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Mid-infrared spectroscopy for large-scale phenotyping of bovine colostrum gross composition and immunoglobulin concentration

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ABSTRACT

Immunoglobulin G is the fundamental antibody for acquisition of passive transfer of immunity in ruminant newborns. Colostrum, in fact, must be administered as soon as possible after birth to ensure a successful transfer of IgG from the dam to the calf. Assessment of colostrum Ig concentration and gross composition via gold standards is expensive, time consuming, and hardly implementable for large-scale investigations. Therefore, in the present study we evaluated the predictive ability of mid-infrared spectroscopy (MIRS) as an indirect determination method. A total of 714 colostrum samples collected within 6 h from parturition from Italian Holstein cows, 30% primiparous and 70% pluriparous, were scanned using a benchtop spectrometer after dilution in pure water. The prediction models were developed by correlating spectral information with the reference measurements: IgG concentration (93.54 ± 33.87 g/L), total Ig concentrations (102.82 ± 35.04 g/L), and content of protein (14.71 ± 3.51%), fat (4.61 ± 3.04%), and lactose (2.36 ± 0.51 mg/100 mg). We found a good to excellent performance in prediction of colostrum IgG concentration and traditional composition traits in cross-validation ($R^2_{CV} \geq 0.92$) and a promising and good predictive ability in external validation with R^2_V equal to 0.84, 0.89, and 0.74 for IgG, protein, and fat, respectively. In the case of IgG and protein content, for example, the coefficient of determination in external validation was greater than 0.84. The other Ig fractions, A and M, presented insufficient prediction accuracy likely due to their extremely low concentration compared with IgG (4.56 and 5.06 g/L vs. 93.54 g/L). The discriminant ability of MIRS-predicted IgG and protein content was outstanding when trying to classify samples according to the quality level (i.e., low vs. high concentration of IgG). In particular, the cut-off that better discriminate low- from high-quality colostrum was 75.40 g/L in the case of the MIRS-predicted IgG and 13.32% for the MIRS-predicted protein content. Therefore, MIRS is proposed as a rapid and cheap tool for large-scale punctual IgG, protein, and lactose quantification and for the screening of low-quality samples. From a practical perspective, there is the possibility to install colostrum models in the MIRS benchtop machineries already present in laboratories in charge of official milk testing. Colostrum phenotypes collected on an individual basis will be useful to breeders for the definition of specific selection strategies and to farmers for management scopes. Finally, our findings may be relevant for other stakeholders, given the fact that colostrum is an emerging ingredient for the animal and human food and pharmaceutical industry.
Key words: colostrum, dairy cattle, animal health, calf, passive transfer of immunity

INTRODUCTION

In most ruminants, newborns are agammaglobulinemic at birth and immunity is exclusively reached passively though a proper administration of colostrum. Successful transfer of immunity relies on both the quality and the quantity of the administered colostrum (Godden et al., 2019; Costa et al., 2021a).

The most important Ig isotypes are 3 in bovine colostrum, G (IgG), A (IgA), and M (IgM), with IgG being the key antibodies of cow colostrum and representing nearly 80% of total proteins (McGrath et al., 2016). Portable small devices to be used on farm are available at a reasonable cost to indirectly assess colostral quality (i.e., to approximate IgG concentration). Refractometers, for example, provide the refractive index (Brix, %) of the analyzed medium, which in colostrum is correlated with total solids and protein content (Soufleri et al., 2019). Various studies have discussed the accuracy of refractometers for cheap and indirect determination of the colostrum narrow-sense quality, i.e., of the IgG concentration usually determined via radial immunodiffusion (**RID**; Quigley et al., 2013; Soufleri et al., 2019). The Pearson correlation coefficient between Brix

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and RID assay reported by Quigley et al. (2013) and Morrill et al. (2015) is 0.75 and 0.79, respectively, and Bielmann et al. (2010) calculated a correlation of 0.71 for the optical refractometer and 0.73 for the digital. These values are very close to the ones reported by Elsohaby et al. (2017): 0.71 (optical refractometer) and 0.72 (digital refractometer). Although strong, the correlation is rather far from unity. In fact, the refractive index is not only given by IgG of colostrum, being more correlated to total solids that include other proteins, lactose, and fat (Morin et al., 2001). Refractometers can be therefore be used for quality screening, but do not allow estimation of a punctual and precise IgG concentration. On the contrary, determination of colostrum antibodies can be successfully achieved through infrared spectroscopy. Very satisfactory prediction accuracies have been obtained by Franzoi et al. (2022) using near-infrared spectroscopy on cow colostrum sampled within 6 h from calving. Moreover, Spina et al. (2021) demonstrated that IgG concentration is strongly correlated with total protein content of colostrum predicted using a mid-infrared spectroscopy (**MIRS**) prediction model developed for protein content of milk. In general, infrared spectroscopy is based on the study of interactions between electromagnetic waves and organic matter, which generates vibrations of the chemical bonds from all constituents of the substrate. Electromagnetic radiation comprises different regions according to the range of wavelengths (O'Donnell et al., 2014). The main difference between near-infrared spectroscopy and MIRS is that the former uses the shorter near-infrared wavelengths (12,500–4,000 cm^{-1}) that enable increased penetration depth (Manley, 2014) and is based on molecular overtone and combination of such vibrations, while the latter works in the range of the electromagnetic spectrum of 4,000 to 400 cm^{-1} and monitors the fundamental vibrational and rotational frequencies (Nunes, 2014). Irrespective of the methodology, the output of the analysis can be visualized in a so-called spectrum, which is a graphical representation of the energy transmitted (or absorbed) as a function of the incident wavelength. While the near-infrared spectrum contains more overlapped bands that make it more difficult to be interpreted (Reid et al., 2005), the mid-infrared spectrum contains more isolated bands and could be potentially more informative (Roychoudhury et al., 2006; De Marchi et al., 2014; Pereira et al., 2019).

To the best of our knowledge, few studies have investigated the ability of MIRS to predict IgG concentration of cow colostrum collected in the field under commercial conditions and none have evaluated the predictability of gross composition and other Ig isotypes. Elsohaby et al. (2017, 2018) developed a calibration model to relate the spectra (4,000 and 400 cm^{-1}) to the colostral IgG concentrations obtained via RID using beef and dairy cows' samples (n = 430). The Pearson correlation between predicted and measured IgG was 0.92 in the validation set (n = 143).

Such a lack is mainly due to the high cost of wet chemical analyses via gold standards and practical sampling difficulties in commercial farms, such as collecting a sufficient quantity of colostrum immediately after birth, correct labeling, and storage. The definition of a strict standardized protocol is reported to be essential to catch variability of quality of the first colostrum secreted by the mammary gland after parturition (Costa et al., 2021b). Accurate MIRS prediction of IgG (in g/L) may boost the adoption of precision feeding strategies in calves to ensure adequate passive transfer of immunity. Major advantages of MIRS are the possibility to simultaneously assess multiple parameters in a single analysis with just one sample, its use for the analysis of milk within the official recording system worldwide, and the constant routine standardization through ring tests. Development of specific prediction equations for bovine colostrum is advisable and can raise the interest of cost-effective on-line monitoring of composition traits and IgG.

