J. Dairy Sci. 108:8697–8709
https://doi.org/10.3168/jds.2025-26414

Feasibility of milk mid-infrared spectroscopy for large-scale screening of hematic traits in dairy buffaloes

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ABSTRACT

Blood profile testing is a valuable tool for monitoring the metabolism, health, and nutritional status of lactating dairy animals, including buffaloes. However, conducting extensive analyses on a large scale is not feasible due to high costs, labor, the need for invasive procedures, and consequent animals' stress. The utilization of the Fourier-transform mid-infrared (FT-MIR) spectroscopy to predict hematic traits from milk spectral information may represent an effective opportunity for the dairy sector, given that buffaloes are under official testing in various countries. This study aims to test the ability of milk FT-MIR spectra to predict the most relevant buffaloes' hematic components. Blood and milk samples were collected once in 9 Italian farms from 308 buffaloes (98 primiparous and 210 pluriparous) at different stages of lactation. The hematic concentrations of urea, creatinine, glucose, total bilirubin, cholesterol, triglycerides, total protein, albumin, globulin, alanine and aspartate aminotransferases, alkaline phosphatase, lactate dehydrogenase, gamma-glutamyl transferase, and creatine kinase were measured using reference analysis to evaluate the predictive ability of FT-MIR. Spectral data (n = 308) were divided into a calibration set (70%) and a validation set (30%). In external validation, R^2 of 0.72, 0.60, 0.56, and 0.69 were achieved for urea, triglycerides, creatinine, and total bilirubin concentrations, respectively, whereas the R^2 of other hematic traits was <0.50. The spectral regions important for the prediction of triglycerides, creatinine, and total bilirubin fell in those associated with milk protein structures (1,570–1,550 cm^{-1}). Although our models were not accurate enough for precise determination of the blood parameters concentration, the predictions of some traits such as urea can be effectively used for herd-level screening (e.g., for nutritional status evaluation). Our results, although preliminary, provide the basis for future large-scale investigations on buffalo health status and metabolism.

Key words: blood biomarker, complex phenotype, animal health monitoring, serum parameters

INTRODUCTION

Italy hosts the vast majority of the European buffaloes (85.27%) and accounts for more than 87.5% of the total buffalo milk supplied in Europe (FAOSTAT, 2024). Based on data reported by the Italian breeders' association, Associazione Nazionale Allevatori Specie Bufalina (ANASB, 2024), the average herd size has increased from 134.1 in 2004 to 228.6 buffaloes in 2024.

The Mediterranean buffalo breed has undergone genetic selection oriented to milk production in the last decades and is nowadays specialized and known worldwide for fresh cheese production (Minervino et al., 2020), in particular, the Protected Designation of Origin "Mozzarella di Bufala Campana." Consequently, the sector has seen significant improvements at various levels within farms and along the chain, which allowed for efficiency boosting and increase in the milk production (De Rosa et al., 2005; Costa et al., 2020a). Most of the high-tech modern sensors and equipment and associated algorithms, such as vacuum and parameters of milking ejection curves, however, are often developed according to the cattle ethology, morphology, and physiology, which are substantially different from buffaloes.

Therefore, early detection of disease and real time monitoring through precision livestock farming devices become difficult. Timely alerts can limit losses, as they prevent the development of a clinical disease. Although diagnosis of clinical metabolic diseases is less frequent in buffaloes than in cows (Fiore et al., 2017; Bertoni et al., 2020), their incidence could still increase due to the intensification of farming systems and, partly, to the genetic selection toward more productive animals. Even though blood testing allows for monitoring metabolic

Received February 3, 2025.
Accepted April 21, 2025.
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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

health and nutritional status, most of the time it occurs when the clinical signs are already evident. Moreover, blood sampling is time-consuming, invasive, and stressful for the animals, and the laboratory analyses with gold standard methods are costly. In this context, there is significant interest in utilizing Fourier-transform mid-infrared spectroscopy (**FT-MIR**) spectra—already in use for buffalo milk composition assessment in Italy and other countries (ICAR, 2017)—to predict complex phenotypes and health biomarkers which are challenging to be measured in the field with standardized and validated protocols (Egger-Danner et al., 2012).

In dairy cows, several studies have been conducted on the prediction of blood metabolic traits (e.g., BHB, nonesterified fatty acid, urea), showing moderate accuracy results (Benedet et al., 2019; Luke et al., 2019; Giannuzzi et al., 2023; Magro et al., 2024). Few studies have focused on protein-related traits and enzyme activities (Giannuzzi et al., 2023; Magro et al., 2024), reporting low to moderate accuracy. However, to our knowledge, no studies have attempted to carry out the same in buffaloes so far. Therefore, with the present study, we tested the ability of milk FT-MIR to predict hematic traits related to metabolic and protein profiles, as well as enzyme activity.

MATERIALS AND METHODS

Between February and September 2022, milk and blood samples were collected from 308 Mediterranean Italian buffaloes (98 primiparous and 210 pluriparous) reared on 9 farms located in Central and Southern Italy. The minimum and maximum number of buffaloes sampled per farm was 25 and 58, respectively. The sampled buffaloes were at different lactation stages (134.55 ± 74.30 DIM) and parity orders (2.89 ± 1.98 lactations). The buffaloes were randomly sampled 1 time in this study and calved in all months of the year; therefore, farms adopting specific fertility protocols to reduce the seasonality of the species (Neglia et al., 2020) and have calvings out of the breeding season were also involved. All the farms were characterized by an intensive farming system with a freestall barn, and TMR feeding, mostly silage-based. Animals with clinical signs of disease (e.g., ketosis, mastitis) or who received medical treatment were not involved in this study.

The study received approval from the Ethical Animal Care and Use Committee of the University of Naples "Federico II" (Protocol number PG/2022/0025539).

Milk Samples

Individual milk samples were taken during the morning milking in correspondence with the monthly official milk testing, following the guidelines of ICAR (2017). Information on DIM, parity, and milk yield (kg/d) were also retrieved. Milk was stored in tubes with bronopol (2-bromo 2-nitro 1,3-propandiol) for preservation. Milk samples were transported to the milk laboratory of the Breeders Association of Basilicata (Potenza, Italy) for FT-MIR analysis and spectrum acquisition through the MilkoScan 7 RM machine (Foss Electric A/S, Hillerød, Denmark). For all buffaloes, fat, protein, casein, lactose content, and urea concentration were determined via buffalo-specific models installed. In this study, spectral information containing 1,060 infrared transmittance data in the region between 5,000 and 900 cm^{-1} was stored for each sample. Additionally, the SCC (cells/mL) value was determined in the same laboratory using flow cytometry (Fossomatic, Foss Electric A/S, Hillerød, Denmark).

