values during the first 50 DIM (96.6 mg/dL), and peak concentrations between 141 and 165 DIM (133 mg/dL). Nonesterified fatty acid concentrations during the cold season were highest within the first 50 DIM (0.52 mmol/L), declined to their lowest values between 76 and 90 DIM (0.23 mmol/L), and subsequently increased again to 0.33 mmol/L between 191 and 215 DIM. Conversely, during the hot season, NEFA concentrations peaked between 91 and 115 DIM (0.33 mmol/L) and declined to 0.24 mmol/L after 216 DIM. γ-Glutamyl transferase concentrations were consistently higher throughout lactation in buffaloes sampled during the cold season compared with those sampled during the hot season.

Association Between ECM Production and Serum Metabolites

The LSM of serum metabolites according to ECM classes are presented in Table 5. Within the energy profile, CHOL and BHB were significantly associated with ECM ($P < 0.05$), with both metabolites increasing as ECM production increased (from 118 to 126 mg/dL and from 0.37 to 0.40 mmol/L, respectively). Within the protein profile, no significant associations were observed among ECM classes ($P > 0.05$). Within the hepatic-muscular profile, GOT, GGT, and CK were significantly associated with ECM ($P < 0.05$), with all 3 metabolites progressively increasing as ECM production increased.

DISCUSSION

The present study aimed to evaluate the main sources of variation in the serum metabolic profiles of lactating Italian Mediterranean buffaloes, focusing on the effects of parity, DIM, sampling season, and ECM produc-

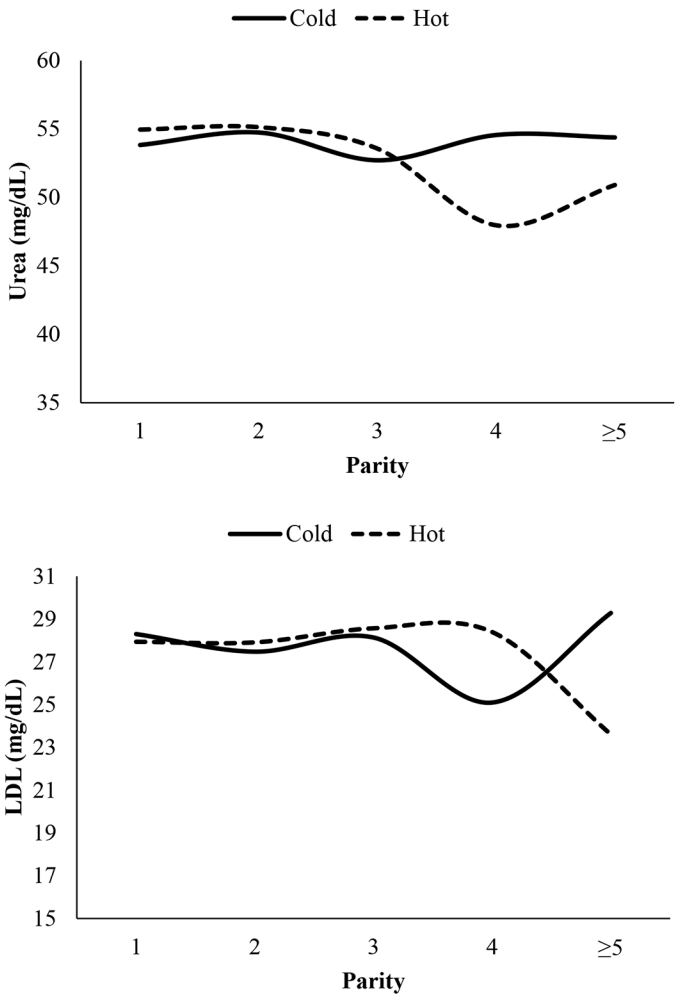

Figure 1. Least squares means of serum urea and low-density lipoprotein (LDL) concentrations in lactating Italian Mediterranean buffaloes: interaction effect between parity and sampling season (hot: spring and summer, or cold: autumn and winter).