Our objective in the present study was thereby to assess the predictive ability of MIRS for colostrum Ig fraction concentrations and gross composition using data referred to individual colostrum samples of Italian Holstein cows.

MATERIALS AND METHODS

Sampling

Ethical approval was not required for the present study as per institutional guidelines/local legislation, since only farmers interacted with the cows. The animals' owners involved in the study signed a written informed consent and are associated with the Veneto Region Breeders Association (**ARAV**, Vicenza, Italy). The whole experiment has previously been described by Costa et al. (2021b); briefly, 714 individual samples of colostrum were collected in 9 commercial Holstein farms located in northern Italy. The animals were 215 primiparous and 499 pluriparous cows, with average parity order of 2.47 ± 1.42. The experiment was designed in such a way to cover variability related to calving season and cow parity (Costa et al., 2021b; Franzoi et al., 2022). After birth, calves were immediately separated from their dams, and the first colostrum was sampled within the first 6 h after calving. The time window was selected considering results from Conneely et al. (2013), who reported that colostrum produced

later than 9 h after parturition had a statistically lower IgG concentration compared with colostrum produced earlier. To avoid presence of hyperimmune colostrum, all the cows included in this study were not vaccinated before calving against common agents such as coronavirus, rotavirus, and *Escherichia coli* (Dunn et al., 2017). The selected farms followed an intensive farming system, with freestall barn, total mixed ration administration, twice-a-day milking, and no access to pasture.

The sampling protocol (Costa et al., 2021a,b, 2022) allowed 120 mL of colostrum from each cow to be obtained. Briefly, sample collection was carried out under farmers' responsibility using plastic sterile tubes with indication of the cow's ID, parity, and calving date (SMIPA srl, Vicenza, Italy). Samples were subsequently stored on farm at −20°C and, by maintaining the cold chain, they were periodically transferred to the laboratory of Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy), where they were kept at freezing temperature until further analyses.

Gross Composition Analysis and Immunoglobulin Quantification

After thawing overnight at 4°C, 40 mL of each sample was lyophilized to measure protein and fat content. The first was quantified by Kjeldahl method 991.20 (AOAC International, 2000), whereas the second was determined according to Verbands Deutscher Landwirdschaftlicher Untersuchungs und Forschungsanstalten (**VDLUFA**) VI C15.2.1 method (VDLUFA, 2013). Lactose quantification was carried out from an aliquot of colostrum by using a Jasco high-performance liquid chromatograph equipped with an Aminex HPX 87H column (300 mm × 7.8 mm, Bio-Rad), and data were interpreted using the ChromNAV software (Version 2.0, Jasco) where the final concentration of lactose was calculated.

The concentration of IgG, IgA, and IgM was assessed with RID as widely described by Costa et al. (2021b) using Bovine Ig RID Kit purchased from Triple J Farms (Bellingham, WA). An overview of the number of samples available is given in Supplemental Table S1 (https://doi.org/10.7910/DVN/RXBC1Z; Goi et al., 2023). To fall within the detection range of the RID assay, colostrum aliquots were diluted with deionized purified water: 1:5 (vol/vol) for IgG and 1:3 (vol/vol) for IgA and IgM. For the calculation of the punctual concentration of the target components, the standard curve obtained for each plate (n = 21 samples) from the 3 reference sera/standards provided by the manufacturer (Triple J Farms, Bellingham, WA) was used. The RID repeatability, described in detail by Costa et al. (2021b), has been tested by means of the intra- and interassay coefficients of variation (**CV**). The first was tested using 4 colostrum samples analyzed in quintuplicate by a single operator: the overall CV was calculated as the average of the individual coefficients of the 4 samples measured in quintuplicate and was equal to 7.56, 2.46, and 3.03% for IgG, IgA, and IgM, respectively (Costa et al., 2021b). The interassay CV was calculated to check for potential analytical errors using the ring diameter of the 3 standards (concentrations can be found in Costa et al., 2021b) measured in 5 different plates. The average CV of the 5 plates was calculated separately for each concentration (high, medium, and low) within each parameter (IgG, IgA, and IgM) by averaging the coefficients. The resulting interassay CV was 4.63, 4.27, and 2.46% for IgG, IgA, and IgM, respectively.

MIRS Prediction Models

Contextually to RID analysis, a representative amount of the thawed colostrum was diluted (1:1, 25 mL in 25 mL) in pure water. Dilution was necessary before spectra collection to reduce the density of the matrix and avoid mechanical issues due to high density or clots. Spectra (Supplemental Table S1) were recorded using the Milkoscan 7 RM spectrometer of the ARAV official milk laboratory (Vicenza, Italy). The benchtop instrument operates at room temperature (20°C) in a wavelength range between 5,011.54 and 925.92 cm^{-1} every 3.85 cm^{-1}, for a total of 1,060 data points recorded, which were collected using FOSS Integrator software (FOSS Electric A/S, Hillerød, Denmark). Spectral data, coupled with the abovementioned RID reference analyses, were used to predict the concentration of IgG, IgA, IgM, and total Ig through the PLS procedure (SAS v. 9.4; SAS Institute Inc., Cary, NC). In addition to Ig, prediction models were also trained for the gross composition traits to obtain MIRS prediction of protein, fat, and lactose.

Before building the model, spectra were independently subjected to standard normal variate (**SNV**) scatter correction, one of the most popular pre-processing treatments of MIRS data (De Marchi et al., 2014). The SNV aims to normalize the spectral data reducing baseline shifting or tilting due to nonspecific scattering of radiation (Manfredi et al., 2018). In fact, to obtain robust prediction models, pre-treatments have great importance to reduce phenomena that can cause a deviation from the linear relation from spectrum and reference value such as scatter from particulates, molecular interactions, and changes in sample size (Rinnan et al., 2009). Spectral regions with low signal-to-noise ratio due to water absorption have been discarded according to the producer (FOSS Electric A/S). Last,