Blood Samples

On the same day, blood was sampled via caudal venipuncture immediately after milking (within 1 h maximum). This procedure required buffaloes to be immobilized in a containment trunk and a preliminary cleaning of the venipuncture site with a disposable paper towel. The 10 mL vacuum tubes adopted were free of anticoagulant agent (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK) and were centrifuged (300 × *g* at room temperature for 15 min) for serum separation directly in the field. The samples were then stored at −18°C until analysis at the Department of Veterinary Medicine and Animal Production, University of Naples Federico II (Certified Quality Management System ISO 9001:2015, Naples, Italy).

On the serum samples, the determination of biochemical traits related to the metabolic and protein profiles, as well as enzyme activity parameters, were assessed using an automated clinical analyzer (SAT 450 AMS Alliance, KPM Analytics, Westborough, MA) with commercially available kits purchased by Spinreact (Barcelona, Spain). In particular, concentrations of urea, creatinine, glucose, total bilirubin (**TBIL**), cholesterol, triglycerides, total protein, albumin, globulin, alanine and aspartate aminotransferases (**ALT** and **AST**, respectively), alkaline phosphatase (**ALP**), lactate dehydrogenase (**LDH**), gamma-glutamyltransferase (**GGT**), and creatine kinase (**CK**) were determined. Finally, the albumin-to-globulin was calculated as the ratio between albumin-to-globulin. All blood traits were available for all buffaloes, except for CK, which was only available for 271 animals.

Mid-Infrared Prediction Models

The software R v. 4.3.3 (R Core Team, 2022) was used for data handling and model development. A diagram

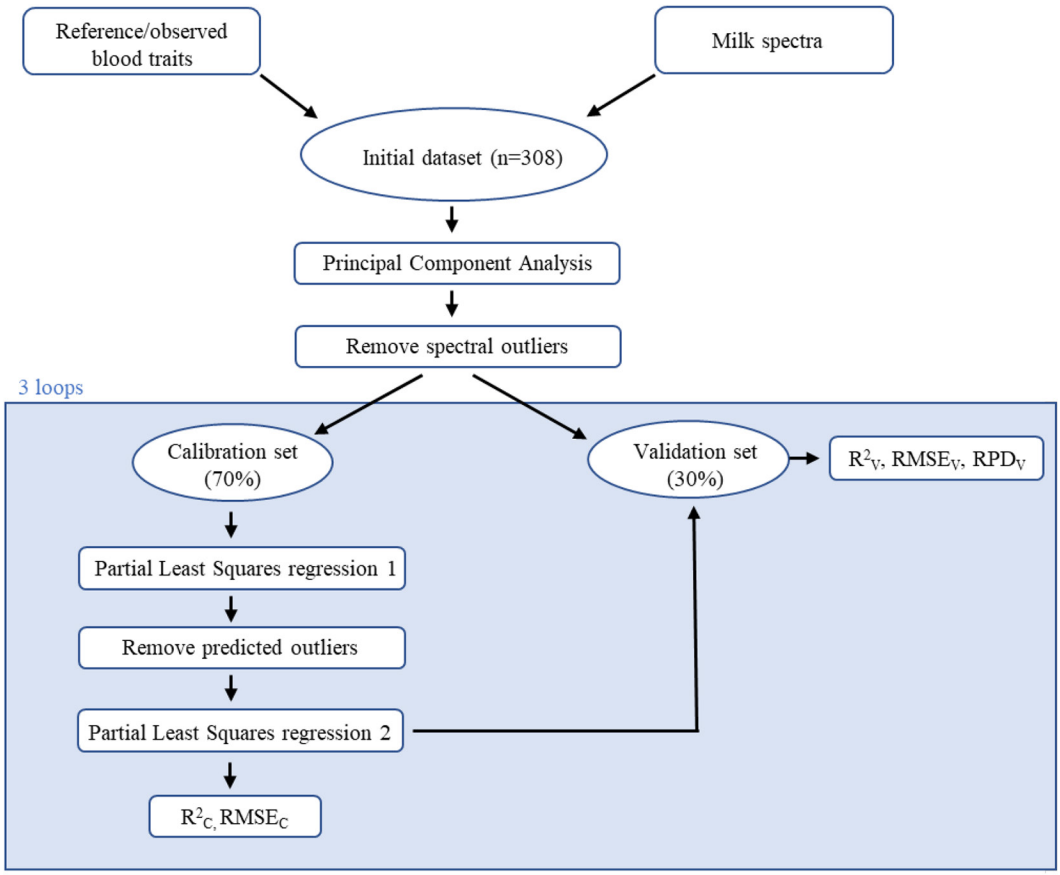

Figure 1. Frameworks and phases for mid-infrared prediction model development. R^2_C = coefficient of determination in calibration; $RMSE_C$ = root mean square error in calibration; R^2_V = coefficient of determination in validation; $RMSE_V$ = root mean square error in validation; RPD_V = ratio of prediction to deviation in validation. These values represent the averages over the 3 loops 3 iterations.

with the main steps implemented for the development of prediction models is represented in Figure 1. Each milk spectrum was paired with its corresponding blood sample to link with the reference hematic traits. In the case of nonnormally distributed traits, that is, CK, TBIL, and ALP, values were preliminarily $\log_{10}$-transformed.

Spectral transmittance values were converted to absorbance using the formula: absorbance = $\log_{10}$(1/transmittance). Spectral regions with significant noise due to water absorption (De Marchi et al., 2014; Grelet et al., 2015; Zhang et al., 2021) were excluded, resulting in a selection of 338 wavelengths distributed across the following intervals: 945.5 to 1,585.6 cm^{-1}, 1,716.8 to 1,929.0 cm^{-1}, and 2,507.7 to 2,970.7 cm^{-1}. After that, a principal component analysis (**PCA**) was used to remove potential spectral outliers through the "PCA" function of R package "FactoMineR" starting from scaled spectra. Five samples/spectra were identified as outliers based on their distance from the main cluster in the 2-dimensional PCA scores plot, leaving 303 records.