Table 4. Least squares means and SEM of serum metabolites according to the effect of sampling season (n = 855)

Serum metabolite[2]	Sampling season[1]		SEM
	Hot	Cold	
Energy profile			
GLU (mg/dL)	70.0	69.7	1.73
CHOL (mg/dL)	120	122	2.73
TRI (mg/dL)	28.4	29.4	1.71
NEFA (mmol/L)	0.27	0.31	0.04
BHB (mmol/L)	0.40	0.38	0.01
Protein profile			
TP (g/dL)	6.81	6.87	0.08
ALB (g/dL)	3.29	3.24	0.03
GLO (g/dL)	3.61	3.64	0.07
A/G	0.88	0.88	0.03
CREA (mg/dL)	1.61[b]	1.65[a]	0.02
Urea (mg/dL)	52.5	54.0	1.97
Hepatic-muscular profile			
GPT (U/L)	41.5	42.1	0.99
GOT (U/L)	122	124	2.49
ALP (U/L)	233	239	21.1
LDH (U/L)	1,318	1,330	27.1
GGT (U/L)	28.6	28.6	0.58
CK (U/L)	192	198	7.52
TBIL (mg/dL)	0.11	0.11	0.01
HDL (mg/dL)	65.8	64.7	1.64
LDL (mg/dL)	27.2	27.6	1.29

[a,b]Means with different superscript letters within a row differ significantly ($P < 0.05$).

[1]Hot: spring and summer; cold: autumn and winter.

[2]GLU = glucose; CHOL = cholesterol; TRI = triglyceride; NEFA = non-esterified fatty acid; TP = total protein; ALB = albumin; GLO = globulin; A/G = albumin-to-globulin ratio; CREA = creatinine; GPT = alanine aminotransferase; GOT = aspartate aminotransferase; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; GGT = γ-glutamyl transferase; CK = creatine kinase; TBIL = total bilirubin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

tion classes, and their significant interactions. Overall, most metabolites across the evaluated profiles showed limited variability and remained within physiological ranges, supporting the hypothesis that Italian Mediterranean buffaloes maintain a relatively stable and well-regulated metabolic status. These findings are consistent with previous studies reporting metabolic resilience in this species (Bertoni et al., 2020; Lisuzzo et al., 2023). Interpretation of metabolic biomarkers in buffaloes remains challenging because species-specific data are still limited, often leading to reliance on knowledge derived from dairy cattle despite substantial physiological and productive differences between the 2 species (Guccione et al., 2016; Fiore et al., 2023; Alterisio et al., 2026). Although both belong to the Bovidae family, buffaloes differ from dairy cows in several relevant aspects, including digestive efficiency, milk production characteristics, and metabolic demands (Bertoni et al., 2020). Buffaloes are more efficient in utilizing low-quality, fibrous forages, likely due to their longer gastrointestinal tract and distinct ruminal microbiota composition (Bertoni et al., 2020; Vastolo et al., 2022). Additionally, compared with dairy cows, buffaloes generally produce lower milk yields while maintaining higher milk fat concentrations (Matera et al., 2022), resulting in different metabolic demands. These species-specific characteristics reinforce the need for caution when applying cattle-based metabolic interpretations to buffaloes and emphasize the importance of developing buffalo-specific knowledge to improve clinical interpretation and herd management.