spectra presenting a Mahalanobis distance >3 from the average spectrum were considered as outliers and removed. The final data set comprised 338 wavenumbers, expressed into absorbance, in the intervals 2,970.7 to 2,507.7 cm^{-1}, 1,929 to 1,716.8 cm^{-1}, and 1,581.8 to 964.5 cm^{-1} for each sample. After spectrum pretreatments, the complete data set was randomly split in a calibration set representing 75% of total samples and in a validation set (remaining 25%), both of which had similar mean and standard deviation of the target trait (IgG, IgA, IgM, total IgG, and gross composition traits) and which were representative of all herds and parities. For each trait, a first PLS regression analysis was performed on the calibration subset by using a self-built macro (SAS software v. 9.4, SAS Institute Inc.) that trained the model with a 5-fold cross-validation. The other subset was used to assess the final performance of the developed calibration model in external validation. To avoid overfitting, the number of latent variables included in the PLS were those that minimize the root mean square error calculated at each iteration of cross-validation (Franzoi et al., 2022). For each model, the standard error of cross-validation ($\mathbf{SE_{CV}}$) was calculated and reference value outliers, detected as samples whose prediction deviated more than ± 2.5 SE_{CV} from the reference value, were excluded. The methodology of PLS regression analysis followed by outliers' elimination was iterated 2 additional times consecutively and the variable importance in projection (**VIP**) scores of the last round of PLS were kept. Residual normality of prediction equations were checked, and a *t*-test was performed using the TTEST procedure of SAS to assess that the bias, calculated as the average difference between predicted and reference values, did not statistically differ from zero. The best prediction equation was identified considering the coefficient of determination of cross- ($\mathbf{R^2_{CV}}$) and external validation ($\mathbf{R^2_V}$), and residual predictive deviation (**RPD**) in external validation.

Diagnostic Accuracy of MIRS-Predicted Traits

Receiver operating characteristic (**ROC**) curves were used to determine specificity (true negative cases), sensitivity (true positive cases), and overall discriminant ability of protein content, IgG, and total Ig predicted through MIRS in external validation. In particular, quality of samples was classified according to the RID IgG concentration as follows: < and ≥70 g/L for low and high quality, respectively. The area under the curve was the parameter used to evaluate the discriminant ability of the proxy (Šimundić, 2009). The data points of ROC curves and calculation of sensitivity and specificity were obtained in SAS software v. 9.4 (SAS Institute Inc.) through a customized script that included the LOGISTIC procedure. Subsequently, the Youden index was calculated to identify the best cut-off value for each trait, namely protein content (predicted via MIRS and measured via gold standard) and MIRS-predicted IgG, IgA, IgM, and total Ig.

RESULTS AND DISCUSSION

The samples used were derived from an experiment carried out between 2019 and 2020. Part of the results from this trial on phenotypic and genetic variability of Ig as well as on near-infrared spectroscopy applications and use of refractometers have already been published (Costa et al., 2021a,b, 2022; Franzoi et al., 2022). For the purpose of the present investigation, the main inclusion criterion was the presence of the mid-infrared spectrum (Supplemental Table S1). Out of the 714 samples originally collected, only those belonging to purebred Holstein animals calving between spring 2019 and spring 2020 and with both mid-infrared spectra and reference available were kept. In some cases, in fact, one or more aliquots were lost and the volume was insufficient (<25 mL) for mid-infrared spectra acquisition (Supplemental Table S1). The number of samples with matching reference measurement and spectrum is reported in Table 1 for each trait. The average parity, ranging from 1 to 8, differed thereby according to the trait considered (i.e., from 2.39 for IgA to 2.48 for IgG and IgM).

Descriptive Statistics

Descriptive statistics of colostrum Ig isotypes and composition traits postediting are summarized in Table 1. The final number of records ranged from 455 (IgA) to 577 for lactose content. The most variable Ig fraction was IgA with a coefficient of variation close to 60% (Table 1), whereas, among composition traits, fat content exhibited the greatest variability (65.94%). According to the averages observed, approximately 91% of total Ig is composed by the G fraction, followed by fractions M and A, which each comprised 5 and 4% of the total Ig, respectively. As expected, there was large variability in the colostrum composition, even if the time window for sampling was narrow (0 to 6 h after calving) compared with other studies where colostrum was sampled as late as 24 h postpartum. The rapid change in both the chemical and the physical properties of bovine colostrum has been widely described in literature, particularly in the case of IgG (Bielmann et al., 2010; McGrath et al., 2016). For instance, Baumrucker and Bruckmaier (2014) reported a wide cow-to-cow variation of the subtype IgG1 (11.8 to 74.2 mg/

Table 1. Overview of descriptive statistics of colostrum[1] Ig isotypes and composition traits measured with the reference method

Trait	N	Mean	SD	CV, %	Range
Ig, g/L					
IgG	531	93.54	33.87	36.21	9.22–198.90
IgA	455	4.56	2.59	56.80	0.13–13.89
IgM	522	5.06	2.37	46.84	0.35–11.39
Total	458	102.82	35.04	34.08	19.67–196.86
Protein, %	559	14.71	3.51	23.86	4.55–25.22
Fat, %	557	4.61	3.04	65.94	0.12–14.95
Lactose, mg/100 mg	577	2.36	0.51	21.61	0.74–4.06

[1]Sampled within 6 h from calving.

mL) and the range observed by Kessler et al. (2020) for RID IgG (12.7 to 185.3 mg/mL) is similar to ours (9.2 to 198.8 g/L). Overall, the concentrations obtained also mirrored those reported by Ceniti et al. (2019) for Simmentals (0.1 to 199.0 g/L) and by Bielmann et al. (2010) for Holsteins (22.4 to 196.9 g/L). Concentrations of IgA and IgM were similar to those obtained in Holstein cows by Gomes et al. (2011). The composition of colostrum is highly variable due to numerous factors, including individual cow, breed, parity, calving season, pre-partum nutrition, dry period length and management, and sampling time postpartum (McGrath et al., 2016; Costa et al., 2023).

Of all the samples with the RID IgG value available, 9% were characterized by a suboptimal IgG concentration (i.e., below 50 g/L). According to McGrath et al. (2016) and Godden et al. (2019), this value represents the threshold to discriminate between low- and high-quality colostrum.

Descriptive statistics for fat, protein, and lactose are consistent with Morrill et al. (2012) who reported average values of 5.6, 12.7, and 2.9%, respectively, and with Kehoe et al. (2007) whose averages were 6.7, 14.9, and 2.5%, respectively. Compared with such authors, fat concentration quantified in the present study was lower, likely due to the analytical method used. Considering the variability, the high CV obtained are consistent with results of Morrill et al. (2012) and Kehoe et al. (2007) who reported a very wide range for both fat (1.0–21.7% and 2.0–26.5%, respectively) and protein content (2.6–20.5% and 7.1–22.6%, respectively). Values reported by Kehoe et al. (2007) are referred to cows fully milked out within 4 h of calving, suggesting that the fat variability is not related to the milking procedure (e.g., empty milking or not) but more likely to the sampling time after calving or other cows' physiological reasons.

Predictive Ability

As in milk, the reference methods to measure colostrum quality are expensive, laborious, and hardly implementable for large-scale acquisition of phenotypes. Previous studies have already highlighted the ability of near-infrared spectroscopy to predict some traits related to colostrum composition, including IgG (Navrátilová et al., 2006; Rivero et al., 2012; Franzoi et al., 2022). Elsohaby et al. (2018) developed a MIRS model for colostrum IgG concentration, but in this study for the first time MIRS has been used to develop and validate prediction models for the 3 Ig fractions (G, A, and M) and gross composition traits. These may be features of interest in contexts where precise quantification is needed, for example, to develop precision feeding strategies (e.g., for calves with poor vitality at birth who may require a particularly excellent colostrum quality), to assess payment system based on the Ig level, and to have phenotypes available for breeding purposes (Costa et al., 2023).