For each blood trait, the complete dataset was randomly divided into a calibration set (70% of total samples) and a validation set (30%) with "createDataPartition" function from the R package "caret" as described in Kuhn (2008). In the 2 sets, mean and SD of the target trait were similar and were representative of parities and lactation stages. Subsequently, models were developed using the partial least squares (**PLS**) regression with the "trainControl" function from the R package "caret" to obtain predictions of the reference/observed values. Model fine-tuning was conducted with the leave-one-out cross-validation and the number of latent variables (**LV**) was set automatically but capped at a maximum of 15 to avoid overfitting. The root mean square error (**RMSE**) in leave-one-out cross-validation was used to evaluate the best model. Spectral data points were scaled and additionally centered, and predicted outliers were defined as observations having a residual between predicted and observed values in calibration more than 2.5 SD from the residual average. External validation was repeated 3

times, each time on a different set of buffaloes, and the reported fitting statistics represent the average over these 3 iterations. The PLS model fit statistics included the R^2 for both leave-one-out cross-validation $\left(R^2_C\right)$ and external validation $\left(R^2_V\right)$, the RMSE, and the ratio of performance to deviation (**RPD**) in validation.

For each of the hematic traits, the most important spectral regions for the prediction were identified using the variables' importance score (**VIP**), calculated as the weighted sum of absolute regression coefficients (Kuhn, 2008) by means of the "varImp" function of the R package "caret." For each trait, VIP >60% was considered and represented graphically.

RESULTS

General Overview

Samples used in the present study belonged to buffaloes that produced on average 13.05 kg/d of milk, with 7.31%, 4.65%, and 3.76% of fat, protein, and casein content, respectively (Table 1). The milk urea concentration was on average 37.46 mg/dL, whereas SCS averaged 4.43, with a median SCC of 125.500 cells/mL.

Regarding metabolic profile, on average, cholesterol was the most abundant trait, followed by glucose and urea, whereas the less abundant metabolic traits in the blood were creatinine and TBIL (Table 2). In contrast, TBIL was the most variable trait with CV of 11.11%, whereas triglycerides were the least variable among the metabolic profile (CV, 13.19%). With regard to enzyme activity traits, LDH was the most abundant enzyme present, with a concentration ~40 and 30 times higher than those of the enzymes present at the lowest concentrations (i.e., GGT and ALT, respectively; Table 2). Among enzyme activity traits, ALP was the most variable trait (CV, 65.95%), whereas LDH was the least variable (CV, 16.33%). Regarding protein profile, the CV ranged from 7.65% for albumin to 16.15% for globulin. The r were calculated within blood traits for completeness (Supplemental Table S1, see Notes). The strongest correlation was calculated within protein and metabolic profile. In particular, triglycerides were negatively correlated with TBIL (−0.65; Supplemental Table S1) and albumin was positively correlated with glucose (0.64; Supplemental Table S1). Correlations were calculated between blood and milk traits also to evaluate whether milk is capable of mirroring blood (Supplemental Table S1). In this case, however, only a few correlations were significant and all of them were weak (<0.21), except for the association between hematic urea and milk urea (0.59; $P < 0.05$).

Table 1. Descriptive statistics of milk traits recorded on 308 buffaloes

Milk trait	Mean (SD)	Range
Yield, kg/d	13.05 (3.75)	4.60–26.10
Fat, %	7.31 (1.88)	2.51–15.28
Protein, %	4.65 (0.44)	3.54–6.18
Casein, %	3.76 (0.40)	2.84–5.28
Lactose, %	4.72 (0.26)	3.75–5.34
Urea, mg/dL	37.46 (14.16)	2.20–80.41
SCS[1]	4.43 (1.83)	−0.32–10.58

[1]Calculated as SCS = 3 + $\log_2$(SCC/100,000).

Table 2. Descriptive statistics of the blood traits analyzed in 308 buffaloes

Trait[1]	Mean (SD)	Range
Metabolic profile		
Urea, mg/dL	60.01 (16.83)	21.00–129.90
Creatinine, mg/dL	1.62 (0.32)	0.90–2.70
Glucose, mg/dL	67.34 (11.92)	25.30–115.00
TBIL, mg/dL	0.27 (0.30)	0.01–4.34
Cholesterol, mg/dL	122.22 (32.50)	38.90–283.00
Triglycerides, mg/dL	18.57 (12.45)	0.20–45.10
Protein profile		
Total protein, g/dL	6.93 (0.72)	4.73–8.73
Albumin, g/dL	3.40 (0.26)	2.55–4.07
Globulin, g/dL	3.53 (0.57)	2.18–5.05
Albumin-to-globulin	0.99 (0.15)	0.59–1.57
Enzyme activity		
ALT, U/L	45.17 (10.97)	17.30–94.00
AST, U/L	130.50 (28.95)	63.60–327.00
ALP, U/L	287.94 (189.90)	19.74–1,528.00
GGT, U/L	30.52 (8.06)	13.00–66.02
LDH, U/L	1,256.22 (205.12)	652.00–1,845.00
CK, U/L	178.00 (76.65)	53.00–793.00

[1]ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase; CK = creatine kinase; GGT = gamma-glutamyl transferase; LDH = lactate dehydrogenase; TBIL = total bilirubin.

Prediction Ability of FT-MIR

The R^2 and RMSE of the prediction models for hematic trait concentrations are shown in Table 3. The number of predicted outliers ranged from 1.60% (albumin-to-globulin) to 4.90% (creatinine), and the number of LV used in the PLS ranged from a minimum of 6 to a maximum of 15.

Overall, the prediction models developed for metabolic profile exhibited the best performance. In particular, the most notable models in terms of R^2 were those for urea concentration and log-transformed TBIL (both R^2 in calibration = 0.76; Table 3, Figure 2). When applied to an external set, these models continued to show a moderate performance (R^2_V = 0.72 for urea and R^2_V = 0.69 for TBIL log-transformed; Table 3). Additionally, prediction models for triglycerides and creatinine concentrations demonstrated fair performance, with an R^2 in the calibration of 0.73 and 0.67, and in the validation of 0.60 and 0.56, respectively (Table 3, Figure 2). Although the cholesterol concentration model performed

Table 3. Performance[1] of the milk mid-infrared spectroscopy models developed for the buffaloes' blood traits