The relative metabolic stability observed in this study is likely influenced by the specific production context of Italian buffalo farming, including climatic conditions, nutritional strategies, genetic background, and herd management practices. Consequently, extrapolation of these findings to buffalo populations raised in other geographical regions should be approached cautiously. Buffaloes

Table 5. Least squares means and SEM of serum metabolites according to the effect of ECM[1] classes (n = 855)

Serum metabolite[2]	ECM class			SEM
	≤4,052	4,052–4,967	>4,967	
Energy profile				
GLU (mg/dL)	69.6	69.5	70.3	1.77
CHOL (mg/dL)	118[b]	119[b]	126[a]	2.87
TRI (mg/dL)	29.2	29.1	28.4	1.72
NEFA (mmol/L)	0.29	0.29	0.29	0.04
BHB (mmol/L)	0.37[b]	0.39[ab]	0.40[a]	0.02
Protein profile				
TP (g/dL)	6.81	6.86	6.84	0.08
ALB (g/dL)	3.22	3.22	3.22	0.03
GLO (g/dL)	3.60	3.64	3.62	0.07
A/G	0.88	0.88	0.88	0.03
CREA (mg/dL)	1.65	1.62	1.62	0.03
Urea (mg/dL)	51.8	54.0	54.0	1.99
Hepatic-muscular profile				
GPT (U/L)	41.5	42.4	41.6	1.02
GOT (U/L)	120[b]	123[ab]	126[a]	2.59
ALP (U/L)	237	223	248	21.6
LDH (U/L)	1,329	1,327	1,315	27.5
GGT (U/L)	27.5[b]	28.1[b]	30.2[a]	0.62
CK (U/L)	185[b]	190[ab]	210[a]	0.04
TBIL (mg/dL)	0.11	0.10	0.11	0.01
HDL (mg/dL)	63.7	65.0	66.9	1.69
LDL (mg/dL)	26.6	27.0	28.5	1.33

[a,b]Means with different superscript letters within a row differ significantly ($P < 0.05$).

[1]ECM was calculated using the following formula adapted for buffaloes (Campanile et al., 2003): ({[fat (g/kg) − 40 + protein (g/kg) − 31] × 0.01155} +1) × milk yield (kg/d).

[2]GLU = glucose; CHOL = cholesterol; TRI = triglyceride; NEFA = nonesterified fatty acid; TP = total protein; ALB = albumin; GLO = globulin; A/G = albumin-to-globulin ratio; CREA = creatinine; GPT = alanine aminotransferase; GOT = aspartate aminotransferase; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; GGT = γ-glutamyl transferase; CK = creatine kinase; TBIL = total bilirubin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

reared under markedly different production systems, such as Murrah buffaloes in India exposed to extreme seasonal temperature fluctuations and monsoon-driven climatic conditions (Airon et al., 2018), populations in the Brazilian Amazon Basin raised under tropical humid environments and forage-based extensive systems (da Silva et al., 2021), or Egyptian buffaloes managed under distinct seasonal feeding systems (Ghanem and El-Deeb 2010), may exhibit different metabolic adaptations. Therefore, the sources of variation identified in the present study should be interpreted within their specific environmental and management frameworks. Serum metabolite concentrations are inherently influenced by multiple interacting factors, including nutrition, climate, management practices, production level, and individual biological variability. Accordingly, the associations observed with parity, DIM, ECM, and sampling season should be interpreted as important physiological contributors within a multifactorial metabolic system, rather than as direct causal determinants.