For all the traits investigated in the current study, the performance of the best MIRS prediction model developed in cross- and external validation are reported in Table 2. In the case of Ig, the samples identified as outliers and excluded from the calibration data set were below 5% and the LV ranged from 3 to 10. The most excellent model in terms of variance explained in cross-validation (Karoui et al., 2006) was the one developed for IgG ($R^2_{CV} = 0.92$), followed by the one for total Ig concentration ($R^2_{CV} = 0.90$). When applied on an external set of samples, the models for IgG and total Ig content showed promising performances ($R^2_V > 0.82$) and the RPD was >2.3, indicating the feasibility of using the prediction equations for rough screening of samples (Williams, 2014). The prediction model developed by Franzoi et al. (2022) for colostrum IgG using near-infrared spectroscopy has a lower R^2_V (0.76) with PLS algorithm, but almost similar coefficient of determination (i.e., 0.83) to that reported in the present study for IgG when applying simulated annealing PLS. The prediction model of IgM and IgA did not reach satisfactory performance (Table 2), as similarly observed by Franzoi et al. (2022) under near-infrared spectroscopy. Through beef and dairy cow colostrum samples, Elsohaby et al. (2018) obtained promising pre-

Table 2. Fitting statistics[1] of modified partial least square regressions in 5-fold cross-validation and external validation for Ig isotypes of colostrum[2]

Ig	n	Outliers, %	LV	$RMSE_{CV}$	R^2_{CV}	n_{ext}	$RMSE_V$	R^2_V	RPD
IgG, g/L	383	4.0	10	9.53	0.92	132	13.39	0.84	2.49
IgA, g/L	329	3.8	5	1.68	0.45	113	2.08	0.33	1.22
IgM, g/L	384	2.0	3	1.65	0.49	130	1.94	0.33	1.21
Total, g/L	328	4.7	8	10.31	0.90	114	14.59	0.83	2.38

[1]n = number of samples used to perform the calibration after the outliers' elimination; LV = number of latent variables; $RMSE_{CV}$ = root mean square error in cross-validation; $RMSE_V$ = root mean square error in external validation; R^2_{CV} = coefficient of determination in cross-validation; n_{ext} = number of samples used to perform the external validation; R^2_V = coefficient of determination in external validation; RPD = residual predictive deviation calculated as standard deviation of reference data (validation) to standard error of predictions.
[2]Sampled within 6 h from calving.

dictability for IgG with MIRS using a device working in a smaller range of wavelength (4,000 and 400 cm^{-1}) compared with that of the present study. However, the sampling protocol adopted by Elsohaby et al. (2018) apparently does not allow to fairly compare their model with ours. In particular, authors did not report the time window considered for sampling, precluding direct comparison of the reference and predicted data (Godden et al., 2019).

The scatter plots of measured versus predicted Ig isotypes and total Ig for the external validation set, and their associated R^2_V, are reported in Figure 1. The mean and the range of measured and predicted traits are reported in Table 3 and demonstrate that MIRS predictions are in line with the gold standard RID even though characterized by a slightly lower variability. Indeed, within a given trait, the coefficient of variation was larger for the reference compared with its predicted counterpart.

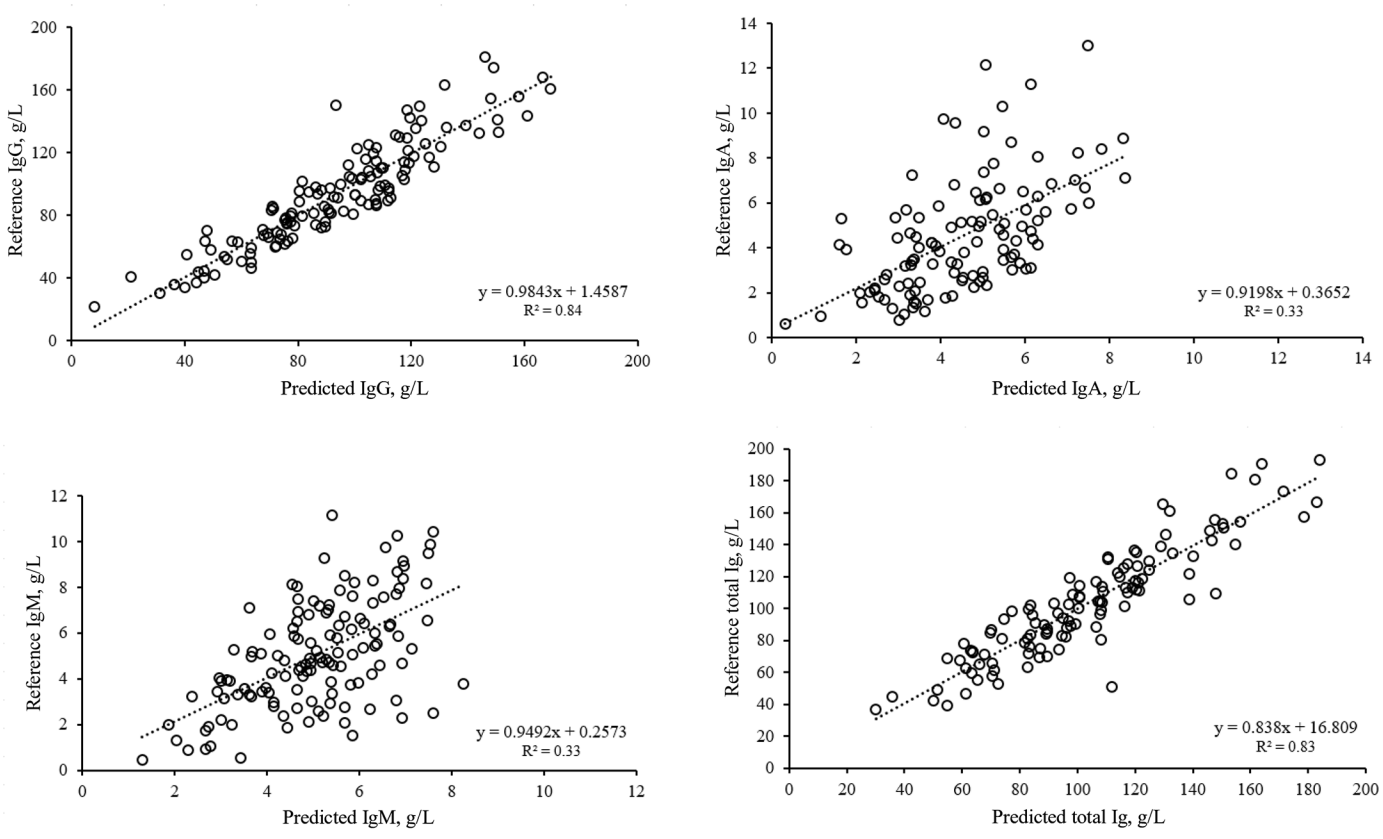

Figure 1. Plot of predicted versus reference values of the Ig isotype concentration and concentration of total Ig of colostrum. Sampled within 6 h from calving; external validation set.