Trait[2]	Outlier, %	No. LV	R^2_C	$RMSE_C$	R^2_V	$RMSE_V$	RPD_V
Metabolic profile							
Urea, mg/dL	4.6	13	0.76	7.11	0.72	8.31	1.87
Creatinine, mg/dL	4.9	14	0.67	0.16	0.56	0.22	1.50
Glucose, mg/dL	3.6	14	0.44	7.63	0.29	8.67	1.23
TBIL, mg/dL	3.0	12	0.73	0.09	0.65	0.11	1.71
$\log_{10}$ TBIL	3.0	11	0.76	0.17	0.69	0.20	1.79
Cholesterol, mg/dL	4.3	15	0.58	18.08	0.24	25.06	1.05
Triglycerides, mg/dL	2.3	9	0.73	6.39	0.60	7.71	1.62
Protein profile							
Total protein, g/dL	3.0	6	0.30	0.57	0.19	0.61	1.10
Albumin, g/dL	2.3	10	0.37	0.20	0.15	0.23	1.09
Globulin, g/dL	2.3	6	0.32	0.45	0.24	0.49	1.10
Albumin-to-globulin	1.6	7	0.29	0.12	0.24	0.13	1.16
Enzyme activity							
ALT, U/L	3.0	10	0.51	6.87	0.35	7.90	1.07
AST, U/L	2.6	9	0.33	19.83	0.15	23.92	0.98
ALP, U/L	4.6	6	0.20	118.21	0.14	127.53	1.07
$\log_{10}$ ALP	3.9	6	0.20	0.20	0.08	0.22	1.04
GGT, U/L	4.6	6	0.36	5.28	0.21	6.08	1.11
LDH, U/L	3.0	11	0.47	138.73	0.26	176.44	1.13
CK, U/L	3.3	10	0.25	47.81	0.07	51.01	0.91
$\log_{10}$ CK	3.6	8	0.37	0.11	0.14	0.13	1.06

[1]R^2_C = coefficient of determination in calibration; $RMSE_C$ = root mean square error in calibration; R^2_V = coefficient of determination in validation; $RMSE_V$ = root mean square error in validation; RPD = ratio of performance to deviation in validation.

[2]ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase; CK = creatine kinase; GGT = gamma-glutamyl transferase; LDH = lactate dehydrogenase; TBIL = total bilirubin.

fairly in calibration (R^2_C = 0.58; Table 3), it exhibited very low performance during validation (R^2_V = 0.24; Table 3). Finally, the glucose model had a low R^2 in both the calibration and validation phases. The RPD in validation for the metabolic profile ranged from 1.05 for cholesterol to 1.87 for urea concentration (Table 3).

Enzyme activities were generally predicted with lower accuracy compared with the metabolic profile. Among the enzyme activity models, the one developed for ALT concentration performed best (R^2_C = 0.51; Table 3), followed by LDH concentration (R^2_C = 0.47; Table 3). When tested on an external sample set, these 2 models showed lower R^2 values (R^2_V = 0.35 and R^2_V = 0.26 for ALT and LDH, respectively). Similarly, the protein profile traits were predicted with lower accuracy, with R^2 values in calibration ranging from 0.30 for total protein to 0.37 for albumin. In validation, R^2 values ranged from 0.15 for albumin to 0.24 for globulin and the albumin-to-globulin ratio (Table 3). The RPD in validation for enzyme activities and protein profile was equal to or lower than 1.13 (Table 3).

Each calibration model demonstrated important wavelengths for the determination of different blood traits. Wavelengths with an importance greater than 60% are presented in Figure 3 for the metabolic profile, Figure 4 for the protein profile, and Figure 5 for enzyme activities.

Regarding the metabolic profile (Figure 3), the wavelengths with the greatest VIP were located between 3,000 and 2,800 cm^{-1} for approximately all traits. In addition, cholesterol, creatinine, and glucose also exhibited important wavelengths in the region between 1,800 and 1,700 cm^{-1}. For triglycerides, creatinine, and TBIL, high VIP were detected from 1,570 to 1,550 cm^{-1}. The most important wavelengths for traits related to protein profiles (Figure 4) and enzyme activities (Figure 5) fell within the same regions as above, that is, 3,000 and 2,800 cm^{-1}, 1,800 and 1,700 cm^{-1}, and 1,570 to 1,550 cm^{-1}.

When only the wavelengths reported in red in Figures 3, 4, and 5 (high VIP) were considered for developing the prediction equation, the models' accuracy reduced substantially in all cases due to the absence of spectral regions whose contribution, despite being small, was additive. For example, the R^2_V of hematic urea reduced from 0.76 (whole spectrum) to 0.62.

DISCUSSION

General Overview

The milk yield of the sampled buffaloes in this study exceeded the average value of 8.74 kg/d reported by AIA (2023) for all national buffaloes monitored in 2023 that refers to the standard lactation length (270 DIM) of first,