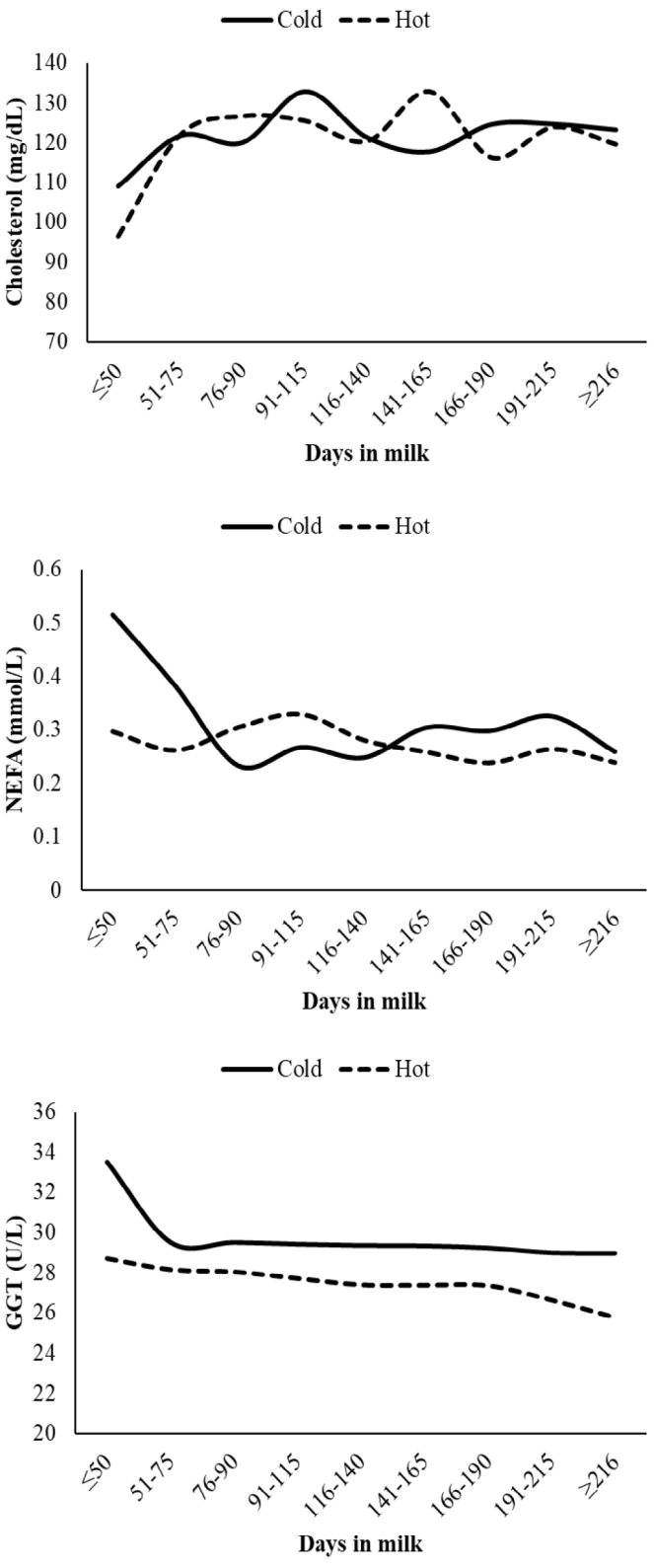

Figure 2. Least squares means of serum cholesterol, nonesterified fatty acids (NEFA), and γ-glutamyl transferase (GGT) concentrations in lactating Italian Mediterranean buffaloes: interaction effect between DIM and sampling season (hot: spring and summer or cold: autumn and winter).

Association Between Parity and Serum Metabolites

Parity-related variations were mainly observed in metabolites associated with protein metabolism and hepatic lipid regulation, whereas NEFA and BHB showed only limited differences among parity classes. These findings indicate that parity was not strongly associated with changes in lipid mobilization or negative energy balance in the studied population. This response differs from that commonly described in dairy cattle, where multiparous cows often show increased NEFA and BHB concentrations because of higher milk yield and greater mobilization of body reserves (Walter et al., 2022). In buffaloes, the limited variation observed for these metabolites may reflect species-specific productive and metabolic features, including lower production intensity compared with dairy cattle (Fiore et al., 2023). Nevertheless, longitudinal studies including individual feed intake, milk yield, and body condition data are required to confirm this interpretation. In contrast, CHOL showed significant parity-related variation, increasing from primiparous to third-parity buffaloes before slightly decreasing in animals with parity ≥5. This trend may reflect age-related modulation of hepatic lipid metabolism and lipoprotein synthesis. Cholesterol is a multifactorial metabolite influenced by hepatic synthesis, energy balance, and peripheral utilization, making its biological interpretation highly context-dependent (Kessler et al., 2014; Gross et al., 2015). The increase observed in younger animals is consistent with progressive enhancement of hepatic metabolic activity associated with increasing production demands, whereas the subsequent decline in older animals may reflect reduced metabolic intensity or adaptive physiological regulation. Similar associations among CHOL, energy status, and hepatic function have been described in dairy cattle (Kessler et al., 2014), although differences in age-related changes in feed intake, production level, and metabolic efficiency between cattle and buffaloes may have influenced the apparent similarity of these patterns.