Table 3. Descriptive statistics of the Ig isotypes of colostrum[1] determined via radial immunodiffusion (RID) and predicted via mid-infrared spectroscopy (MIRS) in the calibration and the validation sets

Ig	Determination	Data set	n	Mean	SD	CV, %	Range
IgG, g/L	RID	Calibration	399	93.55	34.11	36.46	9.22–198.90
		Validation	132	93.50	33.28	35.59	21.48–181.10
	MIRS	Calibration	383	92.40	31.55	34.15	7.31–177.64
		Validation	132	93.51	30.98	33.13	8.06–169.35
IgA, g/L	RID	Calibration	342	4.56	2.61	57.24	0.13–13.89
		Validation	113	4.54	2.53	55.73	0.61–13.01
	MIRS	Calibration	329	4.34	1.57	36.18	0.00–8.65
		Validation	113	4.54	1.59	35.02	0.32–8.37
IgM, g/L	RID	Calibration	392	5.06	2.37	46.84	0.35–11.39
		Validation	130	5.07	2.36	46.55	0.47–11.17
	MIRS	Calibration	384	5.06	1.65	32.61	0.37–9.19
		Validation	130	5.07	1.42	28.01	1.29–8.25
Total, g/L	RID	Calibration	344	102.77	35.18	34.23	19.67–196.86
		Validation	114	102.97	34.77	33.77	37.02–192.66
	MIRS	Calibration	328	101.33	31.73	31.31	16.68–186.89
		Validation	114	103.10	32.07	31.11	30.04–184.16

[1]Sampled within 6 h from calving.

Table 4 reports the fitting statistics of developed calibration models for protein, fat, and lactose content. The reference outliers (about 5%) were excluded before development of the MIRS model of protein and lactose, and for both traits the developed model was quite satisfactory in terms of R^2_V, being 0.89 and 0.86 for protein and lactose, respectively. Due to the high variability of fat content already discussed (Table 1), the number of outliers removed was close to 10%. Nevertheless, even the R^2_V for fat indicated that the developed prediction model can still be considered sufficient for screening and to approximate quantitative prediction (Williams, 2007; Grelet et al., 2021). In support of this, Figure 2 shows the linear relationship between the measured and predicted values of colostrum gross constituents in the external validation set. Navrátilová et al. (2006) obtained similar R^2_{CV} for colostrum protein and lactose content prediction models using near-infrared spectroscopy; with regard to fat, however, the model developed was characterized by a greater R^2_{CV} compared with the present study. However, it was not possible to compare the R^2_V as no external validation was performed. Overall, the RPD value for the developed prediction models in the current study indicates a promising feasibility of MIRS to assess the level of protein and lactose in bovine colostrum. Further implementation of the models, coupled with the use of other algorithms (Gottardo et al., 2015; Frizzarin et al., 2021), could likely result in an increased predictive ability, particularly for fat content. The range of measured and predicted values is reported in Table 5 for both the calibration and the validation sets; as mentioned for the Ig fractions, MIRS predictions fell within the range of measured traits even if they tended to be slightly less variable. In the external validation set, for example, the minimum protein and fat content was smaller than the minimum of their respective reference. With regard to lactose content, only small differences were seen (Table 5). In particular, in external validation, the range of the gold standard (1.16–4.03 mg/100 mg) and the range of MIRS (1.47–4.17 mg/100 mg) were almost the same. The same is valid for the calibration set (Table 5), whose ranges where 0.74–4.06 (gold standard) and 0.97–4.24 (MIRS). Overall, such findings confirm that the models provided robust predictions that fell within the range of values we expected. To assess a punctual determination of constituents for commercial purpose/industrial use, however, we recommend increasing the sample size

Table 4. Fitting statistics[1] of modified partial least square regressions in 5-fold cross-validation and external validation for the colostrum[2] gross composition traits

Trait	n	Outliers, %	LV	$RMSE_{CV}$	R^2_{CV}	n_{ext}	$RMSE_V$	R^2_V	RPD
Protein, %	399	5.0	12	0.81	0.94	139	1.16	0.89	2.97
Fat, %	374	10.5	5	0.78	0.92	139	1.57	0.74	1.96
Lactose, mg/100 mg	410	5.3	10	0.14	0.91	144	0.19	0.86	2.63

[1]n = number of samples used to perform the calibration after the outliers' elimination; LV = number of latent variables; $RMSE_{CV}$ = root mean square error in cross-validation; $RMSE_V$ = root mean square error in external validation; R^2_{CV} = coefficient of determination in cross-validation; n_{ext} = number of samples used to perform the external validation; R^2_V = coefficient of determination in external validation; RPD = residual predictive deviation calculated as standard deviation of reference data (calibration) to standard error of predictions.

[2]Sampled within 6 h from calving.