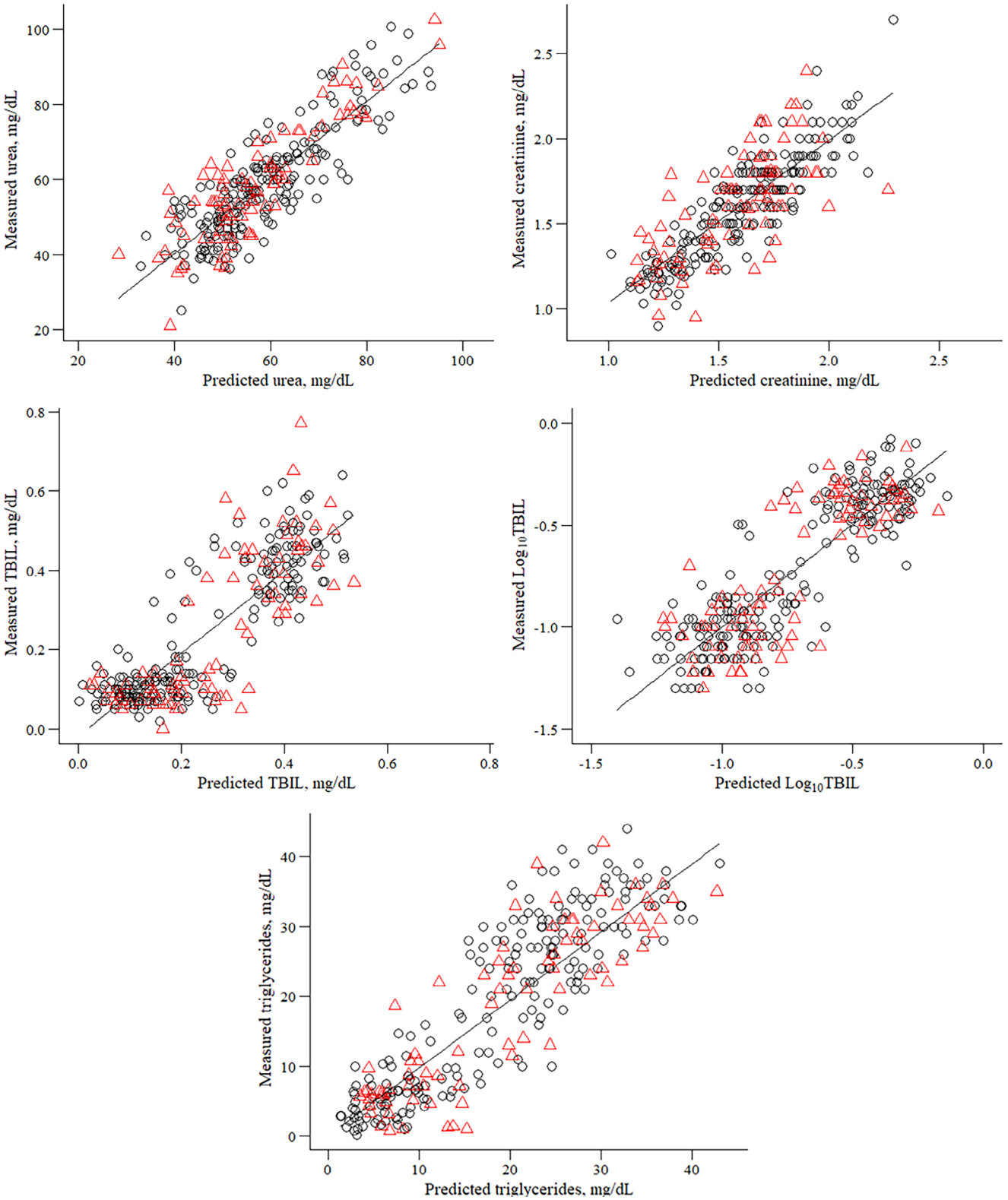

Figure 2. Plot of mid-infrared predictions (x-axis) and measured values (y-axis) for the blood traits (only prediction models with R^2 in validation >0.55 are presented, namely: blood urea, creatinine, total bilirubin [TBIL] and triglycerides). Calibration set is reported in black (●), and validation set in red (▲).

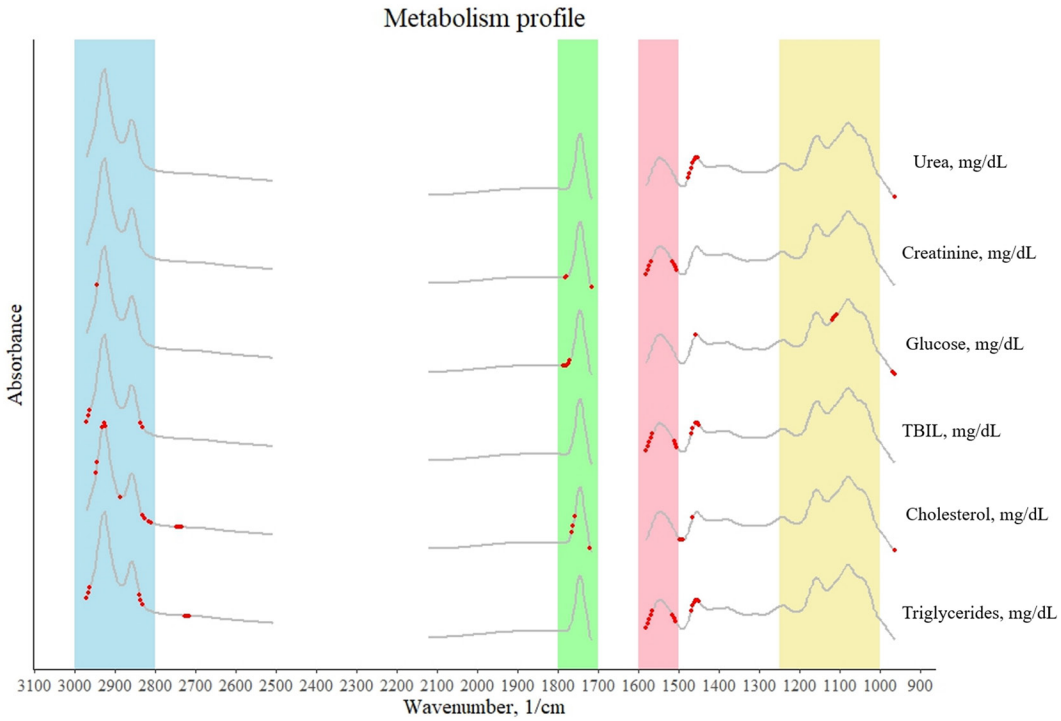

Figure 3. Total wavenumbers used for prediction of blood traits related to metabolic profile, including total bilirubin (TBIL). The red dots represent the most important spectral wavenumbers (>60% of variance explained for each trait). The blue and green bands are known as the milk region "Fat B" and "Fat A," respectively (Iñón et al., 2004), and the pink and yellow bands represent the spectral window associated with milk protein and lactose (Grelet et al., 2015).

second, and third parity. The 9 farms that joined the study had an average herd size of 678 animals in 2022, which is higher than the national average of that year (218.80; AIA, 2025). In the last years, individual productivity level has constantly increased (Costa et al., 2020b), and often, farmers opt for extended lactations.

Conversely, the fat and protein content were slightly lower than those reported by Costa et al. (2020b) in 876,299 test-day records from 70,156 Italian buffaloes reared in Central and Southern Italy between 2013 and 2017. Additionally, SCS was greater than those (2.71 in 2013 and 3.11 in 2017) reported by Costa et al. (2020b) and those (3.92) observed by Bobbo et al. (2024) in 14,571 records from 1,501 animals raised in South Italy.

In terms of blood, discussion and proper comparison with the existing scientific literature was rather difficult. In fact, it was not possible to retrieve hematic reference intervals for adult buffaloes or lactating dairy buffaloes. Veterinary medicine university textbooks rarely address buffaloes, focusing primarily on cattle. Although these ruminants belong to the same subfamily (*Bovinae*), they exhibit distinct physiological and genetic traits, which complicates direct comparisons. In addition, only a few studies have been conducted on hematic parameters in buffaloes (Abd Ellah et al., 2014; Patel et al., 2016); however, these studies often involve small numbers of animals and farms in populations far removed from the Mediterranean region.