Parity was also associated with progressive remodeling of the protein profile, characterized by increasing TP and GLO concentrations alongside declining ALB and A/G values. This pattern likely reflects a physiological shift in hepatic protein synthesis toward GLO fractions, potentially driven by cumulative antigenic exposure and sustained productive demands rather than impaired hepatic function. Because ALB and GLO share hepatic synthetic capacity, increased immunoglobulins and GLO synthesis with advancing age may lead to relatively lower ALB concentrations (Bobbo et al., 2017; Ferreira et al., 2021). The increase in GLO more than compensated for the decrease in ALB, resulting in an overall rise in TP, consistent with observations in dairy cattle (Bobbo et al., 2017). In contrast, urea and CREA did not differ significantly across parity classes, supporting the concept that these metabolites are more strongly influenced by nutritional factors (Bradford, 2021) than by productive stage alone. However, the significant parity × sampling season interaction for urea suggests that nitrogen metabolism may be modulated by the combined influence of physiological maturity and environmental conditions. The lower urea concentrations observed in greater-parity animals during the hot season may reflect greater sensitivity to heat-induced alterations in feed intake and nitrogen utilization, as previously reported in dairy cattle (Gao et al., 2017; Bradford, 2021).

Hepatic-muscular biomarkers also exhibited significant parity-related variation, although values remained within physiological ranges. Alanine aminotransferase, GOT, LDH, and HDL were generally higher in early parities and progressively declined in older animals, suggesting age-related modulation of hepato-muscular metabolic activity rather than pathological impairment. Interpretation of lipoprotein fractions in ruminants should nevertheless be approached cautiously, as these parameters are strongly influenced by hepatic export capacity and lipid turnover dynamics (Kessler et al., 2014). Among liver transport indicators, only LDL showed a significant parity × sampling season interaction, suggesting seasonal modulation of lipid transport mechanisms. In dairy cattle, heat stress is known to reduce lipid mobilization and transport as an adaptive strategy to minimize metabolic heat production (Baumgard and Rhoads, 2013; Bernabucci et al., 2014; Gross et al., 2015), which may explain the reduced LDL concentrations observed in older buffaloes during the hot season. Conversely, higher LDL concentrations in mature buffaloes under cold conditions may indicate more efficient lipid turnover under favorable environmental circumstances. Consistently, optimal environmental conditions have been reported to enhance metabolic efficiency by modulating lipid metabolism and reducing plasma LDL concentrations in dairy cows (Kessler et al., 2014). This effect may be further enhanced by the high lipid demand of the mammary gland, which reaches its peak functional capacity at full productive maturity (Fiore et al., 2017b). However, the elevated LDL concentrations observed in the oldest parity group may also reflect population heterogeneity and age-related declines in metabolic efficiency, insulin sensitivity, and peripheral lipid utilization, as described in older dairy cattle (Liu et al., 2024). Enhanced hepatic very-low-density lipoprotein export, a compensatory mechanism against hepatic lipid accumulation, may further contribute to this pattern (Gross et al., 2015). Among additional hepatic-muscular indicators, ALP activity increased from parity 1 to parity 3 before stabilizing, likely reflecting maximal metabolic demand associated with skeletal remodeling and hepatic