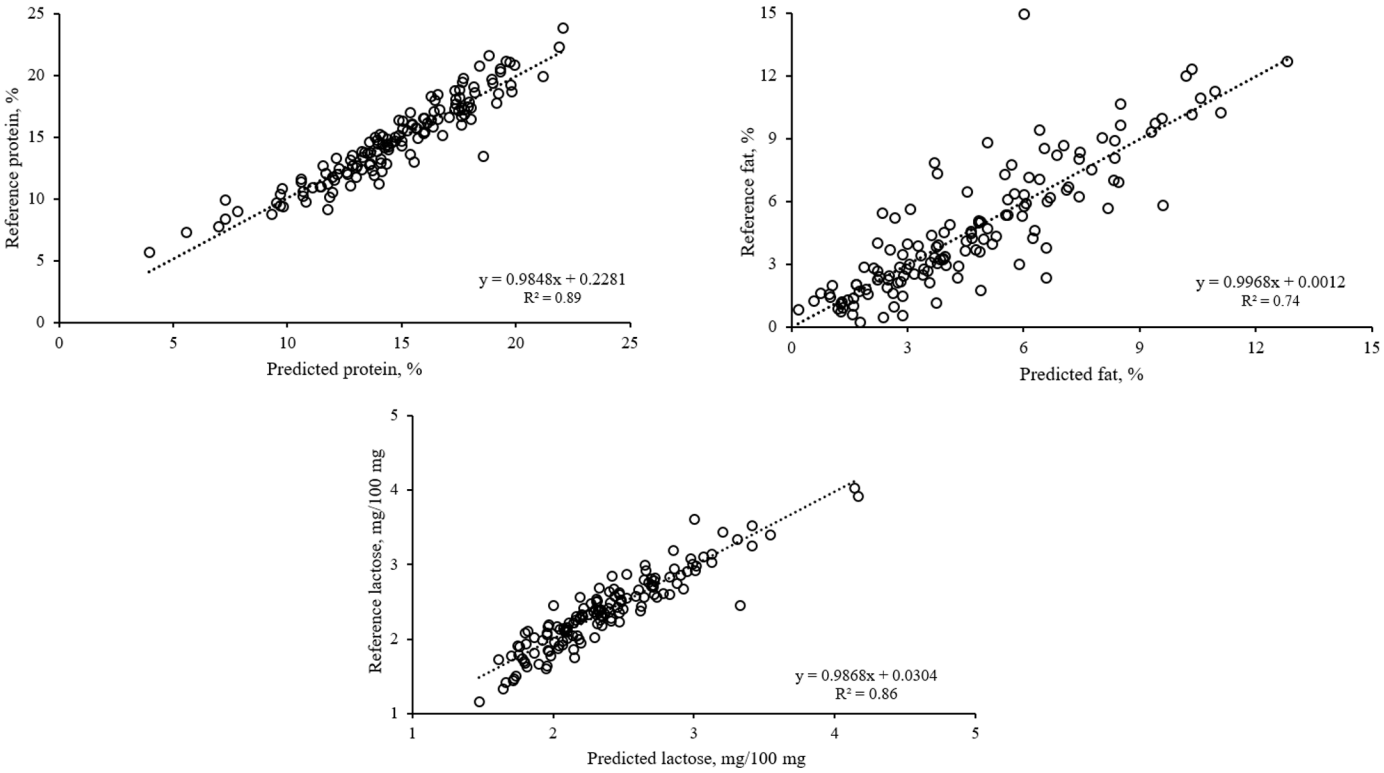

Figure 2. Plot of predicted versus reference values of the gross composition trait concentrations of colostrum. Sampled within 6 h from calving; external validation set.

and variability (e.g., breed, country, farming system) to improve the representativeness and robustness of the calibration sets.

VIP Scores

To compare the spectral regions significant for predicting IgG, total Ig, and protein content, we extrapolated the VIP scores (Figure 3) to identify the most important wavenumbers (i.e., those with a VIP score greater than 1; Caponigro et al., 2023). In fact, the VIP score measures the significance of a component in regression. As expected, the significant regions of the 3 traits overlaps in some spectral wavenumbers. With regard to IgG and total Ig, this was expected since the former accounts for the major percentage of the latter; VIP scores presented peaks for IgG which are always included by those of total Ig. The peaks are located in regions known to be important for protein compounds (i.e., around 1,550 cm^{-1} due to C–N and

Table 5. Descriptive statistics of colostrum[1] gross composition traits determined via gold standard[2] and predicted via mid-infrared spectroscopy (MIRS) in the calibration and the validation sets

Trait	Determination	Data set	n	Mean	SD	CV, %	Range
Protein, %	Gold standard	Calibration	420	14.71	3.53	24.00	4.55–25.22
		Validation	139	14.71	3.45	23.45	5.71–23.80
	MIRS	Calibration	399	14.79	3.32	22.45	4.69–23.77
		Validation	139	14.71	3.30	22.43	3.94–22.07
Fat, %	Gold standard	Calibration	418	4.60	3.04	66.09	0.12–14.95
		Validation	139	4.63	3.07	66.31	0.24–14.93
	MIRS	Calibration	374	4.38	2.70	61.64	0.29–14.10
		Validation	139	4.64	2.66	57.33	0.00–12.83
Lactose, mg/100 mg	Gold standard	Calibration	433	2.36	0.51	21.61	0.74–4.06
		Validation	144	2.37	0.51	21.10	1.16–4.03
	MIRS	Calibration	410	2.36	0.45	19.07	0.97–4.24
		Validation	144	2.37	0.47	19.83	1.47–4.17

[1]Sampled within 6 h from calving.
[2]Protein, fat, and lactose content were determined via Kjeldahl, base hydrolysis plus accelerated solvent extraction, and HPLC, respectively.

N–N stretching; Grelet et al., 2015) and N-H bending deformation (Ribadeau-Dumas and Grappin, 1989). Moreover, Grelet et al. (2015) and Ribadeau-Dumas and Grappin (1989) stated that another absorption band of the peptide bond exists around 1,650 cm^{-1}. Soyeurt et al. (2012) reported regions between 1,700 and 1,500 cm^{-1} and from 1,450 to 1,200 cm^{-1} to be associated with protein absorption bands. However, the most significant wavenumber identified also covered a relevant range for the carbohydrate functional group (1,200 to 900 cm^{-1}; Caponigro et al., 2023), lactose content (1,250 to 1,000 cm^{-1}) and fat content (1,450 to 1,390 cm^{-1}; Grelet et al., 2015). These similarities confirm that antibodies represent most of the protein contained in the first colostrum. However, in terms of dissimilarities, certain wavenumbers in some regions were useful to predict protein content but not Ig, and vice versa (Figure 3). This indicates that, despite being highly correlated, the IgG and the total protein content cannot be considered as the same trait.

Diagnostic Accuracy

Sensitivity and specificity of MIRS-predicted IgG and total Ig are graphically presented in Figure 4 and refer to the external validation set. In the case of IgG (n = 132), the AUC was excellent (0.978) and not far from the AUC of total Ig (0.918; n = 114). These values denote an excellent discriminant ability of the 2 MIRS-predicted traits (Šimundić, 2009). We also tested the discriminant ability of measured and predicted protein content (Figure 5); their AUC were very satisfactory, equaling to 0.952 and 0.957 (n = 127), respectively. Overall, these results are presented as complementary information about the model performance to classify colostrum samples according to their quality. Based on the false positives and false negatives observed (Table 6), MIRS-predicted traits, namely IgG, total Ig, and protein content, correctly classified 90.1%, 84.2%, and 92.2% of the total samples. In the case of protein content measured via gold standard, this percentage was 92.9%. True positives, however, tended to be classified more accurately than true negatives (Table 6), as suggested by the non-zero amount of false positives, i.e., negative samples (low-quality) incorrectly classified as positives (high-quality). The optimal cut-off value, the point on the ROC curve that maximizes sensitivity and specificity (Table 6), was found at 75.4 g/L in the case of IgG and at 95.09 g/L in the case of total Ig (Figure 4). Finally, the cut-offs for MIRS-predicted protein content and protein content determined through the gold standard were 13.32 and 12.65%, respectively, indicating that samples with a MIRS-predicted protein content of 13.32% have to be considered of low quality.

Ideally, colostrum with IgG greater than 50 g/L should be used for the first calf's meal as the absolute amount of antibodies delivered would be sufficient if a conventional volume (~4 L) is administered in the first 12 h of life (Buczinski and Vandeweerd, 2016; McGrath et al., 2016).