The concentrations of the traits were in most cases consistent with those typically observed in lactating buffaloes (Abd Ellah et al., 2014; Patel et al., 2016; Fiore et al., 2017). For example, the average concentrations of cholesterol, triglycerides, glucose, and urea were comparable to those reported by Fiore et al. (2017) in 93 Italian buffaloes in early-lactation Italian buffaloes (5–70 DIM).

However, the average TBIL was lower than that reported by Patel et al. (2016) in Indian buffaloes.

Hematic traits related to the protein profile were within the reference ranges established by Abd Ellah et al. (2014) in 132 healthy buffaloes raised in Egypt within 8 wk postpartum. Similarly, these values aligned with those reported by Patel et al. (2016) for pregnant and nonpregnant lactating buffaloes reared in India.

Enzyme activity–related blood parameters, on average, were higher than those reported by Abd Ellah et al. (2014) and Patel et al. (2016). However, buffaloes in those studies were sampled from different countries, farming systems, and milk production levels. Djoković et al. (2013) demonstrated in dairy cows that enzyme activity markers such as AST, ALT, and GGT can pro-

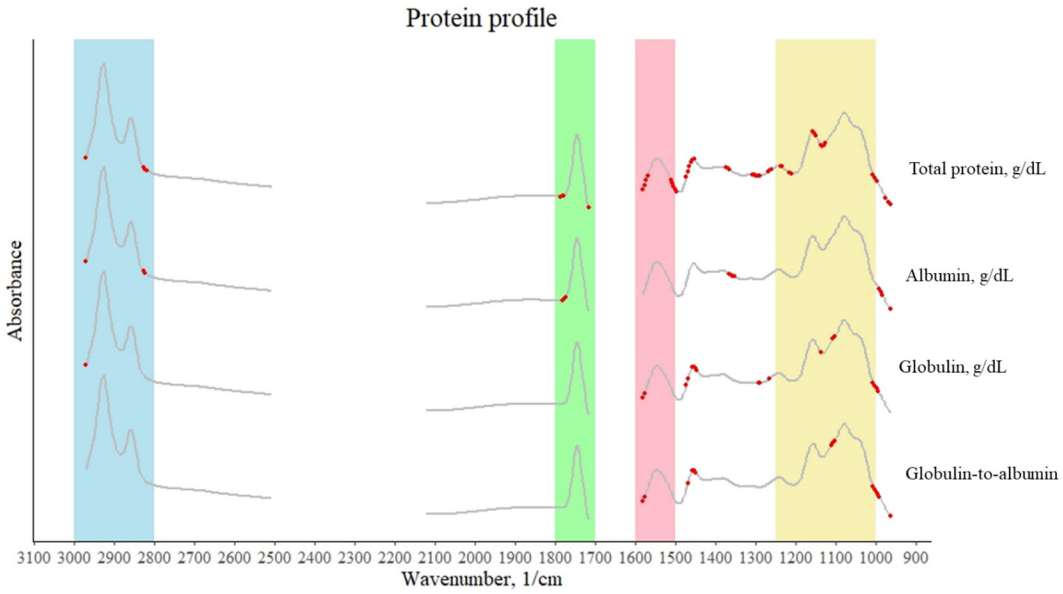

Figure 4. Total wavenumbers used for prediction of blood traits related to protein profile. The red dots represent the most important spectral wavenumbers (>60% of variance explained for each trait). The blue and green bands are known as the milk region "Fat B" and "Fat A," respectively (Iñón et al., 2004), and the pink and yellow bands represent the spectral window associated with milk protein and lactose (Grelet et al., 2015).

vide insights into liver function and production potential. Moreover, it cannot be excluded that buffaloes may have subclinical disease; only animals with visible clinical signs were not sampled. To date, no reference values for blood parameters specific to Mediterranean buffaloes raised in Italy for milk production have been established.

Prediction Ability of FT-MIR

Although FT-MIR has been already applied in dairy cows for the prediction of various blood parameters, it is important to highlight that buffaloes and cows are morphologically and physiologically different animals, with their intrinsic characteristics, such as metabolic activity, body reserves partition, mammary gland structures and compartments, nutritional requirements, and energy intended to milk synthesis and secretion. Moreover, the 2 species differ in terms of FT-MIR spectra and milk composition (Ravinder et al., 2021). For this reason, buffalo-specific prediction models are commercialized and installed in the machineries of laboratories analyzing buffalo milk. Similarly to what happens for cattle, in Italy, buffaloes' prediction models for fat, protein, casein, and lactose content are periodically updated, and all laboratories under Associazione Italiana Allevatori are subjected to periodic ring testing to ensure data quality and comparability (AIA, 2024). Given that the literature on hematic parameters in buffalo is scarce, comparing the FT-MIR models of this study with other articles on buffalo was not possible. In some cases, parallelism can be observed with findings of cattle.

As in cows, blood urea concentration in this species was reasonably predicted from milk spectra (Luke et al., 2019; Magro et al., 2024). In particular, Magro et al. (2024) obtained an R^2 of 0.89 in leave-one-out cross-validation and 0.86 in external validation in 249 Holstein cows. Similarly, Luke et al. (2019) reported an R^2 equal to 0.90 both in cross-validation and random validation in 773 Holstein cows in early lactation.

Conversely, creatinine and TBIL in buffaloes were predicted with greater accuracy compared with the results reported by Giannuzzi et al. (2023) in dairy cows, who found an R^2 of 0.60 and 0.48 in random cross-validation and 0.46 and 0.45 in herd-out cross-validation, respectively. Additionally, triglycerides levels in buffaloes were predicted with better accuracy than reported by Benedet et al. (2019) in early lactation dairy cows, where an R^2 of 0.16 was found in cross-validation. Predictive abilities for cholesterol and glucose in buffaloes were generally similar to those reported by Benedet et al. (2019) and Magro et al. (2024) for early lactation in dairy cows. Finally, blood traits related to protein profile (i.e., total protein, albumin, globulin, albumin-to-globulin) and enzyme activities were predicted with low accuracy, as also reported in dairy cows (Benedet et al., 2019; Luke et al., 2019; Magro et al., 2024). Across the protein profile, in this study, albumin-to-globulin was the trait with the best performance in validation (Table 3). Improving its