activity during early maturity (Cozzi et al., 2011; Taio et al., 2025). In contrast, CK concentrations were highest in primiparous buffaloes and progressively declined with advancing parity. Creatine kinase is widely recognized as a marker of skeletal muscle activity or injury (Hoffmann and Solter, 2008). Although parity-related CK variation has not previously been evaluated in buffaloes, the observed pattern aligns with findings in dairy cattle (Harris et al., 2007; Val-Laillet et al., 2009; Cozzi et al., 2011) and may be associated with greater social stress, competition, or muscular activity in younger animals housed with more mature individuals. However, in the absence of direct behavioral or welfare measurements, this interpretation remains speculative.

Association Between DIM and Serum Metabolites

Lactation stage emerged as a major source of variation in energy-related metabolites, reflecting dynamic physiological changes in energy balance across DIM. The overall pattern, characterized by greater lipid mobilization during early lactation followed by progressive decline and stabilization, suggests a transition from an initial catabolic state toward restored metabolic homeostasis after peak milk production. This trend is consistent with previous findings in Mediterranean buffaloes (Fiore et al., 2018) and parallels patterns described in dairy cattle, where early lactation is typically associated with intensified lipid mobilization followed by gradual recovery of energy balance (Xu et al., 2018; Churakov et al., 2021; Rico and Barrientos-Blanco, 2024). Within this context, decreasing NEFA concentrations and stabilization of BHB may indicate reduced dependence on adipose tissue reserves as lactations progresses, whereas the increase and subsequent stabilization of CHOL likely reflect progressive recovery of hepatic lipoprotein synthesis and lipid transport capacity, as previously described in buffaloes (Fiore et al., 2017a; 2018). The maintenance of these metabolites within physiological ranges throughout lactation further supports the presence of relatively stable metabolic regulation, likely sustained by moderate production intensity and adequate nutritional management (Rico and Barrientos-Blanco, 2024), although a mild and transient negative energy balance during early lactation cannot be excluded. The lower CHOL concentrations observed during late lactation may additionally reflect increased CHOL utilization by steroidogenic tissues to support endocrine activity during pregnancy and the periparturient period, as suggested in dairy cattle (Arfuso et al., 2016). Seasonal conditions appeared to further modulate these dynamics, as suggested by significant DIM × sampling season interactions for NEFA and CHOL. Under heat stress conditions, reduced feed intake and suppression of lipolytic activity are recognized adaptative responses aimed at minimizing endogenous heat production in ruminants (Baumgard and Rhoads, 2013; Bernabucci et al., 2014). Accordingly, the lower NEFA concentrations observed during early lactation in buffaloes sampled during the hot season, compared with those sampled during the cold season, are consistent with reduced adipose mobilization. Similarly, lower CHOL concentrations may reflect diminished hepatic lipid metabolism secondary to reduced nutrient intake, as reported in dairy cattle (Gao et al., 2017). The greater variability observed across DIM during the hot season may indicate less coordinated metabolic adaptation under thermal stress. Overall, these findings suggest that DIM-related metabolic adaptations in Italian Mediterranean buffaloes involve coordinated modulation of energy metabolism in response to both physiological and environmental pressures.

In contrast to energy profile, protein biomarkers exhibited relatively limited variation across DIM and lacked clear directional trends, indicating possible tighter physiological regulation. This relative stability, previously reported in buffaloes (Bertoni et al., 1993), suggests that circulating protein fractions are less sensitive to metabolic fluctuations during lactation than energy-related metabolites. Urea represented the primarily exception, showing lower concentrations during early lactation followed by gradual increases as lactation progressed. This pattern likely reflects progressive increases in feed intake and rebalancing of the dietary energy-to-protein ratio after peak lactation, thereby improving nitrogen utilization efficiency as animals transition toward metabolic stability. Similar patterns have been described in both dairy cattle and other buffalo populations (Grasso et al., 2004; Piccione et al., 2012). Creatinine also exhibited limited sensitivity to DIM-related physiological variation, although the slight increase observed during late lactation may reflect modest shifts in muscle metabolism or changes in energy partitioning associated with reduced production demands and altered body reserve dynamics (Premi et al., 2021).