We tested the ability to discriminate samples base on the quality level by using 70 g/L of IgG as a threshold, as the conventional cut-off (50 g/L) seemed too strict, with a very limited number of samples (n = 12) in the low-quality group. The 50 g/L would lead to very high AUC (0.979), a cut-off of 67.62 g/L, and 95.5% of samples classified correctly (data not shown). In particular, 117 high-quality samples would be the true positives and 9 low-quality samples the true negatives; the remaining amount would be given by the 3 false positives and the 3 false negatives (data not shown).

Practical Considerations

The IgG and gross composition determination via MIRS may be useful to standardize pooled colostrum at industry level (Franzoi et al., 2022), to collect the narrow-sense quality phenotypes at the individual level for selective breeding (i.e., IgG), or to further aid farmers in setting up an internal farm colostrum bank (Costa et al., 2021a, 2022). The list of items analyzable by MIRS-based instruments of milk laboratories or dairy plants may be potentially extended with colostrum. Considering that this secretion is only produced at calving, just 1 sample/year would be analyzed for each lactating animal. This number can be even lower if exclusively colostrum of elite/selected cows is taken (e.g., for colostrum bank or for selling). In most of the countries with a milk recording service, farmers already pay a fee to analyze the milk of their cows 9/10 times per year. Given these considerations and bearing in mind the increasing importance of colostrum in different research fields (Costa et al., 2023), we expect the cost of colostrum MIRS analysis to be almost negligible for farmers in the near future. Devices like refractometers and colostrometers provide indirect measure of total solids content and the relationship with the IgG is strongly time-dependent (h from parturition), making the measurements obtained from different farmers and tools not comparable and often imprecise. The MIRS prediction of colostrum composition, instead, requires the same personnel and instruments already in use for the official milk analyses worldwide, under the specific rules and standard guidelines of the International Committee for Animal Recording (ICAR), without any need of additional resources. Farmers willing to analyze the colostrum of all or certain cows only need to freeze or refrigerate the bottles/tubes and then deliver them to

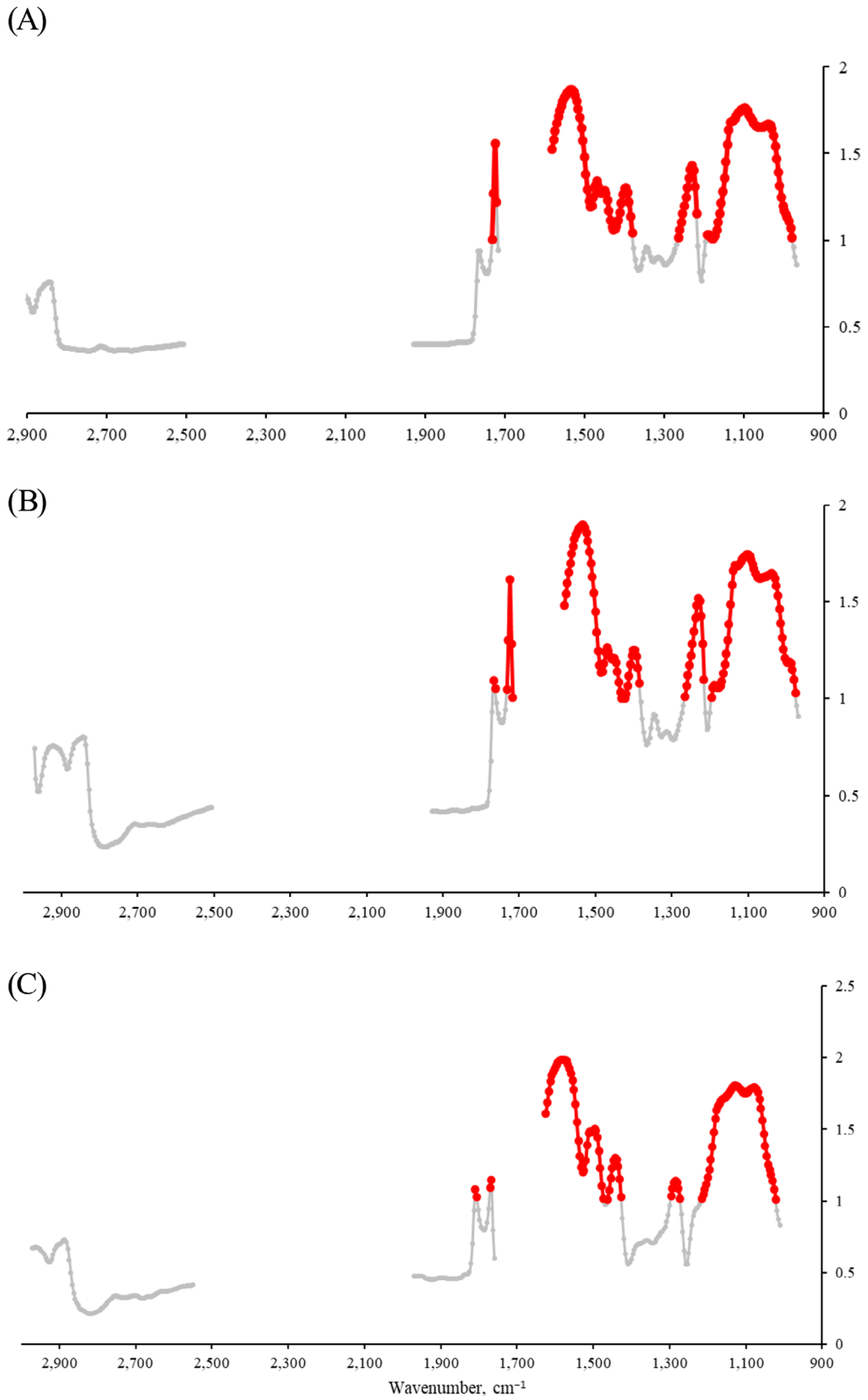

Figure 3. Total wavenumbers used for prediction of (A) IgG concentration, (B) total Ig concentration, and (C) protein content with identification of the most important wavenumbers (red dots).