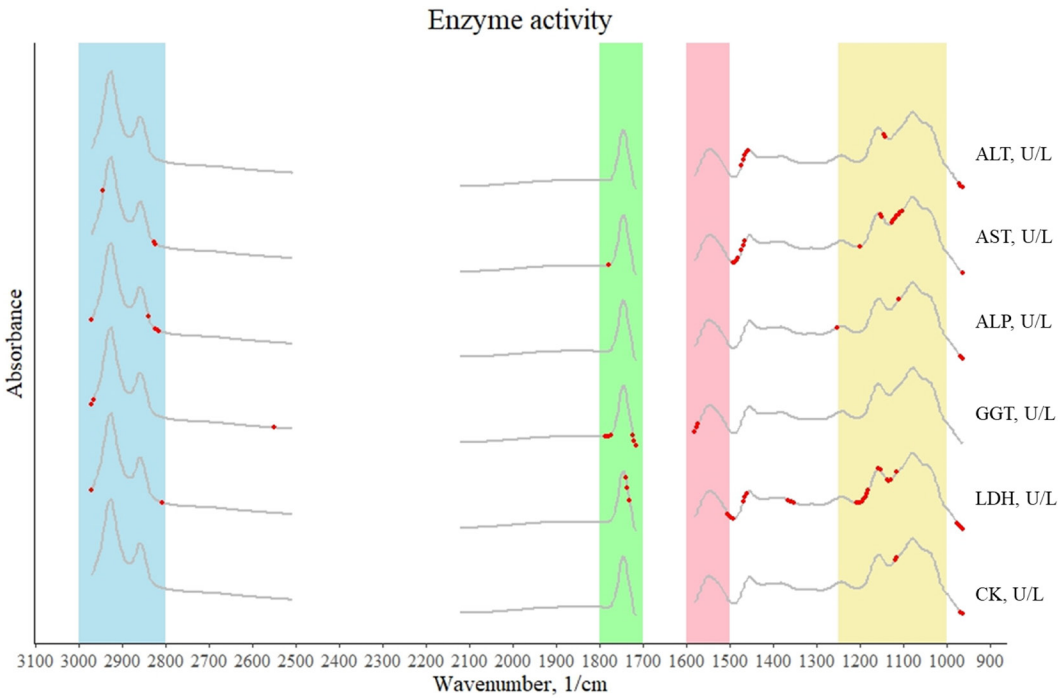

Figure 5. Total wavenumbers used for prediction of blood traits related to enzyme activities (ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase; CK = creatine kinase; GGT = gamma-glutamyl transferase; LDH = lactate dehydrogenase). The red dots represent the most important spectral wavenumbers (>60% of variance explained for each trait). The blue and green bands are known as the milk region "Fat B" and "Fat A," respectively (Iñón et al., 2004), and the pink and yellow bands represent the spectral window associated with milk protein and lactose (Grelet et al., 2015).

performance would be important because the albumin-to-protein ratio is a concise indicator of the blood protein profile and holds greater significance than the total protein concentration (Kaneko, 2008).

Predicting complex traits such as blood components using the milk spectra solely is challenging because it involves indirect prediction. Therefore, we expect blood traits to be predicted with lower accuracy compared with compounds directly present in the milk. However, the alveolar milk and the circulating blood are closely related to each other. In fact, the exchange of soluble and cellular components in the mammary gland is regulated by the blood-milk barrier, whose permeability selectively transfers components necessary for milk synthesis (Wellnitz and Bruckmaier, 2021). In dairy cows, a close linkage between blood and milk urea has been reported (Spek et al., 2012), which is corroborated by the positive significant correlation between milk and blood urea levels found in this study (0.59). Moreover, triglycerides in blood are used by mammary gland cells to produce milk fat, whereas the circulating glucose is the precursor of lactose (Costa et al., 2019). Hepatic enzymes are naturally present in cow milk due to the spontaneous diffusion of low-molecular-weight enzymes from blood, or following the disruption of mammary epithelial integrity (Giannuzzi et al., 2023). Low concentrations in blood are difficult to predict from milk spectra, particularly when the concentration is absent or significantly reduced in milk. Indeed, even if there is a direct prediction of phenotype, for example, milk traits from milk spectrum, the punctual concentration of components/molecules present in a very low concentration in the analyzed matrix is challenging and makes the trait hard to predict due to the weak signal(s) within the spectrum. In fact, FT-MIR predictive performance for quantitative traits is enhanced if absorption peaks are strong, as highly concentrated components exhibit stronger absorbance values in spectral regions where their chemical bonds are located (Soriano-Disla et al., 2014).

Another key point to consider when predicting hematic traits using milk spectrum is the distance between the blood and the milk sampling, which can play a crucial role and affect the goodness of prediction equations. Although milk is still expected to reflect blood composition, there is no exact parallelism, and there could be a delay in some cases for certain traits. For example, Aernouts et al. (2020) demonstrated that in dairy cows, the morning plasma level of nonesterified fatty acid is predicted with a significantly higher accuracy when using evening (R^2 = 0.61) rather than morning (R^2 = 0.50) milk spectra.

According to the general classification proposed by Grelet et al. (2021) for cow milk FT-MIR models, the R^2 obtained in the validation model for urea, triglycerides, creatinine, and TBIL do not allow for punctual determination of the concentration, but it can be still considered as good enough for comparing groups, isolating outliers, and detecting extreme values (Grelet et al., 2021).

Important Wavelengths

Despite having the same wavelength range, the spectrum of buffalo milk differs from that of cow milk due to its distinct compound concentrations (Spina et al., 2022) and, due to the lack of studies on specific spectral regions in buffalo milk, it is rather challenging to compare the results with the literature. In contrast, some studies on cow milk have identified the most important regions for certain compounds based on the chemical bounds absorption. Regarding the metabolic profile (Figure 3), apart from urea and creatinine, the important spectral data points for approximately all parameters belonged to a region attributed to fat in cow milk, that is, the "fat B" interval (3,000 to 2,800 cm^{-1}) described by Iñón et al. (2004). In particular, the prediction model for cholesterol showed key wavelengths at peaks of 2,932 and 2,924 cm^{-1}, which corresponded with C–H stretching of $-CH_3$ and $-CH_2$ in bovine milk (Grelet et al., 2015). In addition to cholesterol, even creatinine and glucose had VIP in the so-called "fat A" region in cow milk (1,800 to 1,700 cm^{-1}, Iñón et al., 2004).