Markers of hepatic and muscular function further supported a pattern of physiological adaptation rather than pathological dysfunction. Enzymes associated with hepatic activity, including GGT, GPT, GOT, and TBIL, showed gradual modulation across lactation, consistent with the transition from elevated metabolic demand in early lactation toward a more stable metabolic condition in mid and late lactation. Importantly, these variations occurred without evidence of hepatocellular damage. This pattern aligns with established observations in ruminants, where improvements in energy balance are commonly associated with normalization of hepatic metabolic function (Cozzi et al., 2011; Fiore et al., 2018; Rico and Barrientos-Blanco, 2024). Environmental con-

ditions also appeared to influence hepatic responses, as indicated by the significant DIM × sampling season interaction for GGT. Lower GGT concentrations observed throughout lactation during the hot season may reflect reduced hepatic metabolic activity associated with lower feed intake and altered nutrient partitioning. In ruminants, heat stress is known to suppress hepatic oxidative metabolism and modify lipid handling as part of broader physiological adaptation mechanisms (Baumgard and Rhoads, 2013; Bernabucci et al., 2014), which may partially explain the reduced circulating GGT concentrations observed under hot conditions.

Association Between Season of Sampling and Serum Metabolites

Among the evaluated metabolites, CREA was the only parameter significantly associated with sampling season, with lower concentrations observed during the hot season compared with the cold season. In ruminants, CREA is generally considered a relatively stable indicator of muscle metabolism and is typically only marginally influenced by short-term physiological fluctuations. However, CREA concentrations may also be indirectly affected by dietary protein sources and variations in feed intake (Bradford, 2021). Given that diet composition remained largely consistent across sampled farms, the seasonal variation observed in CREA is unlikely to be primarily attributable to nutritional differences. Instead, higher environmental temperatures during the hot season may have reduced feed intake, as previously reported in lactating dairy cattle (Gao et al., 2017), potentially contributing to modest alterations in muscle or protein metabolism. Although recent studies indicate that elevated temperature–humidity index can negatively affect milk yield and quality in Mediterranean buffaloes (Albenzio et al., 2024), the clinical and metabolic consequences of thermal stress in this species remain only partially understood. Overall, the limited seasonal effect observed in the present study suggests that sampling season exerts a relatively minor influence on the metabolic profile of Italian Mediterranean buffaloes, with detectable changes emerging primarily within selected components of the protein profile.

Association Between ECM Total Production and Serum Metabolites

The association between ECM production classes and serum metabolites provided important insight into the metabolic adaptations supporting increased productive performance in Italian Mediterranean buffaloes. The relative stability of major energy-related indicators, particularly GLU and NEFA, suggests that higher production levels were not associated with marked negative energy balance or excessive lipid mobilization. This pattern supports the hypothesis that buffaloes can sustain greater milk production through efficient metabolic regulation without extensive reliance on body reserves, differing from the more pronounced metabolic challenges often observed in high-producing dairy cattle (Cozzi et al., 2011; Tessari et al., 2020; Lisuzzo et al., 2023). In contrast, the increase in CHOL and the moderate rise in BHB observed with increasing ECM likely reflect enhanced hepatic metabolic activity and oxidative energy metabolism required to support elevated productive demands. In dairy cattle, similar increases have been associated with intensified hepatic lipid metabolism and ketone body turnover during increased lactational output, often representing physiological rather than pathological adaptations when maintained within normal ranges (Gross et al., 2015; Benedet et al., 2019). Comparable patterns previously reported in Mediterranean buffaloes further support this interpretation (Fiore et al., 2023; Lisuzzo et al., 2023), suggesting that these changes represent coordinated metabolic adjustments rather than indicators of metabolic distress.