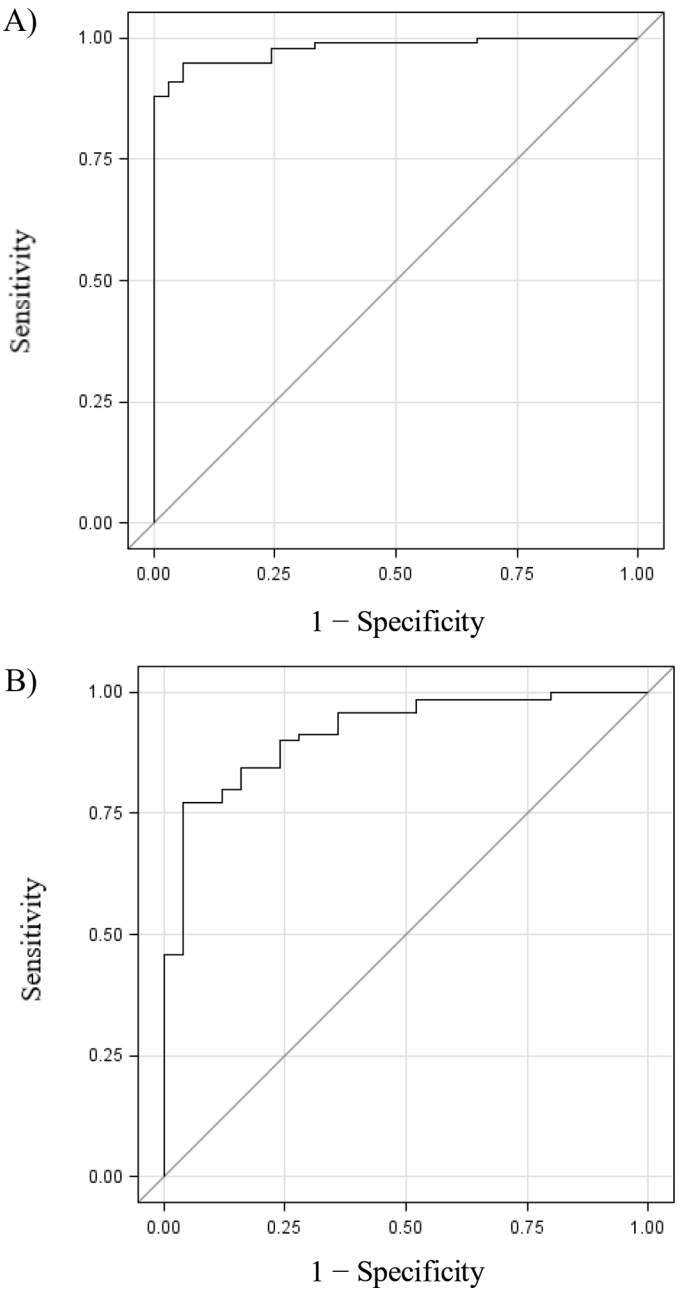

Figure 4. Graphical output of the receiver operating characteristic analysis to assess the discriminant ability of infrared-predicted (A) IgG concentration (n = 132) and (B) total Ig concentrations (n = 114) of samples present in the external validation set with the gold standard IgG concentration available. Low and high quality: < and ≥70 g/L of IgG (determined via gold standard), respectively.

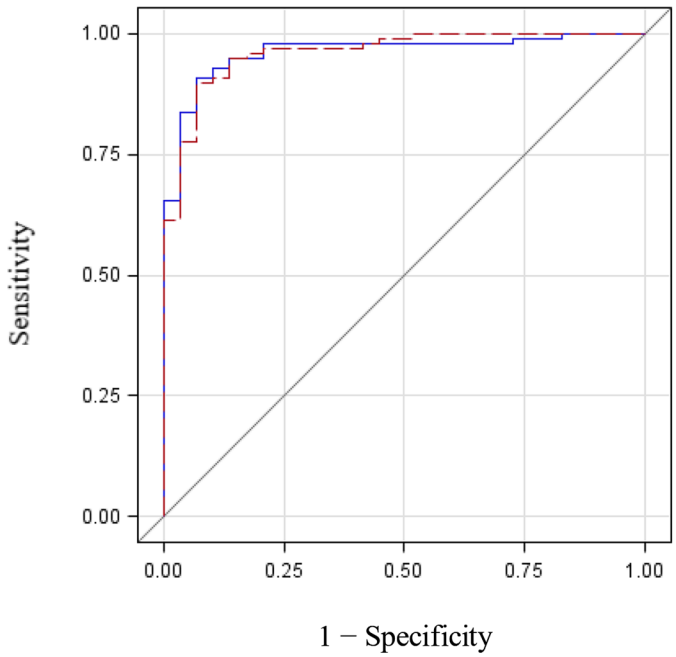

Figure 5. Graphical output of the receiver operating characteristic analysis to assess the discriminant ability of protein content measured via gold standard (blue solid line) and predicted via infrared spectroscopy (red dashed line) in the 127 samples present in the external validation set with gold standard IgG concentration available. Low and high quality: < and ≥70 g/L of IgG (determined via gold standard), respectively.

the laboratory, possibly with the support of the person in charge of the monthly official milk analysis.

The MIRS analysis can allow identification of the top-quality colostrum samples that could be used when precision feeding strategies are needed to ensure a successful transfer of antibodies to calves born with low vitality and weak suckling.

Finally, it is important to highlight that more flexible MIRS models can be developed in future for commercial dissemination using colostrum collected even after 6 h from parturition for calibration (e.g., from 0 to 12 h after calving).

CONCLUSIONS

In the present study, we attempted to predict Ig concentration and gross composition of first colostrum of dairy cows using MIRS, a technology that requires less than 30 mL of sample for spectra collection and handles more than 200 samples per hour. This technology showed good to excellent performance in predicting concentration of colostral antibodies and traditional composition traits in cross-validation and demonstrated a promising and good predictive ability in external validation. In particular, the performance was outstanding in the case of IgG concentration, the key parameter for the definition of colostrum quality in cattle. In addition, we demonstrated that through MIRS-predicted IgG and protein content it is possible to accurately identify low-quality samples by using specific cut-offs: 75.40 g/L

Table 6. Confusion matrix for the discriminant ability of the MIRS-predicted traits and gold standard protein content of samples in the external validation set with the gold standard IgG concentration available[1]

Predicted classification	Actual classification[2]		AUC	Cut-off
	Positive	Negative		
MIRS				
IgG concentration (n = 132)			0.978	75.40 g/L
Positive	92%	17%		
Negative	8%	83%		
Total Ig concentration (n = 114)			0.918	95.09 g/L
Positive	88%	27%		
Negative	12%	73%		
Protein content (n = 127)			0.961	13.32%
Positive	94%	15%		
Negative	6%	85%		
Gold standard				
Protein content (n = 127)			0.962	12.65%
Positive	94%	12%		
Negative	6%	88%		

[1]The area under the curve (AUC) is specified together with the best cut-off identified. Low and high quality: < and ≥70 g/L of IgG (determined via gold standard), respectively. MIRS = mid-infrared spectroscopy.

[2]The percentage of true positives and the percentage of true negatives are the first and the second element in the diagonal, respectively.

and 13.32%, respectively. Considering the importance of having punctual IgG concentration for management, genetic, and analytical purposes, this study paves the way toward the implementation of dedicated models for routine MIRS analysis of bovine colostrum. As for milk, MIRS represents a valid and low-cost phenotyping tool for colostrum to be used for (1) genetic purposes on the animal side and (2) for quality assessment at farm and industry level (food and pharma).

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