For triglycerides, creatinine, and TBIL, high VIP were observed in the 1,570 to 1,550 cm^{-1} range, a region typically associated with protein structures in bovine milk (Karoui et al., 2011; Grelet et al., 2015). Furthermore, urea displayed key wavelengths between 1,540 and 1,479 cm^{-1}, whereas cholesterol and TBIL showed prominent wavelengths at 1,493 cm^{-1}, corresponding to CH-bending in bovine milk (Karoui et al., 2011). Glucose displayed notable wavelengths in the region associated with C–O stretching (Grelet et al., 2015), attributed to lactate and monosaccharides in bovine milk (Sivakesava and Irudayaraj, 2001).

In relation to parameters associated with protein profiles (Figure 4), total protein exhibited important wavelengths within both the "fat A" and "fat B" regions (Iñón et al., 2004), regions associated with protein structures in bovine milk (Grelet et al., 2015), as well as wavelengths related to C–O stretching vibration of alcohols functions and C–O–C ether stretching (Grelet et al., 2015). Albumin was notably present in both the "fat A" and "fat B" regions, whereas globulin and albumin-to-globulin showed important wavelengths in the "fat B" region, in a region associated with protein structures and lactose in bovine milk (Iñón et al., 2004; Grelet et al., 2015).

For parameters related to enzyme activities (Figure 5), important wavelengths in the "fat B" region were observed for AST, ALP, GGT, and LDH. In contrast, the "fat A" region contained relevant wavelengths for AST, GGT, and LDH. In addition, GGT and LDH exhibited important wavelengths in regions associated with protein structures in bovine milk (Grelet et al., 2015), whereas ALT, AST, LDH, and CK showed important wavelengths related to C–O, C–C, and C–H stretching vibration, and C–O–C ether stretching (Grelet et al., 2015).

Overall, the spectral regions where VIP are concentrated are few. In all cases, we selected only the wavelengths that explained for each trait the 60% of the total variance. Indeed, even if the importance could be considered as small for some regions/wavelengths, their contribution is additive, and still all of them contribute to the total goodness of the model, as per the infinitesimal model principle.

Practical Application in the Field

In cattle, the milk urea concentration is used at the bulk milk level to evaluate the supply of protein in the diet, nitrogen efficiency, rumen activity, and ammonia metabolism (Kume et al., 2008). Regardless of the feeding and management, physiologically, buffaloes have a higher concentration of blood urea than cows. This is probably related to both the lower CP requirements of buffaloes compared with cattle and the higher efficiency in nitrogen recycling via saliva and reducing kidney clearance (Campanile et al., 2010; Neglia et al., 2014). Additionally, the ammonia production and buffering capacity of buffalo saliva is higher than that of cows, contributing to differences in nitrogen metabolism (Gandra et al., 2011). In contrast, triglycerides concentration is an indicator of energy metabolism (Puppel and Kuczyńska, 2016), and creatinine is informative of muscle activity and renal function. In addition, in cattle, the TBIL can be used to evaluate the liver functions (Kaneko, 2008).

In this study, even if extreme values were removed, animals at-risk (either with high or low concentrations of a certain molecule) were still included. For example, considering that the threshold of CK in cattle is equal to 200 U/L (Whitaker, 2004), in the present study 50 buffaloes presented CK greater than this value, indicating a likely at-risk. Moreover, buffaloes calving throughout the year were sampled, including those from farms implementing specific fertility protocols aimed at reducing the seasonality of the species. Therefore, the models developed using these data are comprehensive and representative of a wide range of conditions. However, in this study, only apparently healthy animals were sampled. Future research is needed to also consider animals with clinical diseases.

Overall, having predicted blood phenotypes available is a valuable opportunity for farmers and other operators for decision making, management adjustment, and early detection of issues. Moreover, the FT-MIR predictions, even if not highly accurate, are routinely used in the cattle sector for population screening and genetic selection at the population level. Predictions, in fact, may be used for genomic selection to identify families in a whole population where animals are at major risk of presenting a certain disease(s) at a certain point in life thanks to evaluation of their polygenic risk score. In the dairy cattle sector, annual global losses associated with diseases are estimated at ~$351 per cow per year (Rasmussen et al., 2024). Having blood biomarkers available in individual milk on a monthly basis is meaningful and, along with the milk data, can be used for informed choice. In addition, it is worth mentioning that for some hematic parameters, such as milk and blood urea, the prediction model works for the bulk tank milk spectrum, too, making the overall farm-level evaluation possible. However, it should be noted that the early detection might not always be fully applicable in this context, as test-day controls are typically conducted 1 time per month. This frequency may limit the ability to anticipate the manifestation of certain diseases in advance. This represents a potential limitation of the study, although it is a general constraint that applies to dairy cows as well. A valid alternative is to sample fresh milk of animals more frequently for hematic traits prediction, as early lactation is the most critical period in terms of metabolic stress, energy balance, and udder health.

Another possible limitation of this study is that blood and milk samples were collected at the same time, which could have affected the FT-MIR model performance. In future studies, it would be important to evaluate the optimal sampling interval between milk and blood for each trait. Moreover, future research should aim to include more blood reference values to improve model accuracy through herd-out validation, better reflecting reality.

CONCLUSIONS

In the present study, we attempted to predict lactating buffaloes' hematic traits using milk spectra to evaluate if predicted blood markers can provide informative figures to the farmer. Prediction models for traits related to metabolic profile (e.g., urea, TIBL, creatinine, and triglycerides) could be considered as good enough for gross comparison and for isolation. However, the findings indicate that predictions of ALT and AST are still far from being reliable for practical use. Availability of predicted blood phenotypes can significantly aid decision making, management optimization, and early detection of health issues in the field. Overall, despite their moderate accuracy, FT-MIR predictions can be effectively applied for routine population screening. Findings suggest that data predicted from the milk spectrum enable the identification of buffaloes at risk with reasonable reliability. Moreover, they may serve as useful proxies for genetic selection purposes once models are validated.

NOTES

This research was funded by the Italian Ministry of Agriculture (MIPAAF–DISR 07) – Programma di Sviluppo Rurale Nazionale 2014/2020 (Rome, Italy). 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 carried out within the Agritech National Research Center 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.28983701. 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: ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; CK = creatine kinase; FT-MIR = Fourier-transform mid-infrared spectroscopy; GGT = gamma-glutamyltransferase; LDH = lactate dehydrogenase; LV = latent variables; PCA = principal component analysis; PLS = partial least squares; R^2_c = coefficient of determination in calibration; $RMSE_C$ = root mean square error in calibration; R^2_V = coefficient of determination in validation; $RMSE_V$ = root mean square error in validation; RMSE = root mean square error; RPD = ratio of performance to deviation; TBIL = total bilirubin; VIP = variables' importance score.

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