The protein profile remained largely stable across ECM classes, indicating preserved hepatic synthetic capacity and relatively consistent nitrogen metabolism regardless of production level. Similarly, the significant but physiologically contained increases in hepatic and muscular enzymes, including GOT, GGT, and CK, are consistent with increased tissue metabolic activity associated with higher productive performance. Comparable responses have been described in high-yielding dairy cattle, where moderate increases in these biomarkers were interpreted as normal physiological adjustments to elevated metabolic demand (Cattaneo et al., 2023; Taio et al., 2025). Overall, these findings suggest that higher ECM production in Italian Mediterranean buffaloes is supported by coordinated metabolic adjustments, particularly involving hepatic and muscular pathways, without evidence of substantial metabolic imbalance. This adaptive capacity may reflect species-specific metabolic resilience and the ability of buffaloes to sustain elevated productive performance under appropriate nutritional and management conditions.

CONCLUSIONS

This study investigated the main sources of variation in serum metabolite profiles of lactating Italian Mediterranean buffaloes. Most metabolites showed limited variability and remained within physiological ranges, indicating a stable metabolic profile in clinically healthy animals. Parity and lactation stage were mainly associated with changes in protein metabolism and hepatic

lipid regulation, whereas energy-related biomarkers remained relatively stable across the evaluated physiological conditions. Season-related interactions were limited, suggesting that environmental conditions had a moderate influence under the production systems considered. Overall, these findings provide useful population-level information to support the interpretation of metabolic status in lactating Italian Mediterranean buffaloes managed under comparable nutritional, environmental, and management conditions. Further research across different production systems and geographical areas is needed to confirm the broader applicability of these metabolic patterns.

NOTES

This research was funded by the Italian Ministry of Agriculture (MIPAAF–DISR 07), Programma di Sviluppo Rurale Nazionale 2014/2020 (Rome, Italy), under the project "Caratterizzazione delle risorse genetiche animali di interesse zootecnico e salvaguardia della biodiversità", Sottomisura 10.2 "Sostegno per la conservazione, l'uso e lo sviluppo sostenibili delle risorse genetiche in agricoltura", project "Bufala Mediterranea Italiana–Tecnologie innovative per il miglioramento Genetico—BIG" Prot. N. 0215513 11/05/2021, CUP ANASB: J29J21003720005; CUP UNINA: J69J21003020005. This study was also carried out within the Agritech National Research Center, Naples, Italy, and received funding from the European Union Next-GenerationEU, Piano Nazionale di Ripresa e Resilienza (PNRR)—Missione 4 Componente 2, Investimento 1.4—D.D. 1032 17/06/2022, CN00000022. This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them. Supplemental material for this article is available at https://doi.org/10.6084/m9.figshare.32253519. The study received approval from the Ethical Animal Care and Use Committee of the University of Naples "Federico II" (Protocol number PG/2022/0025539). The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: A/G = albumin-to-globulin ratio; ADFom = ADF exclusive of residual ash; ALB = albumin; ALP = alkaline phosphatase; ANASB = National Buffalo Breeder Association; aNDFom = amylase-treated NDF exclusive of residual ash; CEL = cellulose; CHOL = cholesterol; CK = creatine kinase; CREA = creatinine; EE = ether extract; GGT = γ-glutamyl transferase; GLO = globulin; GLU = glucose; GOT = aspartate aminotransferase; GPT = alanine aminotransferase; HC = hemicellulose; HDL = high-density lipoprotein; HSD = herd sampling day; IQR = interquartile range; LDH = lactate dehydrogenase; LDL = low-density lipoprotein; NEFA = nonesterified fatty acid; Q1, Q3 = quartiles 1 and 3, respectively; TBIL = total bilirubin; TP = total protein; TRI = triglyceride; uNDF = undigested NDF.

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